# Biodeterioration of Aluminium Hot Roll Mill Emulsions

by

Peter John Ramsden

Submitted in partial fulfilment of the
requirements for the degree of
Master of Science
in the
Department of Microbiology and Plant Pathology,
University of Natal,
Pietermaritzburg

September 1998

**ABSTRACT**

An in-depth study of the biodeterioration of the Hulett Aluminium hot roll mill emulsion, Prosol, was conducted. Samples of the emulsion in use at the hot roll mill were taken from various areas of the emulsion reticulation system in order to identify regions of highest microbial contamination. Standard plate count techniques and diagnostic procedures were employed to quantify and identify the microorganisms in these samples. In some of the highly contaminated areas of the emulsion reticulation system, microorganisms exceeded $1x10^7 CFUml^{-1}$ emulsion. A range of bacteria was identified which included members of the genera: *Bacillus; Pseudomonas; Escherichia; Enterobacter; Sporosarcina; Micrococcus; Aeromonas*; *Chromobacterium* and *Desulfovibrio.* Various fungi, including several yeasts, were also isolated and some of the filamentous spore-forming types were identified as *Aspergillus* spp.; *Penicillium* spp. and a *Cladosporium* sp.

A visual scale was developed to assess the growth rate of the isolated microorganisms on a range of specific media containing various emulsion components as carbon and energy source. Although the results obtained by using this scale were not conclusive, a few biodegradable components were nonetheless identified. It was found that mixed cultures of the above microorganisms had a greater biodeteriorative effect on the emulsion than did any of the pure cultures when applied separately. This suggested complex microbial interactions were involved in the breakdown of the emulsion.

A laboratory-scale model system representative of the Hulett Aluminium hot roll mill was designed and constructed to carry out a series of tests on unprotected and biocide-treated emulsions. A range of biocide concentrations were tested from which the minimum biocide inhibitory concentration was calculated. It was shown that microorganisms exposed to sub-lethal doses of the biocide Busan (active component glutaraldehyde) over a prolonged period of time, exhibited greater levels of tolerance and resistance to the biocide than did those microorganisms not previously exposed. It was deduced that less frequent, shock doses of biocide are more effective in the control and eradication of emulsion degrading microorganisms than are frequent, low level doses of the same biocide. In addition to the

biocide studies, three imported so-called 'biostable' emulsions were evaluated as possible replacements for the susceptible Prosol. Of these three imported emulsions, two *viz*. HRF3 and Houghton Biostable were shown to be more resistant than Prosol to biodeterioration.

After assessing the current hot roll mill management practices, a number of recommendations were made, including: the improvement of plant hygiene; education of the mill workers; improvement of emulsion monitoring; improvement of down-time management and improvement of biocide dosing regimes. Recommendations are also made for minimizing potential microbial growth in the new hot roll mill currently under construction at the Hulett Aluminium processing plant at Pietermaritzburg, South Africa.

# PREFACE

The experimental work described in this dissertation was carried out in the Department of Microbiology and Plant Pathology, University of Natal, Pietermaritzburg.

These studies represent original work by the author and have not been submitted in any form for any degree or diploma at any other University. Where use has been made of the work of others it is duly acknowledged in the text.

## ACKNOWLEDGEMENTS

Professor F.M.Wallis - Whose tireless dedication and perseverance helped me when I needed it most.

Staff of Hulett Aluminium - Dr. A. Pitchford and Mrs. M. Hemingway. Its here!

Mum and Dad - Who have helped and supported me over the many years of varsity life.

Rose - Who helped me through the bad times and gave me endless encouragement.

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# CHAPTER 1. INTRODUCTION

Aluminium is a versatile, lightweight metal used in a multitude of applications throughout the world. Aluminium processing involves several stages. Depending on the final product one of these stages is rolling. During this process ingots of solid, refined aluminium are repeatedly passed through either a hot or cold roll mill to produce a semi-processed product. This thesis describes experiments conducted during investigations of the biodeterioration of emulsions, particularly Prosol, used at the Hulett Aluminium plant, Pietermaritzburg, South Africa, during the rolling of aluminium, in a hot roll mill.

The Hulett Aluminium hot roll mill is located within the company's processing plant in the Camps Drift Industrial area of Pietermaritzburg. This mill is the largest of its kind in Africa, and by far the most productive. It provides rolled aluminium for the local market as well as for expanding foreign markets. In order to meet demands for processed and semi-processed aluminium products, a R2 billion development has been initiated in which a new hot roll mill, as well as other aluminium processing facilities, will be built.

## 1.1 The Hulett Aluminium hot roll mill

Although relatively simple, the process of rolling aluminium may vary markedly from one hot roll mill to the next. Figure 1 is a diagrammatic representation of the Hulett Aluminium hot roll mill operating in Pietermaritzburg.

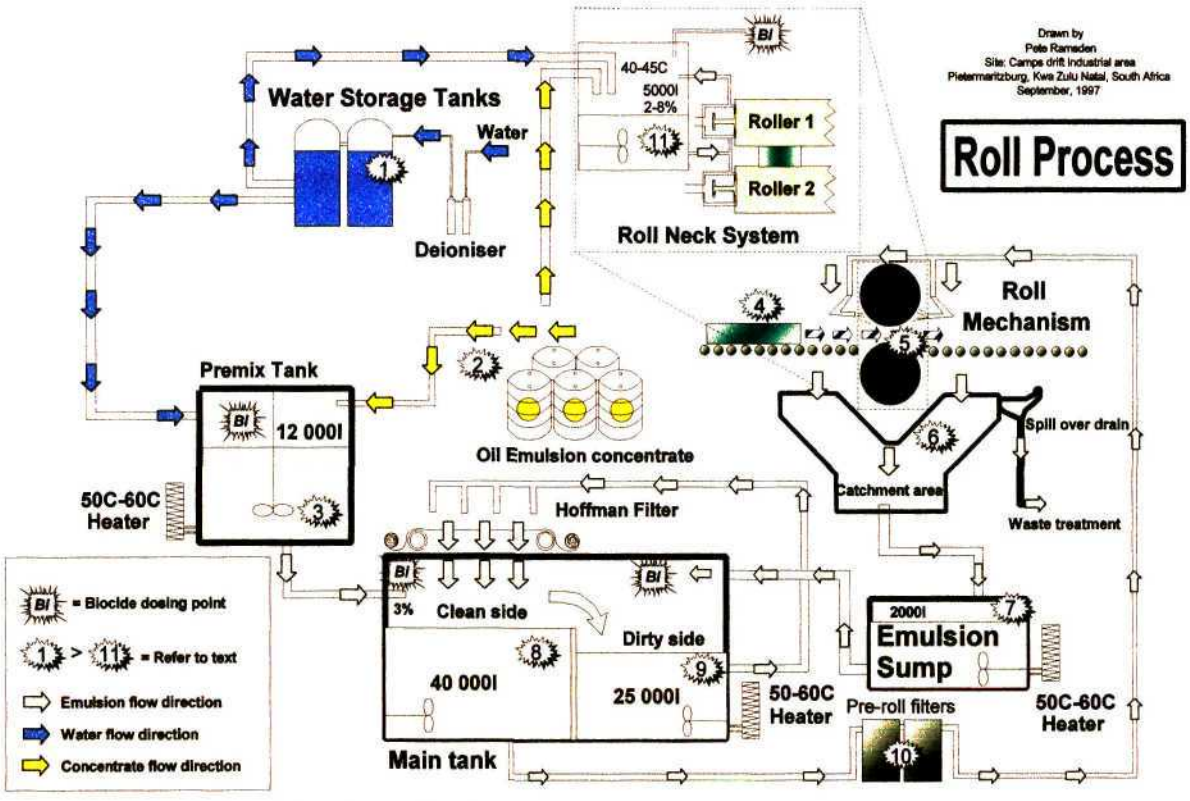


**Figure 1: Hulett Aluminium hot roll mill - Summarized Emulsion Reticulation System**

Deionized water (1) together with oil emulsion concentrate (2) are pumped into a 12000ℓ premix tank (3) where they are mixed and held until the emulsion is fed into the roll mechanism.

A heated aluminium ingot (4) is moved by crane onto a roller conveyer and is passed repeatedly between two large steel rollers (5). After each passage it becomes progressively thinner with concomitant increase in length. In order to process the aluminium, it must be malleable. To achieve this condition the ingots are passed through the steel rollers at approximately 450°C. At this temperature, however, the ingots would normally stick to the steel rollers, thereby damaging them and lowering the quality of the finished product. This problem was overcome with the development of oil-water based emulsions for aluminium rolling. The oil and water components provide the lubrication required to prevent the aluminium ingot from sticking to the rollers, and the water maintains the steel rollers at the required temperature.

The emulsion sprayed onto the rollers can run either directly into the catchment area (6) below

the rollers or, alternatively, onto the ingot and then into the catchment area.

From the catchment area the emulsion drains into a 2000ℓ sump tank (7) where it is maintained at 50-60°C with constant circulation within the tank. Impellers are used to keep the emulsion 'tight' ie. the oil droplets remain small. Without circulation the oil phase would separate from the water phase and the emulsion would become 'loose'. Should the emulsion become 'loose', both its lubricating and cooling properties would decrease markedly. The emulsion is pumped from the sump tank into the main emulsion storage tank. The emulsion storage tank is divided into two components, a 'clean side' (8) and a 'dirty side' (9). The emulsion being pumped from the sump tank enters the 'dirty side', and from there, it is passed through a Hoffman filter into the clean side of the main tank. The Hoffman filter removes slivers of metal and other particulate matter from the emulsion. The 'dirty' and 'clean' sides of the tank are separated by a unidirectional spill-over wall, so that once the clean side is full, the emulsion spills over into the dirty side. The level of emulsion in the clean side is maintained by adding emulsion from the premix tank (3).

From the clean side of the emulsion storage tank, the emulsion is passed through two in-line, aluminium-fines filters (10) and subsequently sprayed back onto the rollers during the roll process, thereby completing the cycle.

Independent of the main tank system is the roll neck system (11). This provides lubrication and cooling to the roll heads. A 5000ℓ tank is filled with a 2-6% emulsion depending on the required rolling conditions.

The roll mill is in operation 24 hrs a day, 7 days a week with the only down-time occurring once every 14 days, when the mill undergoes planned maintenance.

## 1.2 Soluble oil emulsions for the rolling of aluminium

A major category of petroleum product spoilage is the infection of soluble oil emulsions (Genner and Hill, 1981). Soluble oil emulsions consist of two phases, *viz.* an aqueous and an oil phase. The aqueous phase is the major phase comprising between 85-99% of the total volume of the emulsion (Genner and Hill, 1981). This percentage is dependent on the nature of the emulsion and the work that it is required to do (Ullbricht, 1994. pers. comm.[1]). The other 15-1% comprises a complex mixture of oils and various additives, including: the emulsifiers (which hold the oil in suspension) (Ellis *et al.*, 1957), mineral waxes, fatty oils, anti-rust agents, coupling agents, anti-foam agents, dyes, extreme pressure additives, synthetic and phosphate esters, amines and biocides (Sabina and Pivnick, 1956; Genner and Hill, 1981; Passman, 1988) as well as a wide array of inorganic anions (sulphates, chloride and phosphates) and cations (calcium, sodium, magnesium, manganese and iron) (Passman, 1988).

Soluble oils are supplied to the user as an oil concentrate (Genner and Hill, 1981). The oil concentrate is added to water and introduced to the system in which it is required. The oil phase is comprised of droplets of oil a few microns in diameter, and the water-oil interface is therefore very large. This large interfacial area is ideal for microbial growth (Genner and Hill, 1981).

## 1.3 Microflora and biodeterioration of soluble oil emulsions

Petroleum products by nature are very diverse. Since the introduction of soluble oil emulsions early this century, their microbial contamination and biodeterioration have been a serious problems (Leder and Russo, 1989). In 1946, Zobell was the first to describe the action of microbes on hydrocarbons (Atlas, 1981). However, the study of soluble oil emulsion microflora had already been initiated in the early 1940's by numerous investigators who had discovered that the breakdown of soluble oil emulsions was related in all instances to increases in microbial numbers (Fabian and Pivnick, 1953). These early investigations laid the ground work for further

[1]Mr. C. Ullbricht, 1994. Hulett Aluminium hot roll mill plant manager, Hulett Aluminium, Camps Drift, Pietermaritzburg.

and more complex studies on the micro-environment of emulsions and the bacteria and fungi which can grow in them. Although present-day emulsions are much more complex than those used in the 1940's, most of the species of bacteria and fungi identified fifty years ago can be found in emulsions today.

In the 1940's and 1950's a host of scientific reports and reviews appeared describing and discussing the microflora in soluble oil emulsions. In these early studies, emphasis was placed on bacterial and fungal identification rather than on control of the microorganisms.

### 1.3.1 Aerobic organisms

Over the years, numerous species of bacteria have been identified in emulsion systems. Early work by Lee and Chandler (1941) and Duffett *et al.*(1943) showed species of *Pseudomonas, Escherichia, Aerobacter* and *Bacillus* to be predominant in emulsion systems. These authors' findings have been confirmed in numerous studies over the past 50 years (Fabian and Pivnick, 1953, Bennett and Wheeler, 1954, Guynes and Bennett, 1959, Genner and Hill, 1981 and Rossmore, 1995).

Pivnick and Fabian (1954) undertook an in-depth study of the enteric coliform group of bacteria and their survival in emulsion fluids. They showed these microbes to exist only in very small numbers, and that the pseudomonads were antagonistic towards members of the Enterobacteriaceae. The identification of bacteria growing in emulsions should, therefore, not be limited to obligate aerobes only, but should include a wide range of organisms, including the coliforms. Pivnick and Fabian (1954) also hinted at the health implications that might be associated with the presence of these coliform bacteria in oil emulsions. This work was followed by Tant and Bennett's (1956) study of the 'pathogenic' bacteria of oil emulsion systems. Although these authors' work was limited in that they did not show the relative proportions of pathogenic bacteria to non-pathogenic bacteria in the more than 100 mill emulsions they investigated, they, nevertheless, found a wide range of pathogenic bacteria. They also tested emulsions from different locations within the work place thereby maximizing the likelihood of finding different microorganisms. They were also the first to suggest that growth rates of the

many bacteria occurring in emulsions should be considered when identification was necessary, since in many earlier studies, the delay between initial sampling and subsequent isolation and identification was too long. During this period the more vigorous species of bacteria, such as the pseudomonads, outgrew and inhibited less vigorous organisms and because of this many authors had failed to identify some of the bacteria present. Thus misleading reports identifying only specific groups of organisms were published. It is therefore imperative that sampling, isolation and identification be carried out in the shortest time possible.

Reports of fungal and bacterial infections of humans arising from contact with emulsions are few and far between. Rossmore (1993, 1995), arguably the world's leading microbiologist currently working on the biodeterioration of metalworking fluids, cites a few cases of Legionnaire's disease which were ascribed to human contact with contaminated emulsions. In these cases the emulsion was in a severe state of decay and bacterial numbers were extremely high. Rossmore (1993, 1995) also states that the biocides used in emulsions are more likely the cause of human diseases, especially allergic contact dermatitis, than are the bacterial and fungal contaminants in the emulsion. Huletts Aluminium have potentially similar problems with their cooling towers (unrelated to the hot roll mill) and expensive testing kits have been purchased for the identification of the Legionnaire's bacterium, *Legionella feelii*. With respect to the hot roll mill emulsion, workers have complained of burning eyes when high concentrations of the specific biocide Tris Nitro, with the active component formaldehyde (Angus Chemical Company, 1993), have been used (Hemingway, 1994. pers. comm.[2]).

Bennett and Wheeler (1954) studied the survival of bacteria in cutting fluids and identified the need for biocides which target specific groups of organisms. They found that a series of 'test organisms' could be used to screen potential inhibitors of microbial growth in these fluids. These organisms, namely, *Pseudomonas aeruginosa, Pseudomonas fluorescens, Proteus vulgaris* and *Aerobacter aerogenes* are still isolated regularly from modern day emulsions (Holtzman *et al.*, 1982 and Passman, 1988). The use of these 'test organisms' would provide a useful marker for the assessment of the condition of an emulsion at any time. Such assessments of the numbers of

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[2]Mrs. M. J. Hemingway, 1995. Hulett Aluminium hot roll mill Research and Development Department, Hulett Aluminium, Camps Drift, Pietermaritzburg.

organisms present, as well as studies aimed at calculating the maximum bacterial concentrations at which an emulsion can maintain efficacy before breaking, would provide an effective detection method. As the pseudomonads are the most vigorous bacterial species occurring in emulsions (Bennett and Wheeler, 1954, Sabina and Pivnick, 1956, Ellis *et al.*, 1957, Holtzman *et al.*, 1982 and Passman, 1988), they are prime candidates to serve as bacteriological indicators of the condition of an emulsion.

Sabina and Pivnick (1956) studied the oxidation of soluble oil emulsions by species of *Pseudomonas*. These authors showed that not only could the species studied (*Ps. oleovorans* and *Ps. formicans*) degrade the oil component of the emulsion, but that they could also degrade the emulsifier holding the oil in suspension. Although their work was limited in that they only studied short term biodeterioration of oil components, using only two organisms, they nevertheless showed the inherent susceptibility of some of the major constituents of an emulsion to microbial attack.

The work of Sabina and Pivnick (1956) was followed by numerous studies on the effects of bacteria on the oxidizable components of oil emulsions. Ellis *et al.* (1957) showed the effects of both pure and, more importantly, mixed cultures of bacteria on the components of a soluble oil. Mixed cultures were shown to have a greater effect on the oil components as together the microbes could break down more of these constituents. Bennett (1962) pointed out that various strains of the same bacterial species differed in their sensitivity to the inhibitors incorporated into emulsion oils. He showed that different strains of *Ps. aeruginosa* displayed a six-fold difference in their sensitivities to various phenolic compounds found in emulsions. This complicates matters as pure cultures employed in emulsion degradation experiments should undergo strain identification so that the results obtained are meaningful.

Mixed cultures are far more representative of the microbial population of any emulsion system at any given time than are pure cultures, as emulsions comprise a complex mix of numerous chemicals. The study of emulsion dynamics with respect to microbial biodeterioration should therefore include both pure and mixed populations of bacteria and fungi. Ellis *et al.* (1957) showed that various species of bacteria degrade individual components of oil emulsions. They

also speculated on the importance of the use of mixed cultures for experimental purposes, stating that the process of biodeterioration of an emulsion is complex and that various species, in whatever ratio they exist, may play very important roles in the overall process. Although a particular microorganism may metabolize only a single component of an emulsion, the breakdown products thereof may interact with other components and microorganisms. A complex interaction between emulsion components, microorganisms and their breakdown products can thus develop. Bennett (1962) showed conclusively that aerobic bacteria were far more susceptible to microbial inhibitors present in emulsions if there were no anaerobic bacteria present. Using *Pseudomonas aeruginosa* and *Desulfovibrio desulfuricans*, he demonstrated that the former was up to five times more sensitive to the effects of inhibitors in the absence of *D. desulfuricans* than it was when this bacterium was present. He also showed that the presence of dead bacterial cells increased the concentration of inhibitors required to control aerobic organisms.

Also of importance is fungal contamination of emulsions. Rossmore and Holtzman (1974) studied the growth of fungi and yeasts in cutting fluids. They identified species of *Fusarium* and *Cephalosporium* in these emulsions and showed that most biocides were bactericidal in nature, and that fungi and yeasts were not necessarily controlled. Hence it is important to formulate biocides with both bactericidal and fungicidal activity.

The following effects have been associated with the biodeterioration of emulsions:

- **Breaking of the emulsion (spoilage) resulting from a lowered pH.** A reduction in pH of the emulsion is sometimes brought about by the accumulation of bacterial and fungal waste products. These waste products include: carbon dioxide (which combines with the water phase of the emulsion to form carbonic acid) and fatty acids produced as a by-product of hydrocarbon oxidation (Ellis *et al.*, 1957). With the lowering of the pH, the emulsifier deteriorates and the emulsion 'loosens', thereby separating into its two phases. 'Loosening' of the emulsion at the

Hulett's Aluminium roll mill has been associated with a lowering of the pH (Hemingway, 1994. pers. comm.[3]).

• **Breaking of the emulsion without lowered pH.** Although this form of biodeterioration is reported less frequently, degradation of critical components of an emulsion (namely the emulsifiers), which may be present in very small amounts, would result in the splitting of the emulsion without a detectable drop in pH (Ellis *et al.*, 1957).

• **Production of foul odours with or without hydrogen sulphide production.** The production of sulphides is associated with sulphate-reducing bacteria (SRB) which metabolize sulphates to sulphides, which then form hydrogen sulphide (Postgate, 1951b). However, sulphides may also arise from microbial decomposition of proteinaceous material which may be present in the emulsion (Ellis *et al.* 1957). 'Rotten egg' smells have been observed in the basement of the Hulett Aluminium hot roll mill (Ullbricht, 1994. pers. comm.[4]). Enumeration of anaerobic bacteria in the sump areas of the mill might therefore be significant.

• **Reduction in the qualities of an oil emulsion and subsequent emulsion instability.** This generally results from direct emulsifier degradation arising from microbial attack (Bennett and Wheeler, 1954). With the degradation of the emulsifier, the oil phase and water phase separate, and both lubrication and cooling properties are adversely affected. This separation can result in either the aluminium ingot being 'picked-up' (a process by which the heated ingot sticks to the steel rollers) or the ingot 'slips-through' the rollers, both of which leave blemishes and non-uniform finishes on the rolled aluminium (Genner and Hill, 1981) thereby reducing the quality of the finished product.

• **Corrosion of metallic surfaces.** With the growth of anaerobic sulphate-reducing bacteria in the emulsion system, sulphates are reduced to sulphides which then react with ferric ions,

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[3]Mrs. M. J. Hemingway, 1995. Hulett Aluminium hot roll mill Research and Development Department, Hulett Aluminium, Camps Drift, Pietermaritzburg.

[4]Mr. C. Ullbricht, 1994. Hulett Aluminium hot roll mill plant manager, Hulett Aluminium, Camps Drift, Pietermaritzburg.

derived from the mild steel piping through which the emulsion is pumped, to produce black precipitates of corrosive iron sulphide (Bennett and Wheeler, 1954; Guynes and Bennett, 1959) which leads to leaks, holes and breakages in the system (Genner and Hill, 1981).

• **In severe cases, worker related health problems, especially skin irritations and dermatitis.** Although only a few instances of worker-related health problems have been reported, it is likely that many cases go unreported (Rossmore 1993, 1995). Most of the health problems can be traced back to the biocides which are used for microbial control (Rossmore, 1995). Biocides should, therefore, be thoroughly investigated before they are introduced into the work environment.

• **Product surface blemishes.** Surface blemishes occur when biofilms, which may be growing in sump tanks, pipes, filters etc., become partly dislodged. This may be due to either the flow of the emulsion over a biofilm, or a change in environmental conditions such as biocide application, which may result in the death of all or part of the biofilm. These free-floating biofilms are sprayed onto the aluminium ingot whilst the ingot is being passed through the rollers. Blemishes on the aluminium ingot occur where the biofilms are squashed into the surface of the aluminium during the rolling process. Such blemishes reduce the overall quality of the rolled aluminium (Genner and Hill, 1981). These blemishes have been know to occur during the rolling process at Hulett Aluminium (Ullbricht, 1994. pers. comm.[5]).

• **Accumulation of microbial slimes which can cause clogging of filters, screens and lines.** The clogging of filters, screens and delivery lines is indicative of biofilm development. This type of clogging does not occur often as massive numbers of bacteria and fungi are required in order to produce large biofilms (Passman, 1988). This has not been reported at the Hulett Aluminium mill (Ullbricht, 1994. pers. comm.[5]).

• **Extensive down-time periods.** With severe contaminations, extensive down-time periods may be needed to open sump tanks, clean filters and unclog pipes (Holtzman *et al.,* 1982). This

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[5]Mr. C. Ullbricht, 1994. Hulett Aluminium hot roll mill plant manager, Hulett Aluminium, Camps Drift, Pietermaritzburg.

occurred at Hulett Aluminium in early 1994 when microbial numbers became excessive as the biocides then in use were not very effective (Hemingway, 1994. pers. comm.[6]). Piping was steam cleaned and biofilm slimes were scraped off drainage canals and sumps. This necessitated a 48hr downtime period with considerable loss of production.

### 1.3.2 Anaerobic organisms

With the discovery of anaerobic organisms in oil-in-water emulsions, Bennett (1957) (cited by Guynes and Bennett [1959]) demonstrated the role of sulphate-reducing bacteria in the degradation of cutting fluids. Sulphate reducers are capable of reducing sulphates, sulphites and thiosulphates by using them as electron acceptors (Postgate, 1951a, 1951b). Hydrogen is required in order to reduce these compounds (Stanier *et al.*, 1987). Hydrogen may, however, be substituted with ferric ions. These ferric ions then combine with the sulphates to form ferrous sulphide. The ferric ions are microbially extracted from the mild steel piping of which all roll mill emulsion reticulation systems are constructed (Ullbricht, 1994. pers. comm.[6]). With the microbial uptake of these ions, corrosion of the piping occurs. With the production of sulphurous compounds, the pH is lowered and further corrosion can occur (Guynes and Bennett, 1959). Anaerobic bacteria have long been associated with the production of hydrogen sulphide and a lowering of the pH of emulsions. The primary anaerobic organism responsible for the biodeterioration of oil-in-water emulsions is *Desulfovibrio desulfuricans* (Guynes and Bennett, 1959). Guynes and Bennett (1959) showed that sulphate-reducing organisms are dependent upon the interaction of aerobes with the emulsion and are unable to reduce emulsion components in the absence of aerobes. Isenberg and Bennett (1959) studied the interaction of anaerobic sulphate reducers with aerobic organisms. They found that aerobic organisms oxidized emulsion components that were initially inhibitory to sulphate-reducing organisms. Following oxidation, these components became less inhibitory, thus permitting growth of the anaerobic organisms. Isenberg and Bennett (1959) also showed that the aerobes produced various compounds needed by the sulphate reducers for growth and that they lowered the redox potential, thereby making conditions more suitable for anaerobic growth.

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[6]Mrs. M. J. Hemingway, 1995. Hulett Aluminium hot roll mill Research and Development department, Hulett Aluminium, Camps Drift, Pietermaritzburg.

## 1.4 Factors affecting microbial growth in soluble oil emulsions

Survival and growth of microbes in aluminium roll mill systems are subject to a number of physical and chemical factors. These include: oil-water ratio; aeration; pH; temperature; diluent (water) hardness or softness (Holtzman *et al.*, 1982) and the presence of biocides (see 1.5).

• **Oil:Water ratio** is the ratio at which the oil concentrate (from the manufacturer) is applied to water in order to obtain a suitable emulsion. This ratio may vary markedly depending on the oil concentrate, and the physical and chemical constraints placed on the emulsion during operation. Fabian and Pivnick (1953) showed the effects of bacteria on emulsions with different oil:water ratios. They tested five emulsions with concentrations of oil ranging from 1% to 10%. It was shown that bacterial generation times decreased with an increase in the concentration of oil. Although the results of these investigations were relatively rudimentary since not enough different concentrations were tested with different cultures of microbes, the results were, nevertheless, significant in that the ratio of oil to water was shown to correlate with the bioresistance capacity of the emulsion to the microorganisms. Generally emulsions with oil to water ratios between 1:50 (2%) and 1:25 (4%) are most readily degraded by microorganisms (Bennett, 1962). With more oil present, ie. at ratios of 1:5 (20%) oil:water, inhibitory compounds (such as phenols) in the emulsion reduce microbial growth significantly (Bennett, 1962). The working concentration of Prosol, the emulsion used at the Hulett Aluminium roll mill, is of the order of 3-4% which is ideal for microbial biodeterioration.

• **Aeration** takes place via various spray nozzles which are directed at the heated aluminium ingot and the steel rollers. Further aeration may occur in emulsion holding tanks where the emulsion is constantly circulated to ensure that it remains 'tight'. Anaerobic regions may develop in return-to-sump pipes or sump tanks where circulation is reduced or absent. It is of prime importance that potentially anaerobic areas be avoided in the design of any future mills, and that these areas be reduced or eliminated in existing facilities.

• **pH** is a measure of the relative numbers of hydrogen ions present in a solution. Most emulsions have an optimal pH between 8.0 and 8.5 but this can vary from 7.5-9.5 (Genner and

Hill, 1981) depending on the chemical composition of the concentrate and the water supply. The pH of the Hulett Aluminium water supply ranges from 7.5-8.0 which is suitable for microbial, particularly bacterial, activity.

• **Operational temperature** is dependent on the nature of the rolling process and the individual roll mill. Aluminium rolling requires higher temperatures (50-60°C) than does steel rolling (40°C) (Genner and Hill, 1981). Usually the temperature of the emulsion varies depending on its location in the roll mill reticulation system. The total volume of emulsion in the system also affects the temperatures attained. Because of the huge volume of liquid in the storage tanks, emulsion heaters are required to maintain the temperatures needed for rolling. In general the temperature of the emulsion will fluctuate markedly within a roll mill system. Temperature gradients may vary from 60°C in the main emulsion holding tanks, to 40°C in the sump tanks, or the emulsion may be at ambient temperature during down-time periods. The potential for both mesophilic (20-45°C) and thermophilic (>45°C) growth is obvious.

• **Diluent hardness/softness** is a measure of the amount of inorganic salts in the water supply. Bennett (1962) and Holtzman *et al.* (1982) showed that the presence of inorganic salts stimulates the growth of aerobic organisms. Bennett (1962) showed that various mills around America were using the same emulsion under exacting conditions, yet some of the mills were experiencing serious biodeterioration problems whereas others were not. After a study of the water supplies to the various mills it was shown that, in some cases, the presence of inorganic salts doubled and trebled the growth rate of certain organisms. The quality of water used to dilute emulsion concentrates is therefore critical in extending the life of the emulsions, as potential emulsion degrading microorganisms can be starved of important minerals if deionized water is used. Bennett (1962) also showed that the presence of inorganic salts may reduce the effectiveness of certain biocides. The Pietermaritzburg roll mill is equipped with a deionizer/demineralizer which should remove some of the salts from the water entering the emulsion reticulation system. However, the reliability of the deionizer/demineralizer is questionable, and during the research phase of this project it was due for repair.

## 1.5 Biocides compatible with soluble oil emulsions

The use of biocides as a protective measure to reduce the rate of biodeterioration of emulsions has been practised for over 50 years (eg. Lee and Chandler, 1941). Before the use of biocides, soluble oil emulsions were discarded after microbial degradation had occurred. However, with the advent of larger and larger roll mills it became a costly and wasteful practice to discard degraded emulsions. Not only was it expensive to replace the lost emulsion but the cost of disposing of the waste became exorbitant. The formulation of new biocides is a complex and expensive process, yet it is necessary for the continued operation of the aluminium rolling industry.

An ideal biocide shows the following characteristics: it is effective against all forms of microorganisms which are present in the emulsion system; it is cost effective; it is non-toxic (at the recommended dosage levels) to the operators of the roll mill; it is non-corrosive; it is compatible with the emulsion (Heinrichs and Rossmore, 1971) and it must be easily quantified (Pivnick and Fabian, 1953). As yet no biocide shows all these characteristics. It is often a trade-off between effectiveness and cost efficiency, as to which biocide is used in a particular system.

Initially biocides were added to the oil concentrate which was shipped and then mixed with water to form the emulsion at the roll mill. This form of biocide addition is far from perfect as the oil concentrates are often left to stand for long periods of time, resulting in a decrease in the potency of the biocide (Genner and Hill, 1981). A far more effective method of biocide use is the addition of the biocide at the site of use, after dilution of the oil concentrate. In this manner the biocide can be purchased immediately prior to use and introduced into the emulsion. The biocide is therefore not left standing for long periods of time. It is also easier to manage the application of the biocide as additions can be made according to the specific needs of the plant.

The types of biocides used in the control of emulsion microflora vary markedly. They include isothiazolones (Leder and Russo, 1989; Law and Lashen, 1991; Heenan and Burrell, 1992), phenols (Rossmore, 1993), formaldehyde (Angus Chemical Company, 1993; Rossmore, 1993), glutaraldehyde and alkylamine condensates (Heenan and Burrell, 1991). Two of these biocides,

namely, formaldehyde and glutaraldehyde, have been used at the Hulett Aluminium roll mill. The mode of action of these biocides will not be discussed here but is well described in the literature (refer to Holtzman and Rossmore, 1976 and Munton and Russell, 1973 respectively).

### 1.6 Monitoring the biodeterioration of soluble oil emulsions

Aluminium roll mills are subject to more rigorous chemical, physical and biological monitoring than any other emulsion utilizing systems (Genner and Hill, 1981). This is because they often provide near ideal conditions for microbial growth. Both chemical and physical monitoring procedures are employed (Passman, 1988). Gas chromatography (Geiger *et al.*, 1983) and other techniques which assess the state of the oils within the emulsion, as well as the emulsifier's capacity to maintain the oil in suspension, are commonly employed. The chemical and biological monitoring of an emulsion may be quite varied depending on the types of information required. Passman (1988) has summarized the chemical and biological processes which are monitored. These include: the production of biofilm; the production of various organic acids; oxygen consumption; organic compound metabolism; emulsifier/demulsifier production; and microbial growth and proliferation. A wide range of tests and methodologies are needed for an in-depth study of these chemical and biological processes, and these are summarised below:

- **Production of biofilm:** measurements include sizes of surface scrapings and dry weight tests (Passman, 1988).

- **Organic acid production:** measurements include pH/alkalinity tests (Passman, 1988).

- **Oxygen consumption:** measured by means of oxygen volumetric respiration tests (Sabina and Pivnick, 1956; Ellis *et al.*, 1957; Geiger *et al.*, 1983).

- **Organic compound metabolism:** measured by gas chromatography (Holtzman *et al.*, 1982; Geiger *et al.*, 1983), high performance liquid chromatography, mass spectrometry (Holtzman *et al.*, 1982) and various radiotracer methods (Passman, 1988).

• **Emulsifier/demulsifier production:** measured using emulsion stability tests (Holtzman *et al.*, 1982).

• **Microbial growth and proliferation:** measurements of growth are conducted on/in specific substrates via viable cell counts (Bennett and Wheeler, 1954; Fabian and Pivnick, 1953) and chemical assays of growth-related processes such as ATP and enzyme activity (Firko and Burrell, 1991).

### 1.7 Aims and Objectives

Hulett Aluminium spends, on average, about R350 000 per year on biocides to treat their hot roll mill emulsion (Pitchford, 1995. pers. comm.[7]). This excludes the following costs: removal of the emulsion by either partial or full dumping depending on the condition of the emulsion; the down-time costs whilst the emulsion is being replaced; the cost of the treatment of the biodeteriorated waste emulsion; the cost of testing for microbial activity and biocide levels; the damage that microorganisms can cause to the roll system (which includes the replacement of pipes due to either biocidal or microbiological corrosion); and the reduction in quality of the rolled aluminium, either as a direct result of microbiological flocs being compressed into the aluminium sheeting, or indirectly arising from the biodeterioration of the emulsion thereby causing slipping and sticking of the aluminium sheeting during rolling.

In previous studies of the Hulett Aluminium roll mill system the following work has been conducted: microbial degradation of different oil emulsions (Snell, 1992; Sundram, 1993); electron microscopy of microbial surface attachment in the oil emulsion reticulation system (Naidoo, 1991; Snell, 1992); partial identification of the microflora present (Naidoo, 1991; Sundram, 1993); the effect of temperature on microbial growth and emulsion breakdown rates (Sundram, 1993); and biocide trials conducted in batch cultures (Naidoo, 1991).

The present study was undertaken to investigate more thoroughly the microflora of the hot roll

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[7]Dr. A. Pitchford, 1995. Hulett Aluminium hot roll mill Research and Development department, Hulett Aluminium, Camps Drift, Pietermaritzburg.

mill oil-water emulsion system. The following **aims and objectives** were identified:

To **isolate and identify** the microflora of the Hulett Aluminium hot roll mill emulsion. To elucidate the patterns of distribution and to identify the various groups of organisms which inhabit the emulsion reticulation system. Isolation and identification will be carried out using various techniques including a range of selective media, physiological tests and light microscopy. Following these distribution and characterization studies, so called 'target organisms' will be used for further studies.

To **identify the susceptible components of the emulsion.** The 'target organisms' will be used to assess their ability to degrade various components of the roll mill emulsion. Following identification of the biosusceptible components of the emulsion, the 'weakest-link' in the emulsion will be identified and modifications of component ratios will be made with the aim of reducing the biosusceptibility of the entire emulsion.

To **develop and construct a laboratory-scale model** which will simulate the emulsion storage and reticulation system of the Hulett Aluminium hot roll mill. This will enable emulsion breakdown and biocide studies to be carried out in the laboratory which should permit the formulation of recommendations that can be extrapolated to large-scale mill implementation at a later stage.

To **conduct a series of biocide experiments** in the model system in order to develop more effective biocide management practices for implementation at the mill and to determine the potential of the emulsion-inhabiting microbial population to develop resistance to the biocide currently being used at the plant.

To **conduct a series of experiments on other emulsions** using the model system in order to identify more suitable alternatives to the standard emulsion presently being used.

To **suggest possible design improvements** (from a microbiological standpoint), for the new hot roll mill envisaged for the plant. The future hot roll mill should provide a less hospitable

environment for the microflora in order for biocide levels to be reduced without affecting emulsion susceptibility. Suggestions regarding the design of the new mill will be proposed, based on experimental data obtained from the model system.

To **develop an overall management strategy** including not only biocide management but also worker hygiene, plant hygiene and operating protocols. Assessment of the current management strategy will be given and on the basis of experimental data obtained from the model system, potential improvements will be suggested.

# CHAPTER 2. IDENTIFICATION OF MICROFLORA

## 2.1 Introduction

Numerous works in the past (Bennett and Wheeler, 1954; Duffett *et al.*, 1943; Fabian and Pivnick, 1953; Lee and Chandler, 1941; Pivnick and Fabian, 1954; Pivnick, 1955; Tant and Bennett, 1956) were aimed at identifying the microflora in soluble oil emulsions. Most of the organisms identified in these early studies are still isolated from emulsions today, even though many of the compounds used in emulsion formulations have changed markedly.

The identification of the microorganisms present in the Hulett Aluminium hot roll mill emulsion (Prosol) served a two-fold purpose. Firstly, it aided our understanding not only of the microorganisms specific to the Hulett aluminium hot roll mill but also of those that occur in other roll mills around the world. Secondly, it facilitated the development of an effective management practice for the efficient use of the emulsion in the operation of the roll mill.

In order to identify a bacterium or a fungus, it must first be grown in pure culture. To obtain such cultures of any organism, two isolation procedures can be followed. The first involves the use of a selective medium. A sample containing assorted organisms is inoculated onto a medium which permits or favours the growth of only a specific organism. The required organism subsequently 'outgrows' the other organisms, or the other organisms may be killed by the selective medium, depending on the composition of the medium and the conditions under which the organisms are cultured. These selective media may be so specific that isolation and identification of an organism can occur in one step. The second method by which a pure culture can be obtained involves the streaking of a sample containing an array of numerous organisms onto a non-selective or partially selective medium. Inoculum taken from the individual colonies which develop is then reapplied to fresh medium, and by doing this repeatedly, pure cultures are eventually obtained (Krueger and Johansson, 1961).

Following the successful isolation of an organism, in pure culture, identification can be carried out. The following are characteristics which are used in the standard identification of a

microorganism: its staining properties; its morphological (both microscopic and macroscopic) characteristics; and the physiological and biochemical traits the organism exhibits (Krueger and Johansson, 1961).

A study of the literature revealed that the following microbes are common contaminants of most emulsions: among the bacteria are species of *Pseudomonas* (Lee and Chandler, 1941 and Duffett *et al.*, 1943), *Bacillus* (Bennett and Wheeler, 1954), *Aerobacter* (Bennett and Wheeler, 1954), *Escherichia* (Pivnick and Fabian, 1954), *Proteus* (Bennett and Wheeler, 1954), whilst amongst the fungi *Fusarium* and *Cephalosporium* (Rossmore and Holtzman, 1974) have been frequently isolated.

## 2.2 Materials and Methods

### 2.2.1 Sampling sites

To improve the chances of isolating as many of the fungi and bacteria found in the roll mill emulsion as possible, various sampling sites, representative of the different ecological niches present in the mill, were identified. These sampling sites were located in areas considered to be potentially high or low in microbial activity. This was in line with Tant and Bennett's work (1956) in which they isolated microorganisms from all parts of the mill they investigated in order to maximize the chances of finding as many different bacteria as possible.

The sampling sites ultimately used were selected only after assessing the plant and plotting a map of the entire mill and the emulsion reticulation system. These sampling sites are shown in Figure 2 and are described below.

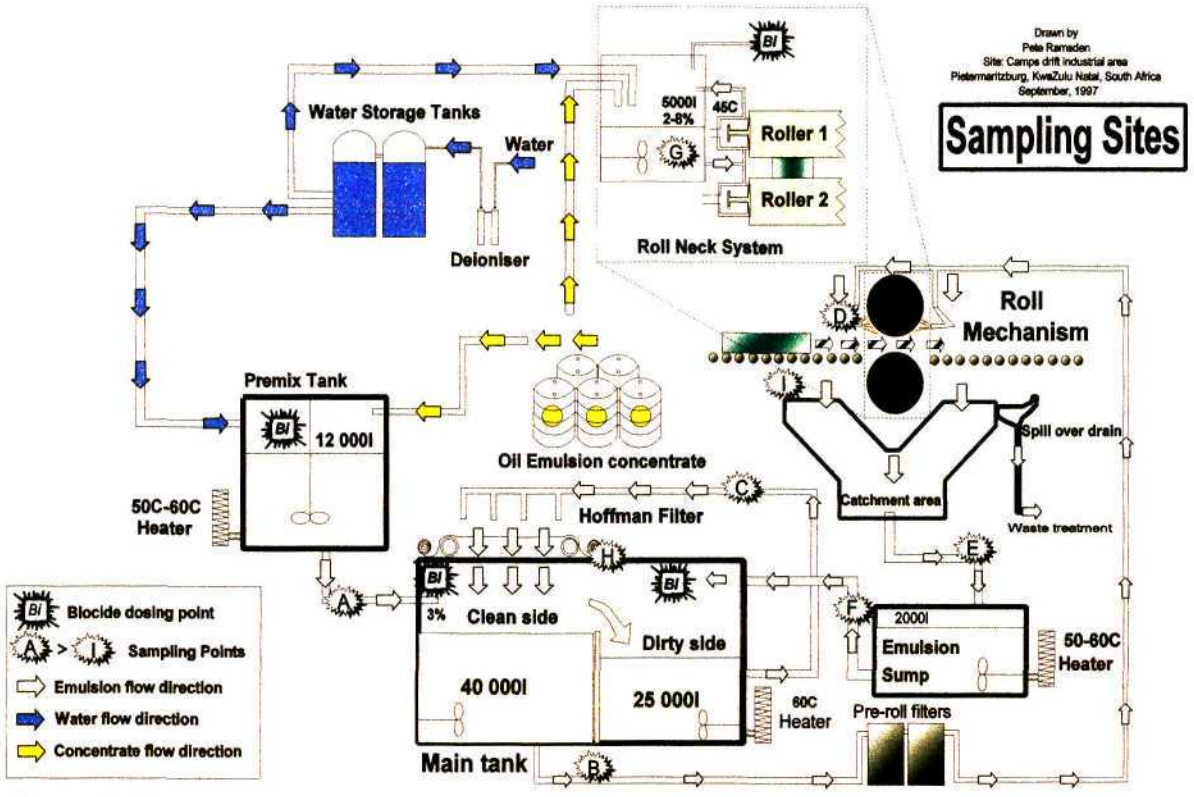


**Figure 2: Hulett Aluminium hot roll mill - Sampling Sites for microorganisms**

•**A.** Premix tank feed to the clean side of the main emulsion tank: selected to assess how early in the system contamination can occur.

•**B.** Out-flow pipe from the base of the clean side of the main emulsion tank: selected to assess the degree of microbial contamination coming out of the clean side of the main emulsion storage tank.

•**C.** Out-flow pipe from the dirty side of the main emulsion tank: selected to assess the degree of contamination coming out of the dirty side of the main emulsion storage tank.

•**D.** Spray point of the emulsion onto the steel rollers and aluminium ingot: selected to assess the degree of contamination being sprayed onto the rollers and heated aluminium ingot.

•**E.** Return-to-sump pipe: selected to assess the degree of contamination after the emulsion has trickled back into the emulsion reticulation system following the rolling process.

•**F.** Out-flow pipe from emulsion sump: selected to assess the level of contamination within the emulsion sump.

•**G.** Roll neck emulsion tank (independent of main emulsion system and kept at 37-45$^{o}$C for optimal efficiency): selected to assess contamination level in the roll neck mechanism and also to determine whether this is a potential source of inoculum.

•**H.** Hoffman filter: selected to assess level of microbial contamination passing into the clean side of the main tank.

•**I.** Bucket of Sludge taken from beneath the conveyer system which moves the aluminium ingot back and forth through the roll mechanism: selected to assess the contamination level of emulsion which runs off the ingot during the roll process, but which does not flow down into the emulsion sump tank.

•***Bi.*** Biocide injection sites.

Initially three samples were taken at five minute intervals from each of the above sites for microbial enumeration and identification purposes.

A total system cleanout was performed just after the initial samples were taken. This process included steam cleaning the sump tank, scraping aluminium deposits from the pipes, blasting biofilms with high pressure water, and running high concentrations of the biocide Busan (active component, glutaraldehyde) through the system. To assess the effectiveness of this system cleanout, three more samples from each of the sampling sites were taken 23 days after the initial samples had been collected. The tests described in Sections 2.2.2 and 2.2.3 were carried out on samples taken (from the hot roll mill) on both occasions.

### 2.2.2 Aerobic organisms

The isolation procedure used is diagrammatically summarized in Figure 3.

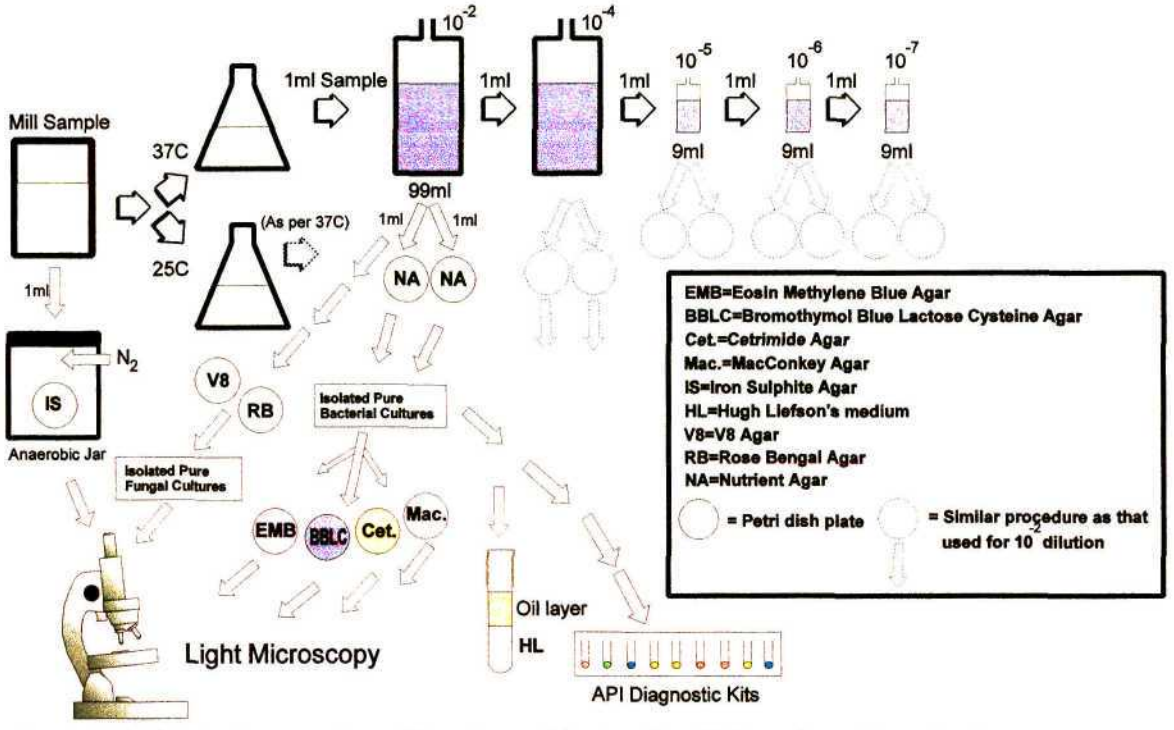


Figure 3: Isolation and purification of hot roll mill fungi and bacteria

In order to quantify accurately the microorganisms in each sample, their numbers had to be decreased significantly. To achieve this a dilution series was set-up. Ringers tablets were added to tap water to produce Ringers solution (an osmotically balanced solution). This technique is in line with that used by Fabian and Pivnick (1953), however, phosphate buffers were not included as the microorganisms would be exposed to the diluent for a short period of time only and, therefore, any pH drop arising from the production of organic acids would not be significant. A dilution series comprising $10^{-2}$, $10^{-4}$, $10^{-5}$, $10^{-6}$ and $10^{-7}$ dilutions was created by placing 99ml, 99ml, 9ml, 9ml and 9ml respectively of the Ringers solution into autoclave resistant bottles with screw cap lids. The bottles were subsequently autoclaved at 121°C for 15 minutes. The bottles were allowed to cool to room temperature on a laminar flow bench. Each mill sample was divided into two equal portions. Both portions were shaken for 2 hrs, one at 25°C and the other at 37°C. After this period, 1ml from each of the samples was aseptically transferred into the first dilution ($10^{-2}$) and passed down the dilution series with vigorous

shaking of each suspension before transfer.

For isolation of bacterial contaminants a one milliliter sample from each dilution was aseptically spread over nutrient agar plates. Three plates were inoculated for each dilution. The plates were incubated at 37°C and 25°C (two of the replicates at 37°C and one at 25°C) for 24 to 48hrs. After incubation the number of colonies on each plate were determined with the aid of a colony counter (WTW Kolnienzählgerät). These values were then used to calculate the numbers of colony forming units per milliliter of sample.

To identify specific species, individual colonies were transferred from the nutrient agar plates to Petri dishes or tubes containing the following media: (see Appendix A for chemical compositions): Eosin Methylene Blue Agar (EMB Agar); Bromothymol Blue Lactose Cysteine Agar (BBLC Agar); Cetrimide Agar (Cet. Agar); MacConkey Agar (Mac. Agar); Iron Sulphite Agar (IS Agar) and Hugh Liefson's Medium (HL Medium).

Cells from each of these plates were transferred to duplicate fresh EMB, BBLC, Cet. and Mac. agar plates, which were then incubated at 25°C and 37°C. For each isolate the source within the roll mill was noted. Five freeze-dried replicates of each selected isolate were made at Allerton Veterinary Laboratory, Pietermaritzburg. These freeze-dried cultures were stored in a dark cupboard at room temperature until required.

Fungi were isolated by plating one milliliter aliquots from each dilution onto Rose Bengal agar (RB agar) and Modified V8 agar (M-V8 agar) [refer to Appendix A].

Identification of the bacteria was based on colony shape and colour, growth patterns and cell morphology. In addition API 20E diagnostic kits were used to identify members of the family, Enterobacteriaceae as well as other Gram negative rods. Gram stains of each culture were made after 24 and 48 hours growth on nutrient agar using Hucker's modification of the procedure (refer to appendix B). The stained cultures were viewed using a Zeiss Axiophot light microscope. The Gram stain reaction was verified using Gregersen's (3% potassium hydroxide) test (refer to appendix B). Identification of the fungal isolates, based on fungal spore bearing

structures as well as mycelial morphology, was carried out with the aid of a Zeiss Axiophot light microscope.

### 2.2.3 Anaerobic organisms

Anaerobic samples were taken from each of the sampling sites using vacuum-sealed test tubes fitted with rubber seals. One milliliter from each sample was transferred to 90mm Petri dishes and covered with pre-autoclaved, warm (42°C) Iron Sulphite agar (IS agar). The inoculated plates were placed in an air-tight anaerobic jar which was flushed twice daily with nitrogen gas. The anaerobic jar was incubated at 37°C and the plates examined twice daily for signs of growth.

Slide preparations of colonies taken from the anaerobic cultures grown on IS agar were viewed using a Zeiss Axiophot light microscope.

## 2.3 Results

### 2.3.1 Sampling Sites

The standard plate count values obtained represent the relative numbers of bacteria and fungi found at the various sampling sites within the roll mill. The following categories are used in Tables 1-6 to denote the level of bacterial and fungal contamination in the emulsion:

TNTC (Too Numerous To Count): denotes plates with microbial numbers in excess of 300 colony forming units per milliliter at any given dilution of emulsion sample.

0: no colony forming units per milliliter at any given dilution of emulsion sample.

#### 2.3.1.1 Aerobic bacterial contamination levels in the hot roll mill prior to the emulsion reticulation system cleanout

Table 1 shows that aerobic bacterial counts were high (in excess of $1x10^6$ $CFUml^{-1}$ of sample)

in all areas of the roll mill except the Hoffman filter (where numbers did not exceed $2x10^4$ $CFUml^{-1}$ of sample) and the clean side of the main emulsion tank and the premix tank, from which no bacteria were isolated.

**Table 1: Levels of aerobic bacterial contamination in the hot roll mill prior to the emulsion reticulation system cleanout**

| | Colony Forming Units per milliliter ($CFUml^{-1}$) emulsion detected on Nutrient Agar | | | | |
|---|---|---|---|---|---|
| Site: | $10^2$ | $10^4$ | $10^5$ | $10^6$ | $10^7$ |
| Premix tank | 0^ | 0 | 0 | 0 | 0 |
| Bucket* | TNTC# | TNTC | TNTC | TNTC | 253 |
| Roll neck | TNTC | TNTC | TNTC | 124 | 14 |
| Hoffman filter | 156 | 2 | 0 | 0 | 0 |
| Return-to-sump pipe | TNTC | TNTC | TNTC | TNTC | 162 |
| Clean side of main tank | 0 | 0 | 0 | 0 | 0 |
| Dirty side of main tank | TNTC | TNTC | 151 | 21 | 2 |
| Sump tank | TNTC | TNTC | 148 | 17 | 1 |
| Sump sludge | TNTC | 265 | 31 | 2 | 0 |

Bucket*: Refers to a bucket of emulsion collected from the catchment area below the ingot conveyer system before system cleanout.

TNTC#: Too numerous to count (refer to 2.3.1 Sampling Sites)

0^: Zero Colony forming units per milliliter of sample (refer to 2.3.1 Sampling Sites)

### 2.3.1.2 Aerobic bacterial contamination levels in the hot roll mill after the emulsion reticulation system cleanout

Aerobic bacterial counts were markedly reduced after system cleanout with contamination occurring only in the roll neck ($4x10^5$ $CFUml^{-1}$ emulsion), return-to-sump pipe ($3x10^4$ $CFUml^{-1}$ emulsion) and sump tank ($1.9x10^3$ $CFUml^{-1}$ emulsion)(Table 2).

**Table 2: Levels of aerobic bacterial contamination in the hot roll mill after the emulsion reticulation system cleanout**

| Site: | Colony Forming Units per milliliter ($CFUml^{-1}$) emulsion detected on Nutrient Agar | | | | |
|---|---|---|---|---|---|
| | $10^3$ | $10^4$ | $10^5$ | $10^6$ | $10^7$ |
| Premix tank | 0^ | 0 | 0 | 0 | 0 |
| Roll neck | TNTC# | 49 | 4 | 0 | 0 |
| Hoffman filter | 0 | 0 | 0 | 0 | 0 |
| Return-to-sump pipe | 179 | 3 | 0 | 0 | 0 |
| Clean side of main tank | 0 | 0 | 0 | 0 | 0 |
| Dirty side of main tank | 0 | 0 | 0 | 0 | 0 |
| Sump tank | 19 | 0 | 0 | 0 | 0 |
| Roll spray | 0 | 0 | 0 | 0 | 0 |

TNTC#: Too numerous to count (refer to 2.3.1 Sampling Sites)

0^: Zero Colony forming units per milliliter of sample (refer to 2.3.1 Sampling Sites)

### 2.3.1.3 Anaerobic bacterial contamination levels in the hot roll mill prior to the emulsion reticulation system cleanout

Highest counts of anaerobic bacteria were obtained from the return-to-sump pipe ($5x10^6 CFUml^{-1}$ emulsion), the bucket ($3x10^5 CFUml^{-1}$ emulsion) and the sump sludge ($6x10^2 CFUml^{-1}$ emulsion)(Table 3). At none of the other sampling sites were anaerobic sulphate-reducing bacteria (SRB) detected.

**Table 3: Levels of anaerobic bacterial contamination in the hot roll mill prior to the emulsion reticulation system cleanout**

| Site: | Colony Forming Units per milliliter ($CFUml^{-1}$) emulsion detected on Iron Sulphite Agar | | | | |
|---|---|---|---|---|---|
| | $10^2$ | $10^4$ | $10^5$ | $10^6$ | $10^7$ |
| Premix tank | 0^ | 0 | 0 | 0 | 0 |
| Bucket | TNTC# | 39 | 3 | 0 | 0 |
| Roll neck | 0 | 0 | 0 | 0 | 0 |
| Hoffman filter | 0 | 0 | 0 | 0 | 0 |
| Return-to-sump pipe | TNTC | TNTC | 41 | 5 | 0 |

Table 3 continued.

| | | | | | |
|---|---|---|---|---|---|
| Clean side of main tank | 0 | 0 | 0 | 0 | 0 |
| Dirty side of main tank | 0 | 0 | 0 | 0 | 0 |
| Sump tank | 0 | 0 | 0 | 0 | 0 |
| Sump sludge | 6 | 0 | 0 | 0 | 0 |

TNTC#: Too numerous to count (refer to 2.3.1 Sampling Sites)

0^: Zero Colony forming units per milliliter of sample (refer to 2.3.1 Sampling Sites)

### 2.3.1.4 Anaerobic bacterial contamination levels in the hot roll mill after the emulsion reticulation system cleanout

No SRB were isolated 23 days after the cleanout of the emulsion reticulation system (Table 4).

**Table 4: Levels of anaerobic bacterial contamination in the hot roll mill after the emulsion reticulation system cleanout**

| | Colony Forming Units per milliliter ($CFUml^{-1}$) emulsion detected on Iron Sulphite Agar | | | | |
|---|---|---|---|---|---|
| Site: | $10^2$ | $10^4$ | $10^5$ | $10^6$ | $10^7$ |
| Premix tank | 0^ | 0 | 0 | 0 | 0 |
| Bucket | 0 | 0 | 0 | 0 | 0 |
| Roll neck | 0 | 0 | 0 | 0 | 0 |
| Hoffman filter | 0 | 0 | 0 | 0 | 0 |
| Return-to-sump pipe | 0 | 0 | 0 | 0 | 0 |
| Clean side of main tank | 0 | 0 | 0 | 0 | 0 |
| Dirty side of main tank | 0 | 0 | 0 | 0 | 0 |
| Sump tank | 0 | 0 | 0 | 0 | 0 |
| Sump sludge | 0 | 0 | 0 | 0 | 0 |

0^: Zero Colony forming units per milliliter of sample (refer to 2.3.1 Sampling Sites)

### 2.3.1.5 Fungal contamination levels in the hot roll mill prior to the emulsion reticulation system cleanout

Table 5 shows the number of fungal colony forming units per milliliter of emulsion sample

(plated onto Rose Bengal agar [RB agar] and modified V8 agar [MV8 agar]). The highest level of fungal contamination of the emulsion reticulation system occurred in the bucket of sludge, the roll neck and return-to-sump pipe. Lowest fungal counts were detected in the dirty side of the main emulsion storage tank, the Hoffman filter and the sump sludge (biofilm). No fungal contaminants were found in the clean side of the main emulsion tank or in the emulsion circulating within the sump tank.

**Table 5: Levels of fungal contamination in the hot roll mill prior to the emulsion reticulation system cleanout**

| | Fungal Colony Forming Units per milliliter ($CFUml^{-1}$) emulsion detected on Rose Bengal Agar and [Modified V8 agar] | | | | |
|---|---|---|---|---|---|
| Site: | $10^2$ | $10^4$ | $10^5$ | $10^6$ | $10^7$ |
| Premix tank | 0^[0] | 0[0] | 0[0] | 0[0] | 0[0] |
| Bucket* | TNTC#[TNTC] | TNTC[237] | 100[21] | 12[2] | 1[0] |
| Roll neck | TNTC[TNTC] | TNTC[TNTC] | TNTC[149] | 89[11] | 9[0] |
| Hoffman filter | 8[6] | 0[0] | 0[0] | 0[0] | 0[0] |
| Return-to-sump pipe | TNTC[TNTC] | TNTC[230] | 290[21] | 26[2] | 3[0] |
| Clean side of main tank | 0[0] | 0[0] | 0[0] | 0[0] | 0[0] |
| Dirty side of main tank | 7[0] | 0[0] | 0[0] | 0[0] | 0[0] |
| Sump tank | 0[0] | 0[0] | 0[0] | 0[0] | 0[0] |
| Sump sludge | TNTC[270] | 9[3] | 0[0] | 0[0] | 0[0] |

TNTC#: Too numerous to count (refer to 2.3.1 Sampling Sites)

0^: Zero Colony forming units per milliliter of sample (refer to 2.3.1 Sampling Sites)

On average, fewer fungal colonies developed on MV8 agar than on RB agar.

### 2.3.1.6 Fungal contamination levels in the hot roll mill after the emulsion reticulation system cleanout

The data in Table 6 indicates that very few fungal colony forming units were present in the emulsion after the system cleanout, with low counts occurring only in the roll neck, the Hoffman filter and the return-to-sump pipe. No other areas showed fungal contamination.

**Table 6: Levels of fungal contamination in the hot roll mill after the emulsion reticulation system cleanout**

| Site: | Fungal Colony Forming Units per milliliter ($CFUml^{-1}$) emulsion detected on Rose Bengal Agar and [Modified V8 agar] | | | | |
|---|---|---|---|---|---|
| | $10^2$ | $10^4$ | $10^5$ | $10^6$ | $10^7$ |
| Premix | 0[0] | 0[0] | 0[0] | 0[0] | 0[0] |
| Roll neck | 9[4] | 0[0] | 0[0] | 0[0] | 0[0] |
| Hoffman filter | 9[3] | 0[0] | 0[0] | 0[0] | 0[0] |
| Return-to-sump | 5[0] | 0[0] | 0[0] | 0[0] | 0[0] |
| Clean side of main tank | 0[0] | 0[0] | 0[0] | 0[0] | 0[0] |
| Dirty side of main tank | 0[0] | 0[0] | 0[0] | 0[0] | 0[0] |
| Sump tank | 0[0] | 0[0] | 0[0] | 0[0] | 0[0] |
| Sump sludge | 0[0] | 0[0] | 0[0] | 0[0] | 0[0] |

0^: Zero Colony forming units per milliliter of sample (refer to 2.3.1 Sampling Sites)

### 2.3.2 Identification of organisms

Based on light microscopic examination of the spore and sporophore morphology of the fungal isolates, the following fungi were identified in the samples taken from the hot roll mill emulsion reticulation system: *Aspergillus* spp.; *Penicillium* spp.; *Cladosporium* sp. and two yeast species. The growth/morphological characteristics of the fungal isolates are described in Table 7.

**Table 7: Characteristics of the fungal isolates found in the hot roll mill prior to the emulsion reticulation system cleanout**

| Genus | Sampling site | Morphological characteristics |
|---|---|---|
| *Aspergillus* sp., possibly *Aspergillus niger* | Return-to-sump,<br>Bucket,<br>Roll neck,<br>Sump sludge. | Conidiophores with sterigmata bearing conidia and arising from a bulbous vesicle. Conidia black. Colonies black in colour on RB agar and MV8 agar. |
| *Aspergillus* sp. | Bucket,<br>Roll neck. | Conidiophores with sterigmata bearing conidia and arising from a bulbous vesicle. Conidia were transparent white. Colonies white in colour on RB agar and MV8 agar. |
| *Penicillium* sp. | Return-to-sump pipe,<br>Bucket. | Brush-like conidiophores with phialides bearing yellow-white conidia. Colonies yellow-white in colour on RB agar and MV8 agar. |

Table 7 continued.

| | | |
|---|---|---|
| *Penicillium* sp. | Bucket,<br>Roll neck. | Brush-like conidiophores with phialides bearing greenish-white conidia. Colonies were greenish-white in colour on RB agar and MV8 agar.. |
| *Cladosporium* sp. | Bucket,<br>Dirty side of main tank,<br>Sump sludge. | Elongate, dark conidia with extended filamentous mycelium. Colonies were dark grey to black in colour on RB agar and MV8 agar. |
| Yeast species 1 | Dirty Side of main tank,<br>Return-to-sump pipe. | Large oval cells. No pseudomycelium present. Colonies were beige in colour on RB agar and MV8 agar. Cell budding evident. |
| Yeast species 2 | Return-to-sump pipe,<br>Roll neck. | Large cellular bodies with some pseudomycelium. Colonies were beige in colour on RB agar and MV8 agar. Cell budding evident. |

Thirty-two bacterial isolates were obtained from the initial samples. These cultures were assigned codes A-Z and A1-A8. Isolates B and L were later discarded as duplicates of isolates H and Z respectively. Characteristics of the bacterial isolates studied are described in Table 8.

**Table 8: Characteristics of the bacterial isolates found in the hot roll mill prior to the emulsion reticulation system cleanout**

| Culture | Sampling site | *Gram/ *KOH | #HL test | ^API kit | Shape | Size (μm) | Morphological and/or physiological characteristics |
|---|---|---|---|---|---|---|---|
| A | Roll neck | *+<br>•+ | + | No | rods | 1.5-2.0 | Centrally located spherical endospore; medium size, circular, flat colony with entire margin on NA |
| C | Return-to-sump pipe | *-<br>•- | - | Yes | rods | 0.9 | Fluorescence on BBLC agar; medium size, circular, flat colony with entire margin on NA |
| D | Sump sludge | *+<br>•+ | + | No | rods | 1.0 | Membranous/Dehydrated colony morphology on NA; medium size, irregular, flat colony with undulate margin on NA |
| E | Roll neck | *+<br>•+ | + | No | cocci | 1.0 | Tetrads formed; orange fluorescent areas on NA, medium size, circular, flat colony with entire margin on NA; centrally placed spherical endospores present |
| F | Clean side of main tank | *+<br>•+ | - | No | cocci | 0.2-0.3 | Very small cells; small size, circular and convex colony with entire margin on NA; centrally placed spherical endospores present, yellow pigment on NA |
| G | Bucket | *+<br>•+ | + | No | rods | 1.5-3.0 | Centrally located spherical endospore; translucent, medium size, circular, flat colony with entire margin on NA |
| H/B | Clean side of main tank | *+<br>•+ | - | No | cocci | 1.0 | Tetrads formed; medium size, circular, raised colony with entire margin on NA; centrally placed spherical endospores present |
| I | Return-to-sump pipe | *-<br>•- | - | Yes | rods | 0.8-3.0 | Fluorescence on Cet. agar; small size, circular, flat colony with curled margin on NA |

Table 8 continued.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| J | Hoffman filter | *- *- | + | No | cocci | 0.4 | Very small coccoid cells; small size, circular, flat and opaque colony with entire margin on NA |
| K | Roll neck | *- *- | - | Yes | rods | 0.7 | Medium size; circular, raised and creamy (mucoid in places) to opaque colony with entire margin on NA |
| M | Dirty side of main tank | *- *- | + | Yes | rods | mixed | Giant cells; medium size, circular, raised and smooth colony with entire margin and metallic sheen on NA |
| N | Sump sludge | *- *- | - | Yes | rods | 1.0-2.0 | Blue green pigments on Cet. agar; medium size, circular, raised colony with entire margin and glossy sheen on NA |
| O | Hoffman filter | *+ *+ | + | No | cocci | 1.0 | Tetrads formed; small size, circular, flat colony with entire margin and glossy sheen on NA; centrally placed spherical endospores present |
| P | Hoffman filter | *+ *+ | + | No | cocci | 0.2-0.3 | Very small cells; small size, circular, flat colony with entire margin and cream appearance on NA; centrally placed spherical endospores present; yellow pigment on NA |
| Q | Return-to-sump pipe | *+ *+ | + | No | rods | 1.0 | Blue green pigments on Cet. agar; large size, circular, flat colony with entire margin and translucent appearance on NA |
| R | Return-to-sump pipe | *+ *+ | + | No | rods | 1.0 | Colony produces strong ammoniacal odour on NA; small size, circular, flat colony with entire margin and translucent appearance on NA |
| S | Return-to-sump pipe | *+ *+ | - | No | rods | 1.0 | Blue green pigments on Cet. agar; small size, circular, flat colony with entire margin and cream appearance on NA |
| T | Roll neck | *+ *+ | + | No | rods | 0.7 | Medium size; circular, raised colony with curled margin and glossy sheen on NA |
| U | Return-to-sump pipe | *- *- | - | Yes | rods | 0.7 | Concentrically ringed translucent colonies on Cet. agar; medium size, circular, flat colony with entire margin and translucent appearance on NA |
| V | Return-to-sump pipe | *- *- | - | Yes | rods | 0.7 | Blue green pigments on Cet. agar; pulvinate colony on BBLC agar and EMB agar, medium size, circular, raised colony with entire margin and translucent appearance on NA |
| W | Dirty side of main tank | *- *- | + | Yes | rods | mixed | Giant cells; small size, circular, raised colony with curled margin and cream appearance on NA |
| X | Return-to-sump pipe | *- *- | - | Yes | rods | 0.7-1.0 | Blue green pigments on Cet. agar; medium size, circular, raised colony with curled margin and opaque appearance on NA |
| Y | Roll neck | *- *- | - | Yes | rods | 1.5-2.0 | Cells surrounded by mucoid capsule; medium size, circular, flat colony with entire margin and pink/cream appearance on NA |
| Z/L | Roll neck | *- *- | + | Yes | rods | 1.0 | Concentrically ringed colonies; medium size, circular, raised with curled margin and cream appearance on NA |
| A1 | Dirty side of main tank | *- *+? | - | Yes | rods | 0.5 | Medium size; circular and flat colony with entire margin and translucent appearance on NA |
| A2 | Sump sludge | *- *- | - | Yes | rods | 1.0-1.5 | Medium size; circular and flat colony with entire margin and translucent appearance on NA |
| A3 | Dirty side of main tank | *- *- | - | Yes | rods | 0.7 | Concentrically ringed colony with yellow pigment on Cet. agar; medium size, flat and circular colony with entire margin and opaque appearance on NA |

Table 8 continued.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| A4 | Dirty side of main tank | *+ •-? | + | No | rods | 2.0 | Gram variable; medium size, flat and circular colony with entire margin and cream appearance on NA |
| A5 | Dirty side of main tank | *- •+? | + | Yes | rods | 1.0-3.0 | Pronounced dark green edge to colony on BBLC agar; very large size, flat and circular colony with entire margin and opaque appearance on NA |
| A6 | Sump sludge | *- •- | + | Yes | rods | 1.0-3.0 | Metallic sheen; medium size, flat and circular colony with entire margin and cream/opaque appearance on NA |
| A7 | Sump sludge | *+ •+ | + | No | rods | 1.2-3.0 | Yellow pigment on Cet. agar; very large size, flat and circular colony with entire margin and translucent brown appearance on NA |
| A8 | Bucket | *- •- | + | Yes | rods | 0.7-1.0 | Colony produces strong ammonium-like odour on BBLC and EMB; ubonate and pulvinate colonies on BBLC and EMB respectively, small size, raise and circular colony with entire margin and cream/brown appearance on NA |

*Gram = Gram stain reaction.
•KOH = Gregersen's test.
#HL test = Hugh Liefson's test.
^API Kit = Isolates subjected to API 20 E diagnostic kit testing.

Eighteen of the bacterial isolates (cultures A, D, E, G, J, M, O, P, Q, R, T, W, Z/L and A4-A8) produced acid when grown in Hugh Liefson's medium in both aerobic and oxygen-limited tubes. The results for the Gram negative rod-shaped isolates that were subjected to API 20 E diagnostic kit identification appear in Table 9.

**Table 9: Probable profiles and identification of Gram negative isolates subjected to API 20 E diagnostic kit testing**

| Isolate Designation | Probable profile | Isolate Identification | Isolate Designation | Probable profile | Isolate Identification |
|---|---|---|---|---|---|
| C | Acceptable-87% | *Pseudomonas* sp. | I | Acceptable-84% | *Pseudomonas* sp. |
| K | Good-75% | *Pseudomonas* sp. | M | Good-75% | *Escherichia* sp. |
| N | Acceptable-85% | *Pseudomonas* sp. | U | Very good-97% | *Pseudomonas* sp. |
| V | Good-93% | *Pseudomonas* sp. | W | Doubtful | Unknown |
| X | Very good-97% | *Pseudomonas* sp. | Y | Good-78% | *Pseudomonas* sp. |

Table 9 continued.

| | | | | | |
|---|---|---|---|---|---|
| Z/L | Doubtful | Unknown | A1 | Good-75% | *Pseudomonas* sp. |
| A2 | Doubtful | Unknown | A3 | Good-78% | *Pseudomonas* sp. |
| A5 | Very good-99% | *Aeromonas* sp. | A6 | Acceptable-87% | *Chromobacterium* sp. |
| A8 | Acceptable-84% | *Enterobacter* sp. | | | |

Only 22 of the 32 bacterial isolates could be identified. These are shown in Table 10. All the isolates (including those which were not identified) were used for further investigations described in Chapter 3.

**Table 10: Identity of aerobic and facultatively anaerobic bacteria found in the hot roll mill prior to the emulsion reticulation system cleanout**

| Culture | Genus ID | Culture | Genus ID |
|---|---|---|---|
| A | *Bacillus* sp.# | C | *Pseudomonas* sp.* |
| E | *Micrococcus* sp. or *Sporosarcina* sp.• | F | *Micrococcus* sp. or *Sporosarcina* sp.• |
| G | *Bacillus* sp.# | H/B | *Micrococcus* sp. or *Sporosarcina* sp.• |
| I | *Pseudomonas* sp.* | K | *Pseudomonas* sp.* |
| M | *Escherichia* sp. | N | *Pseudomonas* sp.* |
| O | *Micrococcus* sp. or *Sporosarcina* sp.• | P | *Micrococcus* sp. or *Sporosarcina* sp.• |
| U | *Pseudomonas* sp.* | V | *Pseudomonas* sp.* |
| X | *Pseudomonas* sp.* | Y | *Pseudomonas* sp.* |
| A1 | *Pseudomonas* sp.* | A2 | *Pseudomonas* sp.* |
| A3 | *Pseudomonas* sp.* | A5 | *Aeromonas* sp. |
| A6 | *Chromobacterium* sp. | A8 | *Enterobacter* sp. |

* various *Pseudomonas* isolates exhibiting dissimilar colonies on nutrient agar
# various *Bacillus* isolates exhibiting dissimilar colonies on nutrient agar
• various *Micrococcus* or *Sporosarcina* isolates exhibiting dissimilar colonies on nutrient agar

Anaerobic organisms were first detected five days after inoculation of the IS agar plates. Black precipitates of iron sulphide were clearly visible surrounding the colonies. There is a high likelihood that these organisms belong to the genus *Desulfovibrio.*

## 2.4 Discussion

### 2.4.1 Sampling sites

Prior to the start of this investigation, there was considerable wastage of emulsion at the Huletts plant, due primarily to biodeterioration of the emulsion by bacteria and fungi.

The system had not been thoroughly cleaned for approximately six months before the first samples were taken. Hence, all areas of the mill suitable for microbial colonization had large microbial populations which facilitated the isolation of a wide range of contaminating bacteria and fungi.

Fungal counts were high before the system was cleaned out. Areas of highest fungal contamination were the cooler areas of the mill, which also supported most of the bacterial growth. These areas included the roll neck (which is 'independent' of the main emulsion reticulation system), the return-to-sump pipe, the bucket of sludge collected from beneath the roll mechanism and, to a lesser extent, the dirty side of the main holding tank and the sump sludge (scraped from the bottom of the sump tank). Difficulties were experienced in homogenizing biofilm sludge from the sump, so it is highly probable that the counts registered for this area were underestimates.

Bacteria were detected in all areas of the mill except for the clean side of the main emulsion storage tank.

In general, the areas in which the emulsion was stagnant were found to have the greatest concentration of both bacterial and fungal contaminants. For example the bucket of sludge contained emulsion that was almost black indicating severe emulsion breakdown and microbial

biofilm deposits. This emulsion was being returned to the system during the rolling process and hence provided a constant source of inoculum. Large pools of emulsion could be seen throughout the roll mill area, especially under the main emulsion storage tank and in the basement where the sump tank is situated. These pools of emulsion afforded excellent conditions for microbial growth. Ambient temperature was approximately 36°C and with circulating air (produced by blowers to direct emulsion droplets away from the mill operators) it was inevitable that a source of airborne contamination was being provided. Another potential source of nutrients for microorganisms present in the emulsion came from the workers who, on occasion, were seen to throw organic waste (including fruit peels) into the system. During cleanout banana skins were found below the roll mechanism! It is, therefore, imperative that the work force be informed of the consequences of introducing any form of organic waste into the emulsion reticulation system.

Before the major cleanout of the roll mill, anaerobic bacteria were found in the overflow feed-back pipe which collects excess emulsion which has trickled away from the main system after rolling. This pipe is an open drainage canal which feeds slowly back into the sump tank. Temperature is around 36°C, and because flowback is very slow, and the drainage canal deep, conditions rapidly become anaerobic and hence ideal for SRB proliferation.

Following the detection of SRB Huletts' management were persuaded to divert the pipe directly to waste management, rather than back into the sump tank and ultimately the entire system. Since that time, SRB have seldom been found in the system and when they have been detected they have been present at very low levels only (less than $1x10^{2}CFUml^{-1}$ emulsion). The newly introduced fortnightly cleanouts of the system have helped to reduce SRB numbers to well below acceptable levels.

Large accumulations of biofilm were identified on the bottom and sides of the sump tank. These biofilms were removed (by spraying them) with pressurized steam during the major mill cleanout. Conditions suitable for microbial growth can develop in the oil sump as temperatures drop to 42°C during down-time and circulation ceases. Bacterial blooms have been known to

occur during this time (Hemingway, 1995. pers. comm.[8]). Bacterial contamination of the emulsion reticulation system is now closely monitored after any down-time period. This ensures rapid detection should contamination levels increase to above acceptable levels, thus enabling biocide concentrations to be increased quickly.

Another source of contamination is the roll neck mechanism. Although the emulsion in this mechanism is independent of the emulsion in the main system, the roll neck mechanism leaks emulsion onto the floor and pipes surrounding the main emulsion system. There is, therefore, a high likelihood of cross-contamination occurring between the two systems.

Samples from the clean side of the main emulsion storage tank showed no microbial growth either before or after the cleanout. This is because it is regularly dosed with heavy applications of biocide. Fresh emulsion (which showed no contamination when tested) is fed directly from the premix tank into the clean side of the main emulsion storage tank. Large amounts of biocide are added to the fresh emulsion and the recycled, used emulsion (emulsion coming from the dirty side which has been filtered [refer to Figure 1]) before it is sprayed onto the rollers. Constant mixing of the emulsion ensures stability. A unidirectional spill-over wall ensures that excess emulsion spills from the clean side into the dirty side of the main tank and not vice versa. Other than the high concentration of biocide, increased temperatures are maintained in this part of the mill, to ensure maximum emulsion efficiency before spraying onto the aluminium ingot and rollers. The temperature seldom drops below 55°C in the clean side of the main emulsion tank. Although some yeasts can grow at this temperature, 55°C is the upper limit at which most true fungi will grow (Stanier *et al.*, 1987). The high temperature, the increased efficacy of the biocide at this temperature, and inhibitory concentrations of certain compounds in the fresh emulsion, are all factors which inhibit microbial growth (both fungal and bacterial) in the clean side of the main tank.

It was interesting that bacteria were found in the dirty side of the main emulsion tank but not

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[8]Mrs. M. J. Hemingway, 1995. Hulett Aluminium hot roll mill Research and Development department, Hulett Aluminium, Camps Drift, Pietermaritzburg.

in the clean side, even though conditions, including temperature, circulation rate and biocide inputs were similar in both compartments. The only difference was that the emulsion had been through the entire system by the time it reached the dirty side. It is probable that the inhibitory concentrations of compounds such as phenols in the fresh emulsion are lowered sufficiently during circulation (by volatilization, complexing with microbial cells or through microbial activity) to permit microbial growth, even at the elevated temperatures found in this part of the system.

### 2.4.2 Microbial identification

Nutrient agar was used as the preferred growth medium in the initial isolation experiments. This medium has been shown by numerous workers (Pivnick, 1955; Sabina and Pivnick, 1956 and Firko and Burrell, 1991) to support growth of the majority of microorganisms associated with hot roll mill emulsion reticulation systems. Other, more selective media, were subsequently included to aid in the identification of the isolates. After a extensive search of the literature particular groups of organisms were specifically targeted for isolation and identification. These groups and the media generally used to isolate members thereof were:

- Pseudomonads (Lee and Chandler, 1941 and Duffett *et al.*, 1943) Cetrimide Agar.
- Coliform bacteria (Pivnick and Fabian, 1954) MacConkey Agar, Eosin Methylene Blue Agar.
- Facultative anaerobes (Wallis, 1994. pers. comm.[9]) Hugh Liefson's medium with a deep overlay of mineral oil.
- Anaerobic sulphate-reducing bacteria (Guynes and Bennett, 1959) Iron Sulphite Agar.

Fungal isolations were performed on Rose Bengal (RB) and modified V8 (MV8) agar (refer to appendix A). RB and MV8 selective media are the preferential growth media for fungi in samples in which high bacterial contamination is evident.

Certain of the bacterial and fungal isolates were identified as belonging in genera previously

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[9]Prof. F. M. Wallis, 1994. Department of Microbiology and Plant Pathology, University of Natal, Pietermaritzburg.

found by other authors in emulsion systems. For example, the present author successfully isolated and identified bacteria belonging to the genera: *Bacillus*, *Pseudomonas* and *Escherichia*. Members of these genera are common contaminants of emulsions and have been isolated and identified by numerous workers (Duffett *et al.*, 1943; Bennett and Wheeler, 1954; and Sabina and Pivnick, 1956). Also identified were: *Enterobacter* sp., *Sporosarcina* sp., *Micrococcus* sp., *Aeromonas* sp. and *Chromobacterium* sp. These were reported as 'new' genera since they have, to the present author's knowledge, not been reported previously in any emulsion systems.

Both *Aeromonas* sp. and *Enterobacter* sp. belong to the enteric group of bacteria and are capable of a fermentative metabolism. Potentially, both can come from human sources (Stanier *et al.*, 1987). *Sporosarcina* sp. and *Micrococcus* sp. are common airborne contaminants (Wallis, 1994. pers. comm.[10]).

Some of the bacterial cultures (namely A, D, E, G, J, M, O, P, Q, R, T, W, Z/L and A4-A8) grew profusely in Hugh Liefson's medium. overlaid with mineral oil, thus indicating that they were facultative anaerobes capable of fermentative metabolism when oxygen was excluded.

Development of bacterial colonies on IS agar under strictly anaerobic conditions, suggested that SRB were present at specific sampling sites in the emulsion reticulation system. Further identification was not required; however, the colonies on iron sulphite agar were black, which is characteristic of the *Desulfo*-bacter group (Postgate, 1951b and Stanier *et al.*, 1987). Light microscopic examination revealed the bacteria to be curved rods, which is characteristic of *Desulfovibrio*. The anaerobic organism *Desulfovibrio desulfuricans* has been identified previously in water soluble oil emulsions (Guynes and Bennett, 1959). It is, therefore, probable that the SRB detected was *Desulfovibrio desulfuricans*, as this bacterium has been identified as the main sulphate-reducing organism occurring in emulsion reticulation systems (Guynes and Bennett, 1959).

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[10]Prof. F. M. Wallis, 1994. Department of Microbiology and Plant Pathology, University of Natal, Pietermaritzburg.

The present author isolated two yeasts using RB and MV8 agar media. Other authors (Rossmore and Holtzman, 1974 and Passman, 1988) have similarly isolated a number of yeasts from various emulsions. Of the filamentous fungal cultures identified, *Aspergillus* sp., *Penicillium* sp. and *Cladosporium* sp. had not previously been reported in the literature available to the present author. They are, however, common contaminants of petroleum products (Alexopoulos and Mims, 1979).

The wide array of bacteria and fungi isolated from the emulsion indicates that conditions at the time of sampling were ideal for optimal microbial growth. The emulsion was 'loose' and the level of microbial contamination was very high. Areas that are usually not accessible for sampling, were available during the cleanout process. These areas included the bottom and sides of the sump and main emulsion storage tanks. These tanks are usually filled with emulsion and because of the high temperatures are difficult to take samples from. Because they were empty during the cleanout process, biofilm scrapings could be taken from the tank surfaces. The wide array of fungi and bacteria present indicated that Prosol could support a complex microbial population, including strict aerobes, facultative anaerobes and obligate anaerobes. However, it is highly unlikely that the isolated microbes obtained all their growth requirements from the emulsion alone. This suggested an environment in which microorganisms relied not only on components from the emulsion for their growth but also on metabolic products synthesized by, or breakdown products resulting from the activity of other microorganisms. Although the study of individual species in pure culture may aid in understanding biodeterioration of the emulsions, it is the interactions occurring between members of the mixed population which are of primary importance. If, for example, a primary degrader could be targeted and controlled (or destroyed) it might be possible to eradicate also all those microorganisms that rely on its products for their growth. So-called 'friendly' emulsions may provide an answer to the problems of biodeterioration. Claims have been made that some emulsions support the growth of bacteria in relatively high concentrations without losing overall performance (Rossmore, 1993 and Pitchford, 1995. pers. comm.[11]). However, more research in the field of 'friendly' emulsions is

[11]Dr. A. Pitchford, 1995. Hulett Aluminium hot roll mill Research and Development department, Hulett Aluminium, Camps Drift, Pietermaritzburg.

needed before any recommendations can be made. Another possible control strategy might be to introduce unrelated compounds to the emulsion thereby stimulating the growth of 'friendly' microorganisms. Such organisms would produce compounds inhibitory to the growth of emulsion degrading bacteria and fungi and in this manner 'protect' the emulsion from biodeterioration.

Also of importance is good management practice in order to reduce the amount of contamination entering the system. From a biodeterioration standpoint conditions at the Hulett Aluminium hot roll mill were ideal at the time the first samples were taken for analysis in early 1994. Pools of emulsion emanating from leaking pipes, pipes feeding contaminated emulsion to the main system, worker hygiene and down-time management were all problems which needed to be addressed. Some of these have already been rectified, and others will hopefully be addressed in the near future (refer to **CHAPTER 6. GENERAL DISCUSSION**).

# CHAPTER 3. OXIDATION OF EMULSION COMPONENTS BY FUNGI AND BACTERIA

## 3.1 Introduction

Emulsions are complex in composition, consisting of a wide variety of compounds which include: oils; emulsifiers (Ellis *et al.*, 1957); mineral waxes; fatty oils; anti-rust agents; coupling agents; anti-foam agents; dyes; extreme pressure additives; synthetic and phosphate esters; amines, biocides (Sabina and Pivnick, 1956; Genner and Hill, 1981; Passman, 1988) and inorganic anions and cations (Passman, 1988).

The study of microbial biodeterioration of hydrocarbon-based substances dates back to work done in the 1940's by Zobell (cited by Atlas, 1981). The oxidation of the soluble oil components of an emulsion, however, was first investigated by Sabina and Pivnick in 1956. They studied the oxidation of soluble oil emulsions by species of *Pseudomonas* and demonstrated that these organisms could degrade both the oil and the emulsifier components of the emulsion. Ellis *et al.* (1957) expanded this research by showing the effect of mixed cultures, as opposed to pure cultures, on the emulsion complex. These authors concluded that mixed cultures have a greater harmful effect on the oil components than do pure cultures. They also showed that various bacterial species were capable of degrading individual components of the emulsion under investigation. These components included a wide range of hydrocarbons as well as emulsifiers which hold the oils in suspension

Hulett Aluminium receives the oil concentrate Prosol, which is then added to water and mixed (in the premix tank) before being introduced into the hot roll mill emulsion reticulation system. Prosol concentrate consists of the following eight components in the specific ratios indicated below: a mix of neutral base oils (46.47%); a mix of naphthenic base oils (30.15%); oleic acid (12.90%); phenol (0.3%); hexylene glycol (1.8%); tricresol phosphate (3.0%); diethanolamine (1.39%) and triethanolamine (3.49%) and a small amount of water (0.5%). Since both the neutral base oils and naphthenic base oils are comprised of hundreds of sub-components, it was

not feasible to study each of these basic constituents separately. The neutral and naphthenic base oils were therefore investigated as their respective complex mixtures.

The present author set out to establish which components of the Hulett Aluminium emulsion, Prosol, were most susceptible to microbial biodeterioration. In order to do this various agar media, containing the different emulsion components as sole carbon and energy sources, were developed. The various pure cultures isolated (refer to Chapter 2) were tested for their ability to grow on these agar media. A gross linear measurement assay was devised to establish which components of the emulsion were susceptible to degradation by each of the pure bacterial and fungal cultures. It was hoped that this experiment would facilitate identification of the most susceptible components, and at the same time aid in the selection of a 'target organism'. Such a 'target organism' may provide the essential first link in a chain of reactions which ultimately lead to the biodeterioration of the emulsion. With the control and/or elimination of this 'target organism', the initial degradation and subsequent 'loosening' of the emulsion would be stopped or at least reduced or retarded, thereby extending the useful lifespan of the emulsion. Future enumeration studies could then be specifically geared towards this 'target organism' and perhaps help to assess indirectly the condition of the emulsion at any given time. In this way a rapid detection method for 'target organism' enumeration might possibly be devised.

### 3.2 Materials and Methods

The following selective media were made up using concentrations of the various emulsion components identical to those in fresh hot roll mill emulsion (refer to Appendix C):

- 1. **Neutral base oil selective medium** (NBO).
- 2. **Naphthenic base oil selective medium** (NapBO).
- 3. **Oleic acid selective medium** (OA).
- 4. **Phenolic selective medium** (PH).
- 5. **Hexylene glycol selective medium** (HG).
- 6. **Tricresol phosphate selective medium** (TCrP).

- 7. **Diethanolamine selective medium** (DEA).
- 8. **Triethanolamine selective medium** (TEA).
- 9. **Prosol selective medium** (PS).

Water agar plates were also made up for use as controls. It was hoped that the fungal or bacterial culture under test would grow to a measurable value, X, after seven days on water agar. This value could then be compared quantitatively to the amount of growth of the same isolate on the various specific media, thereby allowing determination of growth rate differences on the various media after the same period of time. The plates were placed on a laminar flow bench for two hours to remove excess moisture. The bacterial and fungal isolates were inoculated separately onto Petri dish plates containing each of the selective media and the water agar control (refer to Figure 4).

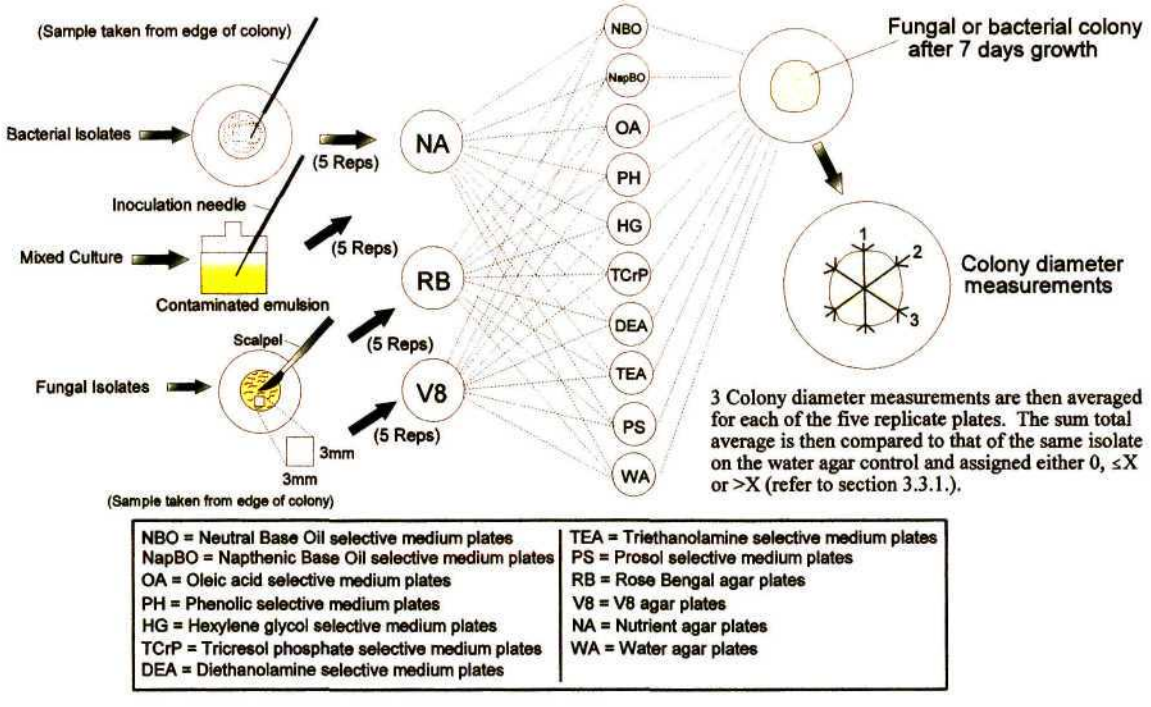


**Figure 4: Procedure for measurement of the ability of fungal and bacterial isolates to metabolize the various components of the emulsion Prosol**

One standardized stab inoculation of each bacterial culture was centrally placed on each of the plates. For fungal cultures, a three millimeter square block of agar overgrown with hyphae was aseptically cut from a parent colony and placed centrally onto the selective medium plates and

water agar control plates. Included for reference was a standard mix of microorganisms obtained directly from a sample of highly contaminated emulsion (with microbial numbers in excess of $1x10^7 CFUml^{-1}$). For each isolate, five replicate plates of each medium were identically inoculated. The inoculated plates were incubated for 7 days at 37°C. After the incubation period, the diameter of each colony was measured from three 120 degree perspectives. For each of the five colonies, the measurements were compared with those on the corresponding five water agar controls.

## 3.3 Results

### 3.3.1. Growth response of aerobic bacterial isolates on the different selective media

The growth response of the aerobic bacterial isolates on the different selective media, is presented in Table 11.

**Table 11: Growth response of aerobic bacterial isolates and a mixed culture on various selective media after 7 days incubation at 37°C**

| | Selective Media | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Culture: Genus Identification | DEA | TEA | NBO | NapBO | OA | TCrP | HG | PH | PS | WA |
| A:*Bacillus* sp. | $<X^6$ | $0^2$ | $0^4$ | $X^{12}$ | $0^4$ | $<X^6$ | $0^4$ | $0^2$ | $>X^{15}$ | $X^{11}$ |
| C:*Pseudomonas* sp. | $X^{11}$ | $>X^{13}$ | $0^3$ | $<X^6$ | $0^2$ | $0^4$ | $X^{10}$ | $0^2$ | $0^2$ | $X^9$ |
| D:Unidentified | $>X^{20}$ | $X^{15}$ | $0^4$ | $0^4$ | $0^2$ | $<X^9$ | $X^{12}$ | $0^2$ | $0^2$ | $X^{13}$ |
| E:*Micrococcus* sp. or *Sporosarcina* sp. | $0^2$ | $0^3$ | $0^3$ | $0^2$ | $>X^7$ | $0^3$ | $0^3$ | $0^2$ | $0^2$ | $X(0)^3$ |
| F:*Micrococcus* sp. or *Sporosarcina* sp. | $0^3$ | $0^2$ | $0^4$ | $0^4$ | $>X^9$ | $>X^6$ | $0^4$ | $0^2$ | $0^2$ | $X(0)^3$ |
| G:*Bacillus* sp. | $>X^{10}$ | $>X^{19}$ | $0^4$ | $0^4$ | $0^2$ | $0^3$ | $X^9$ | $0^2$ | $>X^{10}$ | $X^7$ |

Table 11 continued.

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| H:*Micrococcus* sp. or *Sporosarcina* sp. | $0^4$ | $0^2$ | $X^5$ | $0^2$ | $0^2$ | $0^3$ | $0^3$ | $0^2$ | $0^2$ | $X(0)^3$ |
| I:*Pseudomonas* sp. | $>X^8$ | $>X^{25}$ | $0^2$ | $0^4$ | $0^3$ | $0^4$ | $0^3$ | $0^2$ | $>X^7$ | $X(0)^4$ |
| J:Unidentified | $0^3$ | $0^2$ | $<X^7$ | $<X^6$ | $0^4$ | $0^3$ | $0^3$ | $0^2$ | $0^2$ | $X^{11}$ |
| K:*Pseudomonas* sp. | $>X^{18}$ | $>X^{10}$ | $0^3$ | $0^4$ | $>X^7$ | $0^3$ | $>X^{10}$ | $0^2$ | $0^3$ | $X(0)^4$ |
| M:*Escherichia* sp. | $>X^{22}$ | $>X^{10}$ | $X^6$ | $0^3$ | $0^2$ | $0^3$ | $0^4$ | $0^2$ | $>X^9$ | $X(0)^4$ |
| N:*Pseudomonas* sp. | $>X^{12}$ | $>X^{16}$ | $X^6$ | $0^4$ | $0^2$ | $X^6$ | $0^4$ | $0^2$ | $>X^{11}$ | $X^7$ |
| O:*Micrococcus* sp. or *Sporosarcina* sp. | $0^3$ | $0^2$ | $X^5$ | $0^4$ | $0^3$ | $0^3$ | $0^4$ | $0^2$ | $0^2$ | $X(0)^4$ |
| P:*Micrococcus* sp. or *Sporosarcina* sp. | $0^3$ | $0^4$ | $X^6$ | $0^4$ | $0^4$ | $0^3$ | $0^4$ | $0^2$ | $0^3$ | $X(0)^4$ |
| Q:Unidentified | $0^2$ | $>X^{21}$ | $X^{11}$ | $X^{10}$ | $0^4$ | $0^3$ | $X^{11}$ | $0^2$ | $0^3$ | $X^{12}$ |
| R:Unidentified | $>X^{11}$ | $X^{10}$ | $X^7$ | $0^3$ | $0^3$ | $X^8$ | $>X^{12}$ | $0^2$ | $0^2$ | $X^8$ |
| S:Unidentified | $>X^8$ | $X^6$ | $>X^7$ | $0^2$ | $X^5$ | $0^3$ | $>X^{16}$ | $0^2$ | $0^3$ | $X(0)^4$ |
| T:Unidentified | $<X^{13}$ | $<X^{14}$ | $<X^7$ | $<X^6$ | $0^4$ | $<X^5$ | $<X^5$ | $0^2$ | $0^2$ | $X^{22}$ |
| U:*Pseudomonas* sp. | $>X^{10}$ | $>X^9$ | $0^4$ | $X^5$ | $0^2$ | $>X^9$ | $0^4$ | $0^2$ | $>X^7$ | $X(0)^4$ |
| V:*Pseudomonas* sp. | $>X^{14}$ | $>X^{17}$ | $<X^7$ | $0^4$ | $0^2$ | $<X^5$ | $<X^5$ | $0^2$ | $0^2$ | $X^{11}$ |
| W:Unidentified | $<X^7$ | $>X^{25}$ | $0^4$ | $0^4$ | $0^2$ | $0^3$ | $0^4$ | $0^2$ | $0^2$ | $X^{12}$ |
| X:*Pseudomonas* sp. | $X^6$ | $>X^{12}$ | $0^2$ | $>X^8$ | $0^4$ | $>X^9$ | $>X^{11}$ | $0^2$ | $0^2$ | $X^5$ |
| Y:*Pseudomonas* sp. | $0^4$ | $0^4$ | $0^3$ | $0^4$ | $0^3$ | $0^3$ | $0^4$ | $0^2$ | $0^3$ | $X(0)^4$ |
| Z/L:Unidentified | $>X^{12}$ | $>X^{11}$ | $0^4$ | $X^5$ | $X^7$ | $0^3$ | $X^7$ | $0^2$ | $>X^{10}$ | $X^5$ |
| A1:*Pseudomonas* sp. | $>X^{28}$ | $>X^{18}$ | $0^4$ | $<X^5$ | $0^2$ | $0^3$ | $0^4$ | $0^2$ | $0^2$ | $X^{13}$ |
| A2:*Pseudomonas* sp. | $X^8$ | $X^7$ | $X^8$ | $0^4$ | $X^9$ | $0^3$ | $X^{10}$ | $0^2$ | $0^3$ | $X^8$ |
| A3:Unidentified | $>X^{17}$ | $>X^{21}$ | $0^4$ | $<X^5$ | $<X^7$ | $0^3$ | $>X^{25}$ | $0^2$ | $0^2$ | $X^{13}$ |
| A4:Unidentified | $>X^{11}$ | $>X^{14}$ | $0^3$ | $0^3$ | $X^{10}$ | $0^3$ | $<X^5$ | $0^2$ | $0^2$ | $X^8$ |
| A5:*Aeromonas* sp. | $<X^9$ | $<X^9$ | $<X^8$ | $X^{10}$ | $0^2$ | $X^{12}$ | $X^{13}$ | $0^2$ | $0^3$ | $X^{12}$ |
| A6:*Chromobacterium* sp. | $>X^{11}$ | $>X^{11}$ | $0^3$ | $0^4$ | $0^3$ | $0^3$ | $X^7$ | $0^2$ | $>X^{10}$ | $X^5$ |
| A7:Unidentified | $X^8$ | $>X^{13}$ | $0^4$ | $0^4$ | $<X^5$ | $<X^5$ | $0^4$ | $0^2$ | $0^2$ | $X^{10}$ |
| A8:*Enterobacter* sp. | $0^4$ | $0^4$ | $0^2$ | $0^3$ | $X^8$ | $0^4$ | $0^4$ | $0^2$ | $0^2$ | $X^6$ |
| Mixed Culture | $X^6$ | $X^6$ | $X^5$ | $X^8$ | $X^7$ | $X^7$ | $X^8$ | $0^3$ | $X^8$ | $X^7$ |

**$X^{\#}$**. The amount of growth, represented by the average diameter of the five replicate bacterial

colonies, that developed on the five replicate water agar control plates after 7 days was assigned the value **X#** (Where # equals the average colony diameter measurement in millimeters). If the average amount of growth of the specific isolate on five replicate plates of a particular selective medium was **similar** (ie. an average diameter difference ≤ two millimeters) to that of the average diameter of the five replicates on water agar it was assigned a **neutral** value **X**.

**>X.** If the average amount of growth of the specific isolate on five replicate plates of a particular selective medium was **greater** (ie. average diameter difference of > two millimeters) than that of the same five replicates on water agar it was assigned a **greater than X (>X)** value.

**<X.** If the average amount of growth of the specific isolate on five replicate plates of a particular selective medium was **less** (ie. average diameter difference of < two millimeters) than that of the same five replicates on water agar it was assigned a **less than X (<X)** value.

**0.** If no growth of the specific isolate on five replicate plates of a particular selective medium occurred on a selective medium it was assigned a **0** value.

Only DEA, TEA, PS and HG selective media supported growth of any of the isolates. DEA supported growth of 11 of the 32 isolates including one species of *Bacillus*, five species of *Pseudomonas* (isolates I, K, N, U and A1), the *Escherichia* isolate, the *Chromobacterium* strain, and the unidentified cultures D, Z/L and A3. The TEA selective medium supported the growth of 14 of the 32 cultures including the majority of the *Pseudomonas* isolates (7/10), one species of *Bacillus*, the *Chromobacterium* isolate and the four unidentified isolates Q, W, Z/L and A3. The HG selective medium supported the growth of one *Pseudomonas* isolate *viz.* isolate K and the unidentified culture A3. The PS selective medium comprised the emulsion concentrate mixed directly with agar and water (*viz.* it was a combination of all of the components used in the other selective media) to the standard Huletts Aluminium roll mill operating concentration of 3%. This medium supported the growth of only eight of the 32 isolates. These isolates included two *Bacillus* isolates *viz.* cultures A and G; three isolates of *Pseudomonas viz.* cultures I, N and U; the *Escherichia* isolate; the *Chromobacterium* isolate; and the unidentified culture Z/L.

The NBO, NapBO and TCrP selective media did not support growth of any of the bacterial cultures examined. The PH selective medium proved to be inhibitory to all of the isolates tested ie. colonies were significantly smaller than on the control.

The pseudomonad group of bacteria showed the greatest versatility in degrading the various emulsion components. This agrees with the findings of Sabina and Pivnick (1956) and Ellis *et al.* (1957).

The mixed cultures grew as much on all of the selective media as on the water agar control plates except that growth was inhibited on the PH selective medium.

### 3.3.2. Growth response of fungal isolates on the different selective media

The results with respect to the growth response of the fungal isolates on the selective media, are presented in Table 12.

**Table 12: Growth response of fungal isolates on the various selective media after 7 days incubation at 37°C**

| Culture: Genus Identification | Selective Media | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | DEA | TEA | NBO | NapBO | OA | TCrP | HG | PH | PS | WA |
| *Aspergillus* sp. | $X^{10}$ | $X^{11}$ | $X^{10}$ | $X^{12}$ | $X^{13}$ | $>X^{17}$ | $X^{12}$ | $0^{5}$ | $X^{11}$ | $X^{12}$ |
| *Penicillium* sp. | $X^{11}$ | $X^{11}$ | $X^{10}$ | $X^{9}$ | $X^{9}$ | $>X^{15}$ | $X^{10}$ | $0^{5}$ | $X^{9}$ | $X^{10}$ |
| *Cladosporium* sp. | $X^{10}$ | $X^{9}$ | $X^{10}$ | $X^{11}$ | $X^{10}$ | $>X^{15}$ | $X^{7}$ | $0^{4}$ | $X^{9}$ | $X^{8}$ |
| Yeast species A | $X^{6}$ | $X^{6}$ | $X^{7}$ | $X^{6}$ | $X^{5}$ | $X^{6}$ | $X^{6}$ | $0^{4}$ | $X^{9}$ | $X^{7}$ |
| Yeast species B | $X^{7}$ | $X^{7}$ | $X^{7}$ | $X^{8}$ | $X^{8}$ | $X^{9}$ | $X^{6}$ | $0^{4}$ | $X^{8}$ | $X^{7}$ |

Of the fungal isolates tested, *Aspergillus* sp., *Penicillium* sp. and *Cladosporium* sp. showed increased growth on the TCrP selective medium. All of the fungal isolates were inhibited on PH selective

medium.

### 3.4 Discussion

Water agar was used as the control medium since it provided minimal amounts of carbon readily available for fungal and bacterial metabolism. It is surprising that none of the isolates tested (even the mixed culture) showed increased growth on either of the base oil supplemented media. This can possibly be attributed to the fact that solid (as opposed to liquid) media were used, resulting in the oils being less readily available for microbial metabolism. The fact that base oils are composed of many hundreds of different compounds may also be of consequence. It is possible that one or more of these components may have been toxic to the tested organisms at the concentrations used.

Surprisingly several of the isolates, namely: A (*Bacillus* sp.); H/B (unidentified); J (unidentified); O (*Micrococcus* sp. or *Sporosarcina* sp.); P (*Micrococcus* sp. or *Sporosarcina* sp.); R (unidentified); S (unidentified); T (unidentified); Y (*Pseudomonas* sp.); A2 (*Pseudomonas* sp.); A4 (unidentified); A5 (*Aeromonas* sp.); A7 (unidentified) and A8 (*Enterobacter* sp.) did not show increased growth, compared to the control, on any of the selective media. This supports a statement by Ellis *et al.* (1957) that firstly, although various bacterial species are capable of degrading individual components of an emulsion, and secondly, that some bacteria may not directly effect the emulsion, but rather have secondary effects on other bacteria, it is mixed cultures that have a greater biodeteriorative effect on the whole emulsion complex. A particular species of bacterium may be able to degrade only a specific component of an emulsion whereas several species of microorganisms in co-culture might be capable of degrading a wider array of components and are, therefore, more likely to cause an increased rate of biodeterioration of the emulsion. Further research into the specific hydrocarbons and other substances which make up neutral and naphthenic base oils is required before assumptions can be made regarding the degradation of the oil components of Prosol.

In general, the emulsion components with the highest microbial susceptibility were the amines,

*viz.* diethanolamine and triethanolamine. This contrasts with the findings of Geiger *et al.* (1983) which showed that triethanolamine is relatively non-biodegradable. However Geiger *et al.* (1983) state that industrial triethanolamine also contains small amounts of diethanolamine (8%) and monoethanolamine (6%). It is possible, therefore, that the cultures tested in the present investigation were growing on the traces of mono- and diethanolamine and not on the triethanolamine. The results obtained from this experiment were unexpected insofar as the pure cultures showed very limited growth on all the selective media used. Even the Prosol selective medium supported the growth of only eight of the 32 cultures tested. A number of possibilities could account for this, namely:

- Conditions in the laboratory were not similar enough to those prevailing at the roll mill for microbial degradation of the emulsion, or its many components, to occur. Most degradation results from a complicated reaction sequence involving physical, chemical and biochemical processes, which are difficult to simulate effectively outside the hot roll mill. An operational model system would, therefore, be more effective for testing the biosusceptibility of the emulsion and its numerous components;

- Pure cultures were not representative of the microbial populations prevailing in the roll mill emulsion *in situ*. Ellis *et al.*'s (1957) experiments with mixed cultures showed that such cultures had greater biodeteriorative powers than did pure cultures. The use of mixed cultures in the present investigation may have proven more successful. There are, however, problems associated with the use of such mixed cultures. Firstly, mixed cultures need to be isolated from the contaminated emulsion. After isolation they need to be applied to the specific media in proportions similar to those found in the roll mill emulsion *in situ*. The correct ratios of various fungi and bacteria are very important, as some species will outgrow others. This was reported in Tant and Bennett's (1956) work, where delay between initial sampling and subsequent isolation produced different populations resulting from the vigorous species, such as the pseudomonads, outgrowing and inhibiting the growth of less vigorous organisms;

- The linear measurement scale used was not accurate enough to detect very small changes in

the growth rate of the cultures tested. The experiments conducted were not designed to show biodeterioration of the compounds used in the specific media but rather to determine if microbial growth rates could be proportionally related to metabolism of the various compounds. As such, the growth response experiment described in section 3.2 was a purely comparative study concerned with establishing the broad metabolic capabilities of the various isolates. With this in mind, it was hoped that an increase or decrease in the rate of growth of an isolate (measured in terms of colony size produced on a substrate in seven days) would relate directly to that isolates ability to metabolize the specific compound in the selective media when compared to the control.

Since the naphthenic and neutral base oils are comprised of a mixture of compounds, some of which are present in very low concentrations, colony diameter measurements might not reflect biodegradation of some of these specific compounds. The linear measurement scale employed did identify increased growth rates of some isolates on some of the specific media. It also clearly showed the inhibitory effects of phenol on microbial activity. However, as the linear measurement scale was rather crude, some of the results recorded as neutral may actually have been either slightly positive or slightly negative. The use of liquid media with direct measurement of either microbial growth or component degradation would have been preferable to the use of solid media. However, the oil and water phases would have separated in liquid culture with growth being limited to the contact area between the two phases. Smaller changes in either microbial numbers or component levels could have been detected using more sophisticated techniques such as gas chromatography, high performance liquid chromatography or spectrophotometry;

- Not all of the sub-components of each of the oil components were tested as potential substrates in selective media. It is, however, almost impossible to test all of these sub-components, as each oil comprises hundreds of sub-components. Each of these sub-components may interact differently with other components and with the microorganisms, thereby complicating the issue even further. Some of the additives in the emulsion concentrate produce specific effects in the diluted emulsion or the environment through which the emulsion

passes. The study of individual components of the oil fraction of any complex oil in water emulsion would therefore require an in-depth analytical study of the individual hydrocarbons in the oils as well as the other components present. This clearly was outside the scope of the present investigation.

A number of conclusions were made from the results of the above experiments. It was hypothesized that a commensal relationship probably exists between the various microorganisms inhabiting the emulsion. It was also shown that some organisms preferentially degrade particular components of the emulsion. This preferential degradation of specific components of the emulsion may provide a 'starting point', in the form of breakdown products, on which other fungi and bacteria could grow. The use of mixed, as opposed to pure, cultures for experimental purposes would be more representative of the dynamics of the roll mill emulsion *in situ*. For this reason further experiments were conducted using emulsion known to be contaminated with a variety of organisms.

# CHAPTER 4. CONTROL OF MICROORGANISMS INHABITING THE HULETT ALUMINIUM HOT ROLL MILL EMULSION

## 4.1 Introduction

A successful biocide needs to be: cost effective; antimicrobial over a period of time (ie. have a reasonable residual activity); non-toxic to workers under working conditions; non-corrosive and compatible with the emulsion (Heinrichs and Rossmore, 1971). The development of effective biocides exhibiting all the above characteristics is a long and arduous process.

Hulett Aluminium spends on average about R350 000 a year on biocides (Pitchford, 1995. pers. comm.[12]). Two biocides were in use at the hot roll mill during the research phase of this investigation. The first, Busan, remains in use today; it has been the biocide of choice at the plant for the past four years and has been relatively successful in controlling the emulsion contaminating microorganisms, provided it is used in conjunction with a second biocide. Alternative biocides, such as Tris Nitro have been used in the past. Unfortunately, because mill workers complained of eye irritations, Tris Nitro was administered only when microbial numbers increased to potentially dangerous levels. It is also exceedingly corrosive to the mild steel piping of the emulsion reticulation system.

The active component in Busan is glutaraldehyde, while in Tris Nitro the active compound is formaldehyde. Both glutaraldehyde and formaldehyde are slowly released from the biocides under alkali conditions. The mode of action of formaldehyde and glutaraldehyde on the cell structure and metabolism of microorganisms is well studied and will not be discussed here [refer to Holtzman and Rossmore (1976) and Munton and Russell (1973)].

Various other biocides are being tested by Buckman Laboratories (a consulting company which

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[12]Dr. A. Pitchford, 1995. Hulett Aluminium hot roll mill Research and Development Department, Hulett Aluminium, Camps Drift, Pietermaritzburg.

provides Busan to Hulett Aluminium) for introduction into the roll mill emulsion. As the cost of formulating new biocides increases annually, it is unlikely that the cost of biocide application to the emulsion will be reduced in the near future. If more efficient biocide management practices were to be initiated, ie. applying the biocide at the right time and in the correct concentrations so as to maximize its antimicrobial capacity, then increased efficiency as well as a reduction in the amount of biocides being used would reduce the amount spent annually on protecting the emulsion. Whilst several researchers have investigated the application of biocides to various microbially contaminated emulsions (Bennett, 1962; Leder and Russo, 1989; Law and Lashen, 1991; Heenan and Burrell, 1992), few have used semi-continuous model systems.

To measure the effectiveness of a biocide, numerous techniques can and should be used. In order to determine the biocide concentration in the emulsion, samples of emulsion are passed through a high performance liquid chromatograph (HPLC). This provides the plant manager with a direct measure of the concentration of biocide in the emulsion at any specific time. Such monitoring is essential for emulsion management. However, HPLC analysis does not show directly the presence of potentially harmful or undesirable microbes within the emulsion.

Plating techniques provide a measure of microbial numbers in the emulsion at any time. Although such information is useful, it may still not be enough to effectively assess the condition of the emulsion. Non-microbial factors can also result in a reduction of quality of the emulsion. Since the condition of the emulsion is of prime importance, as this directly affects the roll process, the condition of the emulsion was monitored regularly using a Malvern laser. This instrument accurately measures the size of the oil droplets within an emulsion. This is achieved by shining a high intensity laser beam through two glass plates, between which the emulsion is being pumped. Using a computer, the amount of absorbence is measured and from this data, the average size of the oil droplets is calculated. The results are presented in both tabular and graphical form. Of primary importance, however, is the **specific surface area** of the oil droplets measured in $\mathbf{m^2ml^{-1}}$ of sample. The specific surface area is a measurement of the total surface area of all the oil droplets in suspension. If the specific surface area is high, the emulsion is regarded as relatively 'tight' with large numbers of

relatively small oil droplets in suspension. Such an emulsion is considered to be in good condition. If the specific surface area is low, the oil droplets are not being held in suspension and the emulsion is referred to as 'loose'. Should an emulsion become 'loose', the oil and water phases separate and, in the case of an aluminium rolling emulsion, the ingot slips through the steel rollers during the rolling process. This causes irregularities in the width and thickness of the aluminium sheeting and therefore reduces the quality of the finished product. Prosol (the emulsion currently in use in the Hulett Aluminium hot roll mill) is considered to be 'tight' when the specific surface area is in the order of $10m^2ml^{-1}$. However, an acceptable surface area may range from 9.5-$12m^2ml^{-1}$ (Pitchford, 1995. pers. comm.[13]).

The following were identified as major aims and objectives of the present investigation:

- To construct a model system appropriate for performing biocide efficiency studies on a laboratory scale for realistic application to the hot roll mill emulsion;
- To conduct a series of biocide experiments which would assess the ability of roll mill emulsion-inhabiting microorganisms to degrade the emulsion at specific biocide concentrations within the range 0-$5000mgl^{-1}$;
- To assess the effect of large microbial populations on the emulsion;
- To study the effect of microbial growth on the pH of the emulsion and to assess the effect of changing pH on emulsion stability;
- To study the effect of changes in pH on the emulsion to assess the potential for emulsion instability at pH extremes;
- To assess potential microbial resistance to the biocide;
- To recommend to Hulett Aluminium management alterations to the biocide dosing regime currently in use in order to maximize biocide efficiency.

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[13]Dr. A. Pitchford, 1995. Hulett Aluminium hot roll mill Research and Development Department, Hulett Aluminium, Camps Drift, Pietermaritzburg.

## 4.2 Materials and Methods

### 4.2.1 The model system

The model was built to simulate as closely as possible the conditions experienced by the microflora at the hot roll mill. The semi-continuous system (refer to Figure 5) consisted of three tanks, each holding approximately 25ℓ of emulsion.

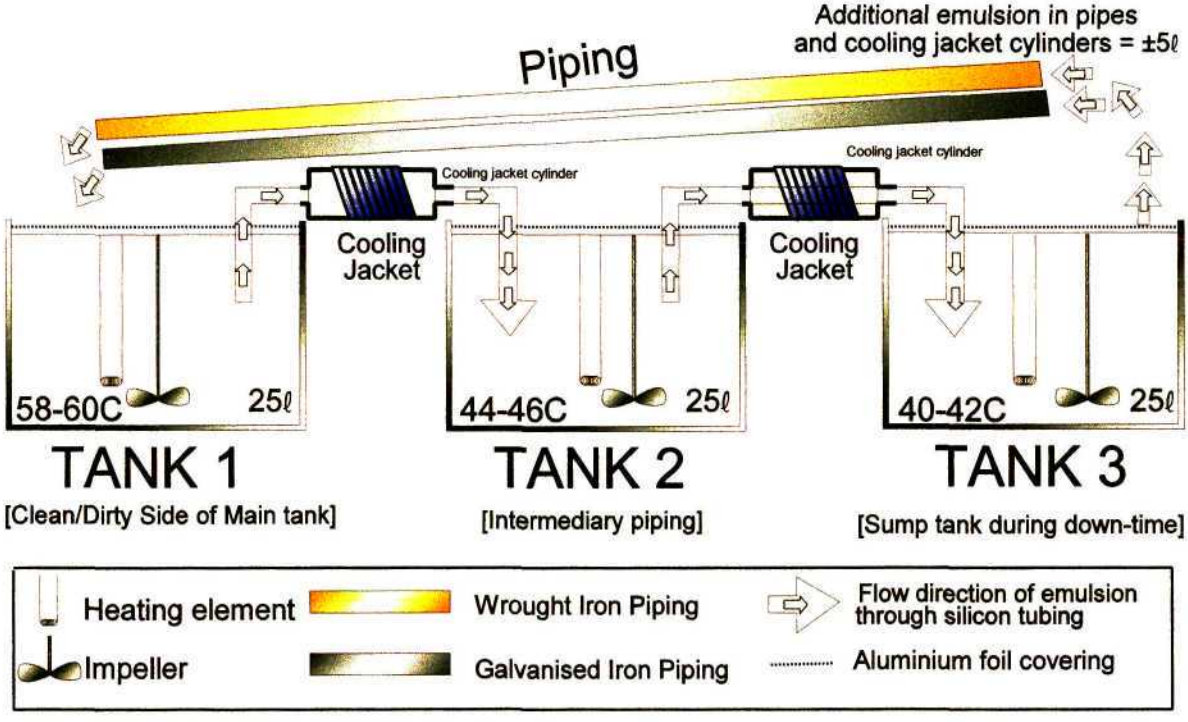


**Figure 5: Model system used for biocide efficiency studies on roll mill oil emulsion**

Initially transfer of the emulsion fluid from one tank to the next was achieved via plastic tubing and a Watson Marlow 604U peristaltic regulatory pump. This plastic tubing became brittle as a result of the heat of the emulsion passing through it and started to leak. Therefore it was subsequently replaced with autoclavable, solvent-resistant silicon tubing. Circulation and temperature control were achieved within the tanks using impellers and heating elements respectively. The impellers originally used were not powerful enough to provide the constant aeration and adequate mixing of the emulsion required to maintain it in a 'tight' form. These were later replaced with stronger impellers capable of maintaining 'tightness' and aeration of the emulsion. Cooling jackets were fitted between the tanks to facilitate reduction in the

temperature of the emulsion whilst it was being pumped from one tank to the next. Two pipes (one galvanized iron and one wrought iron) were incorporated into the system in order to: assess the corrosiveness of long term exposure to increased biocide levels; facilitate gravitational, trickle flow-back of the emulsion from Tank 3 to Tank 1; add ferric ions into the emulsion system, thereby simulating the hot roll mill emulsion reticulation system.

The tanks were maintained at the following temperatures: Tank 1, 58-60°C; Tank 2, 44-46°C and Tank 3, 40-42°C.

### 4.2.2 Biocide experiments

Each experiment involving the model system (except where otherwise stated) was conducted according to the following methodology:

i). Emulsion concentrate (Prosol) was added to each of the tanks and diluted, with tap water, to the normal roll mill concentration of 3% v/v (concentrate/water). Since the water-deionizer at the Hulett Aluminium hot roll mill was not functioning during the experimental stage of this investigation, it was decided that non-deionized tap water should be used to mimic the conditions at the plant.

ii). Biocide (Busan) was added (except during the control run) to each of the tanks at the levels specified in Experiments 1-5.

iii). Inoculum consisted of a mixed population of microorganisms, freshly isolated from the hot roll mill, together with a laboratory-grown, mixed population. Final microbial concentration was in the order of $1x10^8$ colony forming units per milliliter inoculum. Except where otherwise stated the laboratory-grown cultures were cultivated in Prosol emulsion (3% concentration) in the presence of 500 milligrams per liter ($mgl^{-1}$) (or parts per million equivalent) Busan. The cultures were incubated at room temperature (24-26°C), in a magnetically stirred 2ℓ Erlenmeyer flask containing one liter of medium and plugged with a

cotton wool stopper and aluminium foil (Hulett's!) covering. Each week, 250ml of the culture was removed and replaced with 250ml of fresh emulsion containing 500mgl$^{-1}$ biocide. The tanks were inoculated as follows: Tank 1, 100ml inoculum; Tank 2, 100ml inoculum and Tank 3, 300ml inoculum. In total, 500ml of inoculum was added to ±80ℓ of emulsion. The final microbial population in Tank 3, immediately after inoculation (day 0) was in the range $1x10^3$-$1x10^4$ CFUml$^{-1}$ emulsion.

Biocide-resistant or susceptible microbes were inoculated into the tanks depending on the experiment. The term '**Resistant microbes**' refers to microorganisms grown in the presence of biocide for an extended period of time (two months) prior to their introduction to the model system. It was postulated that these resistant microbes would show a similar resistance to the biocide as those microorganisms found in the emulsion at the hot roll mill. '**Susceptible microbes**' were those cultured in the absence of the biocide for an extended period of time (two months), before inoculation into the model system containing one of the specified biocide concentrations (see sections 4.2.2.1-4.2.2.5). It was considered that microbial cells that had not been exposed to biocide for an extended period prior to inoculation into the model system would display reduced resistance to the biocide. This increased sensitivity of the microbes to the biocide would in effect increase the anti-microbial efficiency of the biocide.

iv). The Watson Marlow pump was set at a flow rate of 1ℓ per 6.5 minutes resulting in full cycling of the emulsion in the system in approximately 9 hrs (this included the emulsion in the cooling jacket cylinders and silicon piping).

v). The tanks were covered with aluminium foil to minimize losses from evaporation. Each day Tank 1 was topped-up to the appropriate level with fresh emulsion containing the relevant biocide concentration. In addition, four liters of fresh emulsion containing the appropriate biocide concentrations were routinely added to the system each day after an equivalent amount of 'used' emulsion had been removed from Tank 1. This ensured that the working volumes and biocide concentrations in all the tanks remained approximately the same throughout the

course of the experiments.

vi). Depending on the experiment, daily pH readings and colony counts were done on samples of the emulsion in Tank 3 (40-42°C). Duplicate samples were stored at 4°C for subsequent Malvern Laser analysis (to be carried out at the end of each experiment)(Refer to Appendix D) and glutaraldehyde analysis (Refer to Appendix D). All samples were stored in glass jars with plastic screw cap lids. The pH was measured with a Crison Micro 2000 pH meter fitted with an oil-emulsion probe (Hanna Instruments). For the colony counts, one milliliter sub-samples were serially diluted ($10^{-2}$ to $10^{-7}$) and the cell populations expressed as the number of colony forming units per milliliter of emulsion. A one milliliter sub-sample from each dilution was transferred to a 90mm Petri dish and thoroughly mixed with molten nutrient agar at 42-45°C. The plates were incubated for 36hrs at 37°C. After incubation, colonies were counted with the aid of a WTW Kolnienzählgerät colony counter. Where high biocide concentrations were used (5000mgl$^{-1}$), colony counts were made only every two days.

vii). At the end of each experiment, the system was thoroughly cleaned. The tanks were washed with 70% alcohol and the silicon tubing and iron piping were cleaned and autoclaved to remove and destroy any microflora present.

viii). A control experiment was conducted to determine the percentage active component (glutaraldehyde) in the biocide Busan. Busan (500mgl$^{-1}$) was applied to emulsion containing no microorganisms and which was well aerated at 50°C. Five samples were taken at ten minute intervals and immediately subjected to glutaraldehyde analysis using a HPLC. The active component released into the emulsion was determined to be approximately 40% of the biocide, Busan.

#### 4.2.2.1 Experiment 1: Emulsion containing 0mgl$^{-1}$ Busan and inoculated with biocide resistant microbes

A standard growth curve of the mixed microbial population in emulsion without biocide was

first established so that comparisons with samples containing biocide at differing concentrations could be made. The model system was set up according to the specifications presented in section 4.2.2.

#### 4.2.2.2 Experiment 2: Emulsion containing 500mgl$^{-1}$ Busan and inoculated with biocide resistant microbes

Experiment 2 was conducted in accordance with the specifications presented in section 4.2.2. Biocide was added to the emulsion to bring the biocide concentration to 500mgl$^{-1}$ on the first day of the experiment. Whenever fresh emulsion was added, it was supplemented with 500mgl$^{-1}$ biocide before introduction to the system. It is **important to note** that mgl$^{-1}$ biocide refers to the concentration of the biocide Busan, added to the emulsion **and not** to the concentration of the active component of the biocide, which is glutaraldehyde. The concentration of glutaraldehyde depends on the amount of biocide added to the emulsion as well as on other factors which influence glutaraldehyde release into the emulsion. These include: pH; aeration; temperature; and microbial contamination of the emulsion.

#### 4.2.2.3 Experiment 3: Emulsion containing 1000mgl$^{-1}$ Busan and inoculated with biocide resistant microbes

Experiment 3 was conducted in accordance with the specifications presented in section 4.2.2. Biocide was added to the emulsion to bring the biocide concentration to 1000mgl$^{-1}$ on the first day of the experiment. Whenever fresh emulsion was added, it was supplemented with 1000mgl$^{-1}$ biocide before being introduced into the system.

#### 4.2.2.4 Experiment 4: Emulsion containing 5000mgl$^{-1}$ Busan and inoculated with biocide resistant microbes

Experiment 4 was conducted in accordance with the specifications presented in section 4.2.2. Biocide was added to the emulsion to bring the biocide concentration to 5000mgl$^{-1}$ on the first

day of the experiment. Whenever fresh emulsion was added, it was supplemented with 5000mgl$^{-1}$ biocide before being introduced into the system.

#### 4.2.2.5 Experiment 5: Emulsion containing 500mgl$^{-1}$ Busan and inoculated with biocide susceptible microbes

In experiments 1-4, microbial populations were repeatedly exposed to fresh emulsion supplemented with the biocide prior to incorporation into the model system. In this experiment the microbes were transferred to fresh biocide-free emulsion every two weeks over a period of 2 months. These 'biocide susceptible' microbes were then inoculated as per section 4.2.2 iii. into the tanks containing emulsion supplemented with 500mgl$^{-1}$ biocide. The first phase of this experiment also served to elucidate the long term effects of microbial growth on the emulsion in the absence of biocide.

## 4.3 Results

All the raw data from experiments 1-5 are presented in Appendix E.

### 4.3.1 Experiment 1: Emulsion containing 0mgl$^{-1}$ Busan and inoculated with biocide-resistant microbes

Graphical representation of the results obtained appears in Figures 6a, 6b and 6c.

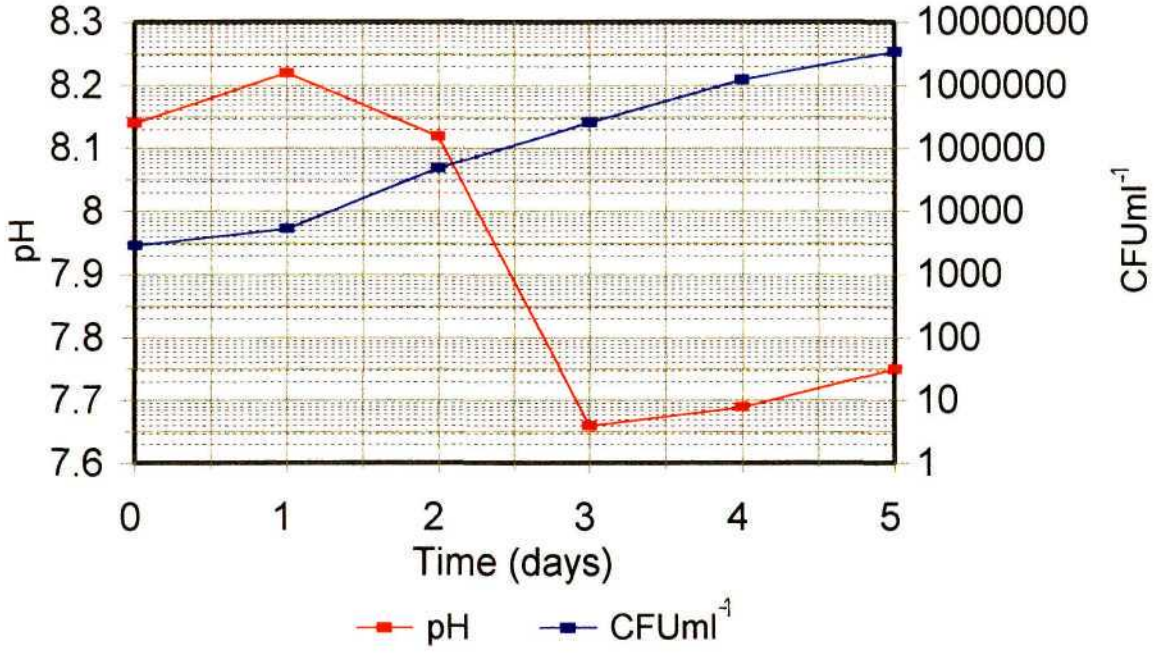


Figure 6a: Relationship between CFUml $^{-1}$ and pH over time in emulsion containing 0mgl$^{-1}$ biocide

The relationship between pH and CFUml$^{-1}$ of emulsion, at 0mgl$^{-1}$ biocide, over time is depicted in Figure 6a. Initial microbe concentration was of the order of 2.9x10$^{3}$ CFUml$^{-1}$ sample. The number of microbes increased steadily until the experiment was terminated on day 5, at which time the population had reached 3x10$^{6}$ CFUml$^{-1}$. The pH remained relatively high (ranging from 8.12-8.22) over the first two days, after which it decreased, reaching a low of 7.66 on day 3. Following this decrease, the pH increased slightly to 7.75 on day 5. The pH started to decline when the CFUs reached 1x10$^{4}$ml$^{-1}$ of emulsion.

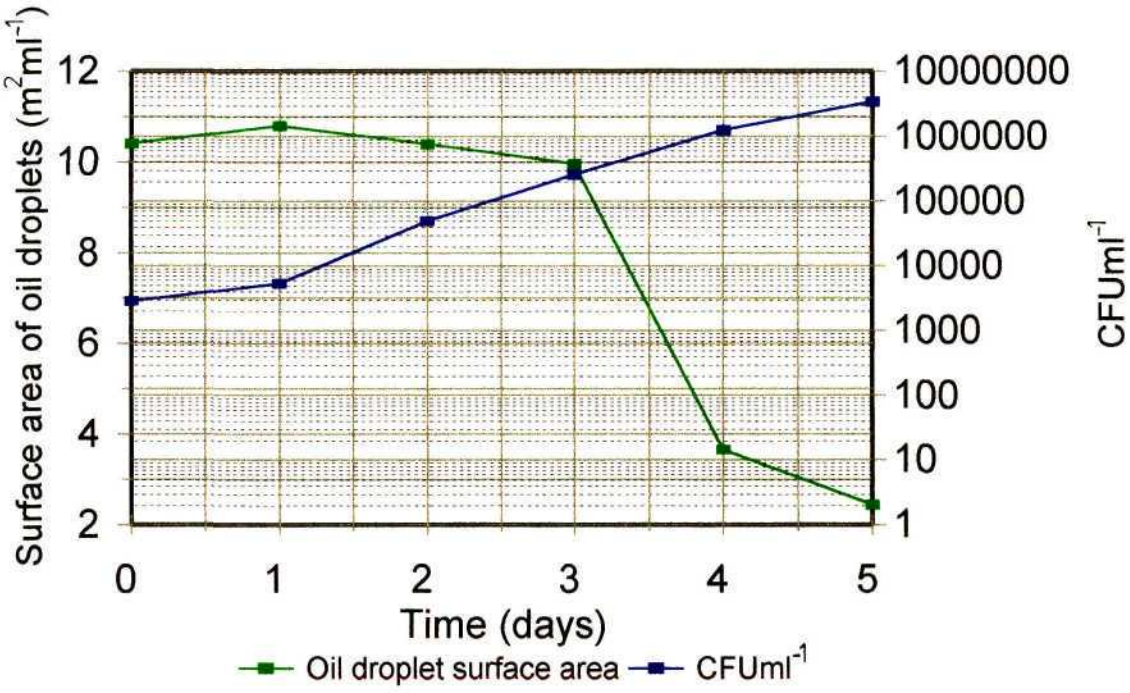


Figure 6b: Relationship between CFUml$^{-1}$ and oil droplet surface area over time in emulsion containing 0mgl$^{-1}$ biocide

Figure 6b shows the relationship between CFUml$^{-1}$ of emulsion and oil droplet surface area over time in the absence of biocide. The emulsion remained 'tight' for 2 days, with the specific oil droplet surface area remaining above 10m$^2$ml$^{-1}$ of emulsion. After this time the emulsion started to 'loosen' until it eventually 'broke' on day 3. 'Loosening' is reflected on the graph by a reduction in oil droplet surface area. From days 1-2, when the emulsion first started to 'loosen', the specific oil droplet surface area decreased from above 10 m$^2$ml$^{-1}$ to just over 4 m$^2$ml$^{-1}$ over the ensuing two day period. The microbial population was of the order of 1x10$^4$-1x10$^5$ CFUml$^{-1}$ when the emulsion first started to 'loosen'.

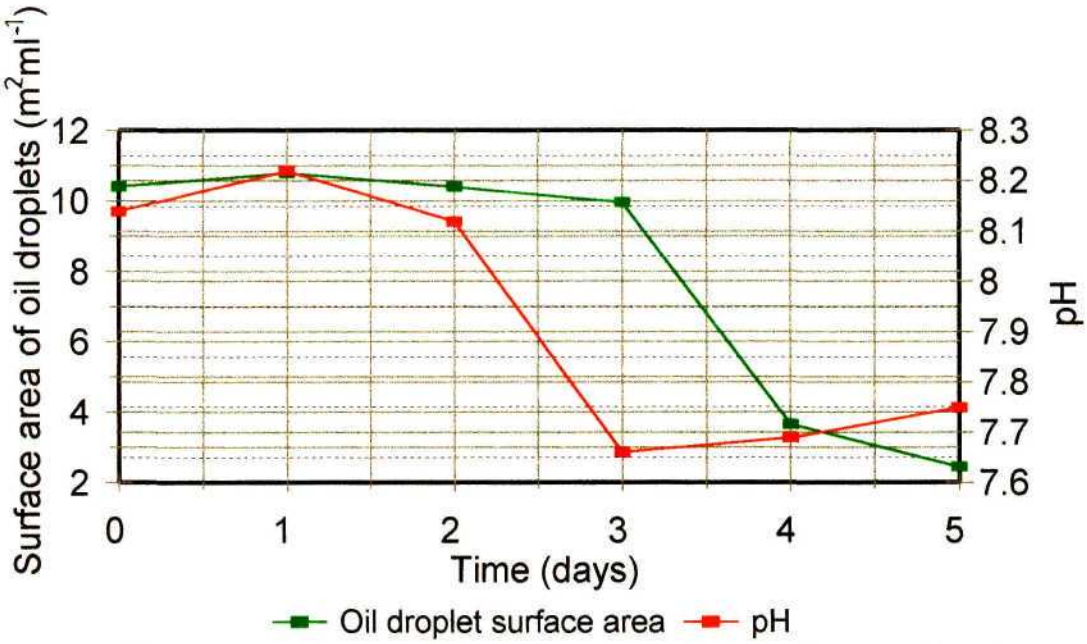


Figure 6c: Relationship between pH and surface area of oil droplets over time in emulsion containing $0mgl^{-1}$ biocide

Figure 6c shows the relationship between emulsion pH and the specific surface area of oil droplets over time. The pH started to decrease on day 1 and by day 3 had dropped to 7.66 from the initial value of 8.14. After day 3 the pH increased slightly to reach 7.75 on day 5 when the experiment was terminated. Emulsion 'loosening' started on days 1-2 as shown in Figure 6b with significant 'loosening' occurring from day 3 onwards. The pH at the onset of 'loosening' was approximately 8.15. A substantial drop of 0.4 pH units is clearly visible prior to the onset of significant 'loosening' of the emulsion on day 3 (Figure 6c).

### 4.3.2 Experiment 2: Emulsion containing $500mgl^{-1}$ Busan and inoculated with biocide-resistant microbes

Graphical representation of the results obtained appears in Figures 7a, 7b, 7c and 7d.

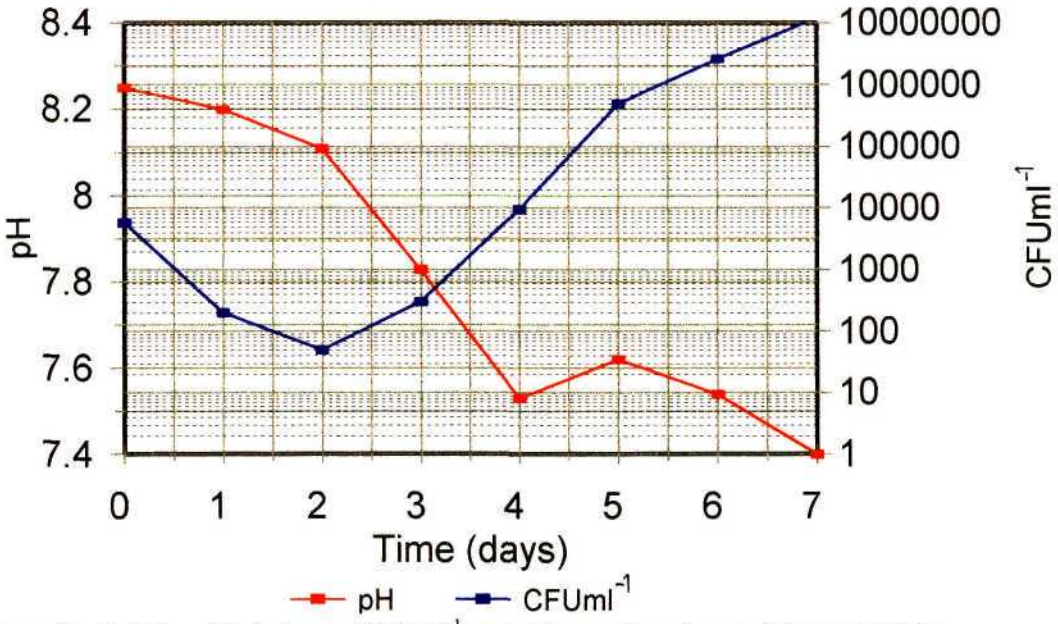


Figure 7a: Relationship between $CFUml^{-1}$ and pH over time in emulsion containing $500mgl^{-1}$ biocide

Figure 7a shows the relationship between $CFUml^{-1}$ of emulsion and pH over time in Prosol-based emulsion containing $500mgl^{-1}$ Busan. Initial microbial counts were of the order of $5.8x10^{3}$ $CFUml^{-1}$ of emulsion. Microbial numbers declined over the initial 2 days reaching a minimum of just over 50 $CFUml^{-1}$ of emulsion on day 2. Following day 2, the microbial population increased steadily until counts exceeded $1x10^{7}$ $CFUml^{-1}$ of emulsion on day 7. The pH dropped soon after inoculation and continued to decrease concomitantly with the increase in microbial population up to day 4. After this, the downward trend in pH became slightly more gradual reaching a value of 7.4 on day 7.

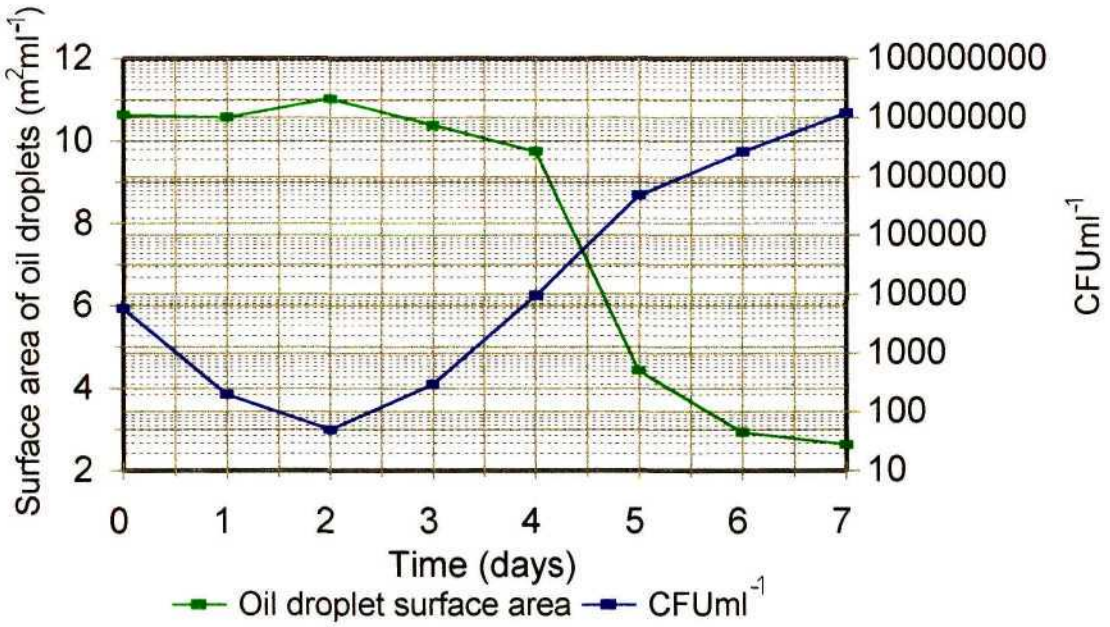


Figure 7b: Relationship between CFUml$^{-1}$ and surface area of oil droplets over time in emulsion containing 500mgl$^{-1}$ biocide

Figure 7b shows the relationship between CFUml$^{-1}$ of emulsion and specific oil droplet surface area over time. The oil droplet surface area was shown to remain high (9.8-11m$^2$ml$^{-1}$) for 4 days. After day 4 the emulsion 'loosened' and breakdown commenced, with complete breakdown (*viz.* no further deterioration) having occurred by day 7. CFUs were of the order of 1x10$^4$ml$^{-1}$ of emulsion at the start of the initial 'loosening' of the emulsion on day 4.

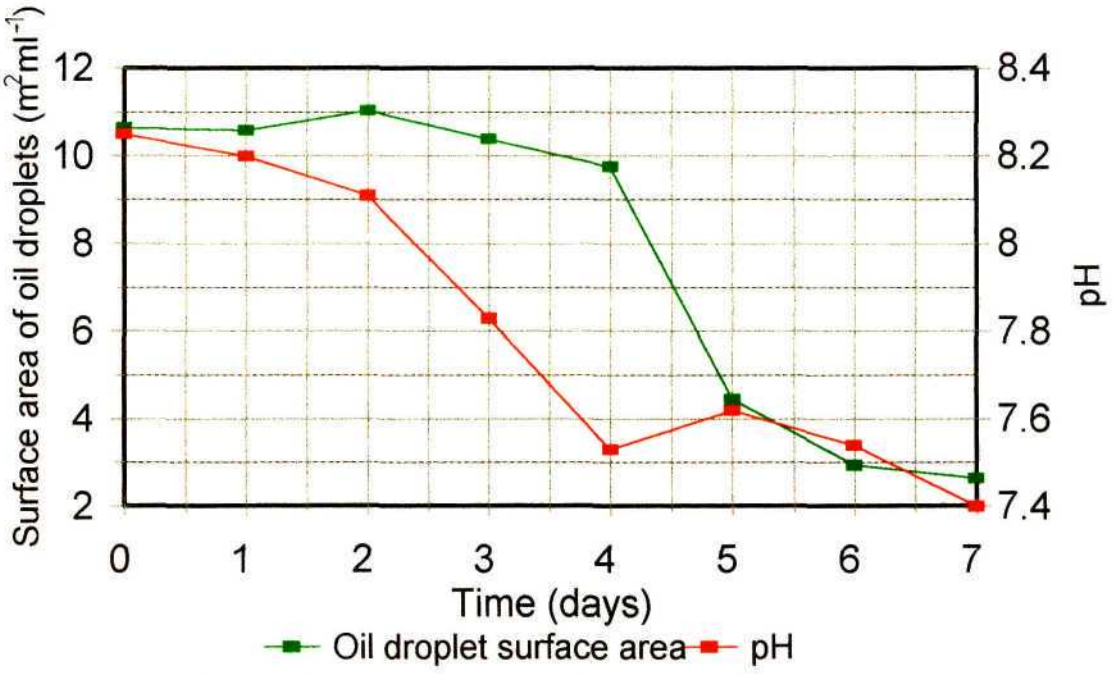


Figure 7c: Relationship between pH and surface area of oil droplets over time in emulsion containing 500mgl$^{-1}$ biocide

The relationship between pH and the specific surface area of oil droplets over time is depicted in Figure 7c. The pH decreased as incubation time increased following a similar trend to that depicted in Figure 6c. Initially the pH was 8.25, however, over the course of the experiment the pH dropped to 7.40. At the time significant 'loosening' of the emulsion commenced, the pH was approximately 7.55.

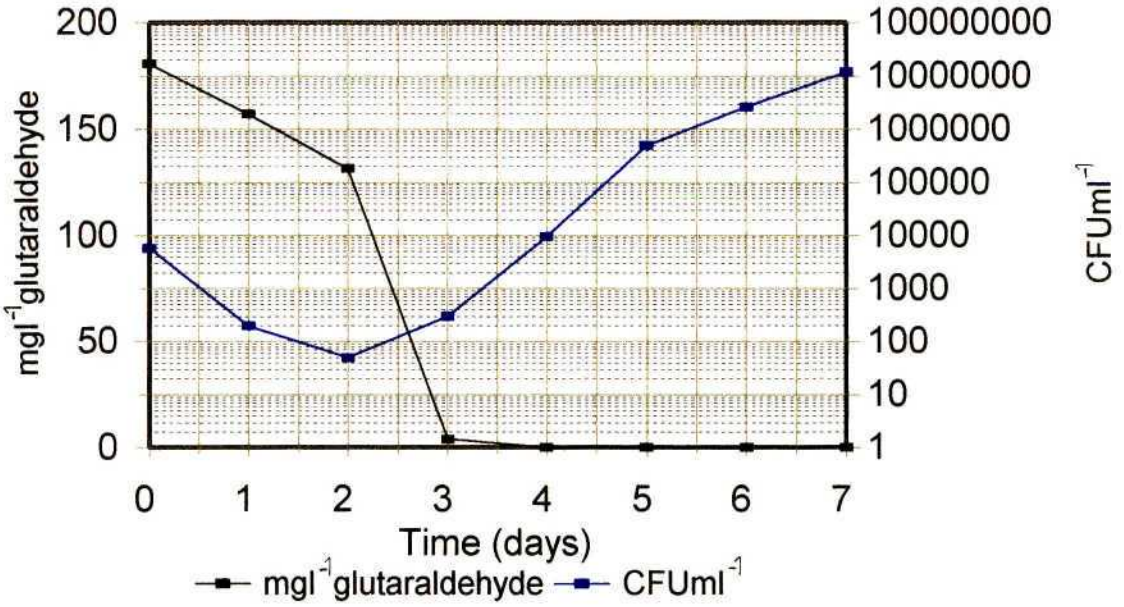


Figure 7d: Relationship between CFUml $^{-1}$ and mgl$^{-1}$ glutaraldehyde over time in emulsion containing 500mgl $^{-1}$ biocide

The relationship between glutaraldehyde concentration and CFUml$^{-1}$ of emulsion over time is depicted in Figure 7d. Initial glutaraldehyde concentration in the emulsion at the time of inoculation was 180mgl$^{-1}$. A rapid reduction in the concentration of glutaraldehyde was observed, with complete disappearance from the system occurring in 4 days. Glutaraldehyde concentration had decreased to 130mgl$^{-1}$ on day 2 when the CFUml$^{-1}$ first started to increase. Approximately 0.5x10$^{2}$CFUml$^{-1}$ were present at the time the increase in microbial growth first became apparent. The microbial population continued to increase, reaching approximately 1x10$^{7}$ CFUml$^{-1}$ emulsion when the experiment was terminated on day 7.

### 4.3.3 Experiment 3: Emulsion containing 1000mgl$^{-1}$ Busan and inoculated with biocide-resistant microbes

Graphical representation of the results appears in Figures 8a, 8b, 8c and 8d.

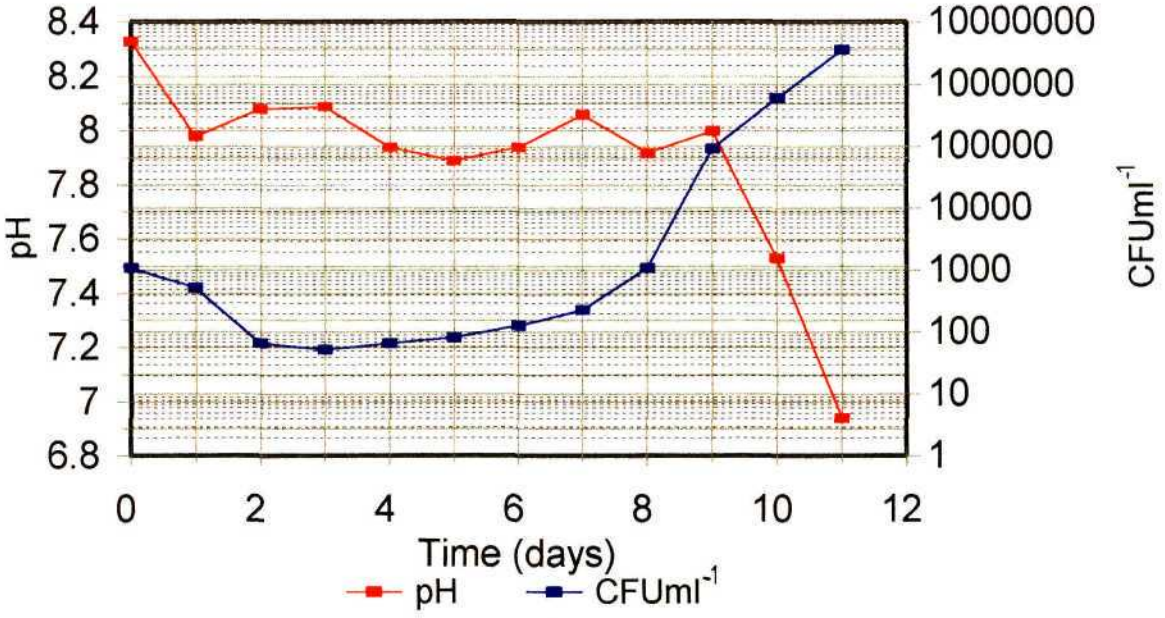


Figure 8a: Relationship between CFUml $^{-1}$ and pH over time in emulsion containing 1000mgl $^{-1}$ biocide

The relationship between pH and viable cell numbers over time is depicted in Figure 8a. Initially microbial numbers were $1x10^3$ CFUml$^{-1}$ of emulsion. For the first 3 days an overall decline in the microbial population from $1x10^3$ to $0.6x10^2$CFUml$^{-1}$ occurred. On day 4 a slight increase in cell numbers was observed. This modest rate of increase in microbial cell numbers continued for 3 days after which a marked increase in population size occurred. This increase is indicated by the steep upward slope of the growth curve from day 7 to the conclusion of the experiment on day 11, when the microbial population was in excess of $3x10^6$ CFUml$^{-1}$. The pH remained relatively stable until day 9 when it decreased rapidly, decreasing from just below 8, when the CFU count was $1x10^5$ml$^{-1}$, to below 7 on day 11 when the microbial population had reached $3.6x10^6$ CFUml$^{-1}$.

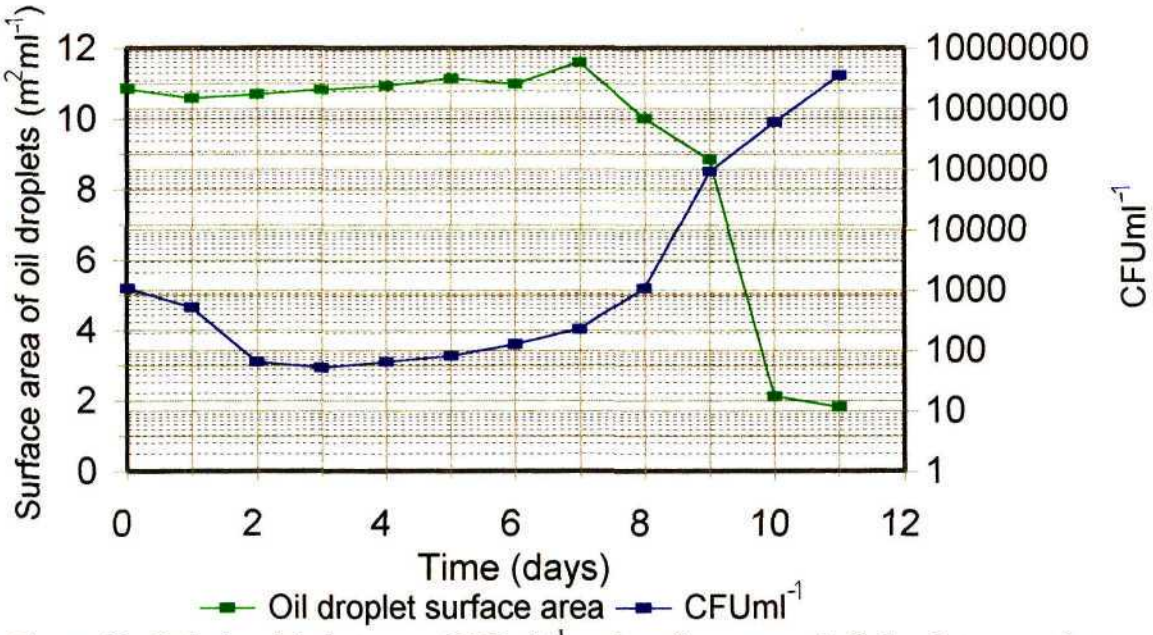


Figure 8b: Relationship between CFUml $^{-1}$ and surface area of oil droplets over time in emulsion containing 1000mgl $^{-1}$ biocide

Figure 8b depicts changes in specific surface area of the oil droplets and viable cell numbers over time. The specific surface area of the oil droplets remained high for 7-8 days after which the emulsion started to 'loosen', with breakdown finally occurring between days 8-11. At the onset of 'loosening' (days 7-8), the number of microbial cells had reached $1x10^3$CFUml$^{-1}$ of emulsion.

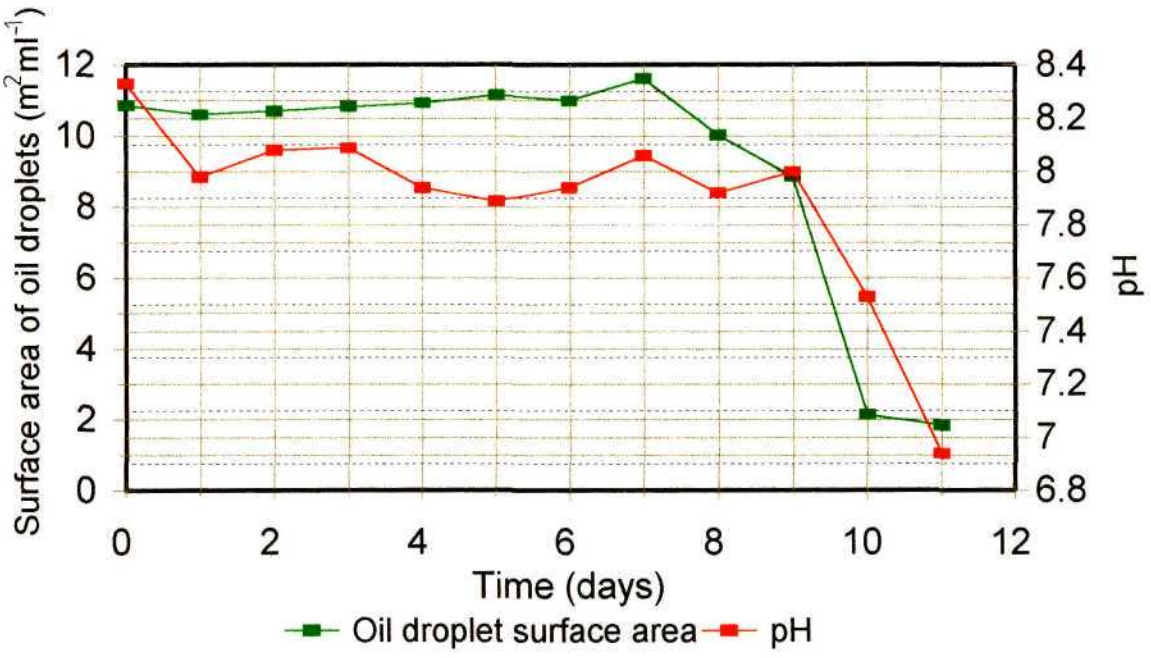


Figure 8c: Relationship between pH and surface area of oil droplets over time in emulsion containing 1000mgl$^{-1}$ biocide

Figure 8c shows changes in pH and specific surface area of oil droplets over time. The pH remained relatively stable, not dropping below 7.89, for 9 days. Following this period of stability the pH fell sharply to 6.94 on day 11. The pH at the time of onset of emulsion 'loosening' (between days 7-8) ranged from 8.06-7.92.

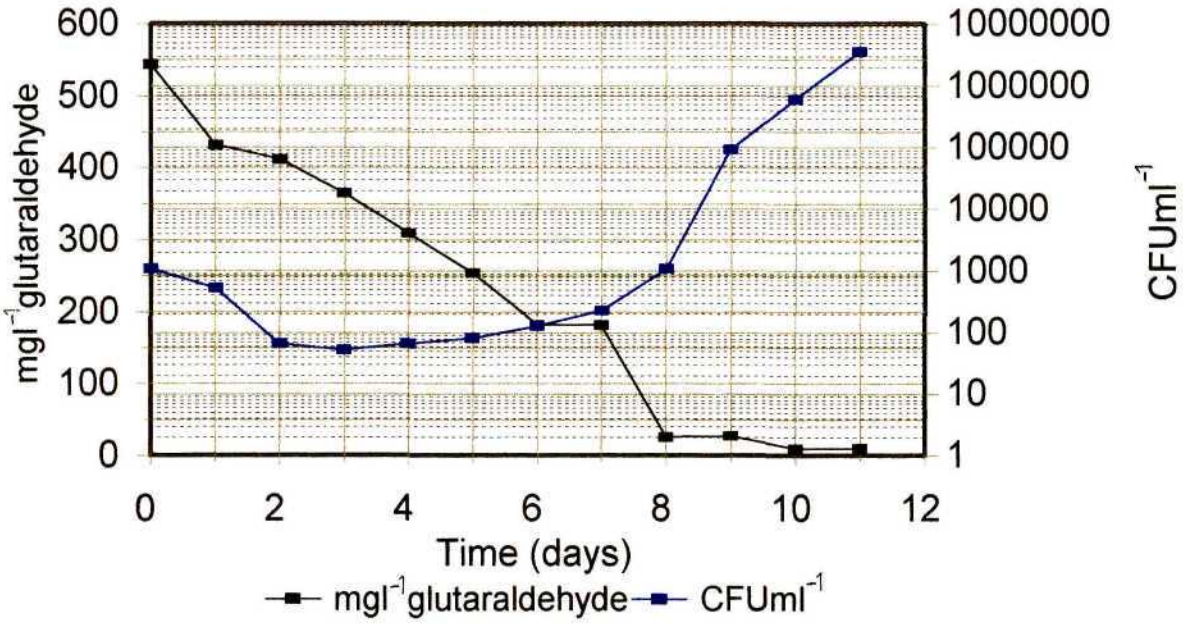


Figure 8d: Relationship between CFUml $^{-1}$ and mgl$^{-1}$ glutaraldehyde over time in emulsion containing 1000mgl $^{-1}$ biocide

The relationship between glutaraldehyde concentration and CFUml$^{-1}$ of emulsion over time is depicted in Figure 8d. The initial glutaraldehyde concentration was 544mgl$^{-1}$. This was three times the concentration of glutaraldehyde found in Experiment 2 (where 500mgl$^{-1}$ Busan was added to the emulsion) and as a result 180mgl$^{-1}$ more glutaraldehyde was detected than was anticipated. This had decreased to 10mgl$^{-1}$ on day 11 when the experiment was concluded. Microbial numbers started to increase gradually on day 4 after an initial drop from 1000 CFUml$^{-1}$ to 0.53x10$^{2}$CFUml$^{-1}$ between days 0 and 3. This increase in cell numbers coincided with the time when the glutaraldehyde concentration had decreased to approximately 310mgl$^{-1}$. A rapid increase in microbial numbers commenced on day 7 by which time the concentration of glutaraldehyde had decreased to 160mgl$^{-1}$. Between day 7 and 11, the glutaraldehyde concentration decreased to about zero while the microbial population increased very rapidly to approximately 3x10$^{6}$CFUml$^{-1}$.

### 4.3.4 Experiment 4: Emulsion containing 5000mgl$^{-1}$ Busan and inoculated with biocide-resistant microbes

Graphical representation of the data obtained is presented in Figures 9a, 9b, 9c and 9d.

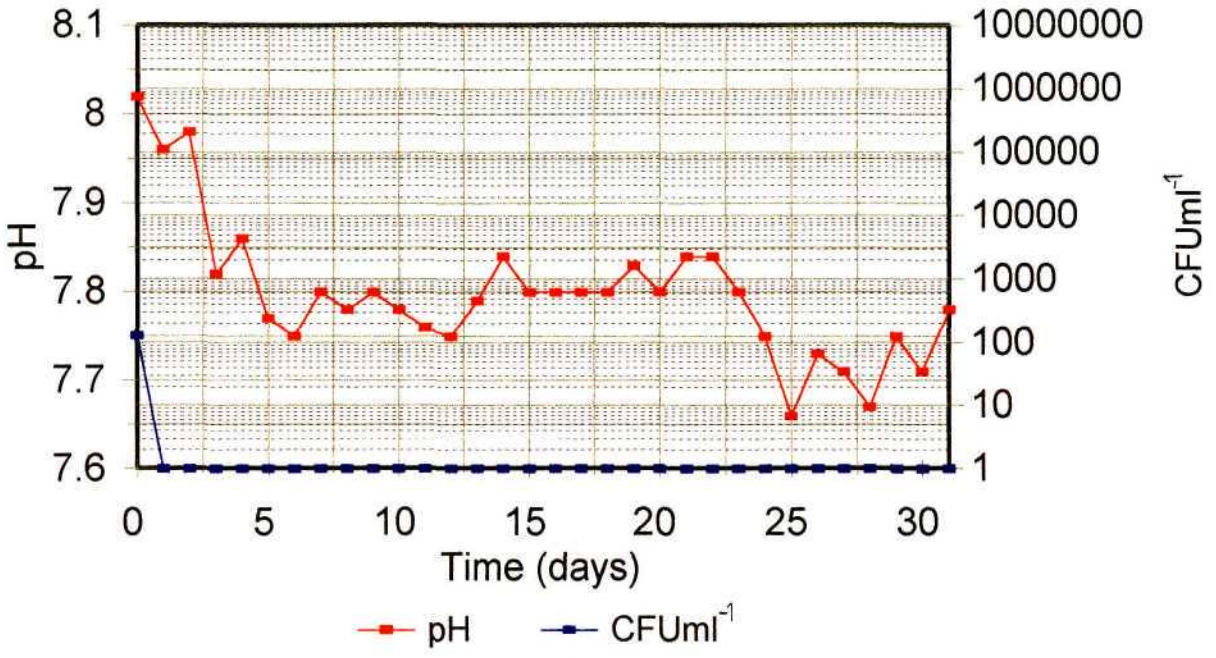


Figure 9a: Relationship between CFUml $^{-1}$ and pH over time in emulsion containing 5000mgl $^{-1}$ biocide

Figure 9a shows the relationship between pH and microbial population over time in emulsion containing 5000mgl$^{-1}$ biocide. The initial microbial population was 1.3x10$^{2}$CFUml$^{-1}$ of sample. No viable cells were detected at any subsequent sampling times. The pH fluctuated erratically, varying from a high value of 8.02 on day 0 to lows of 7.66 on day 25 and 7.67 on day 28. A stable period from days 4 through 22 occurred with the pH varying only slightly between 7.86 and 7.75.

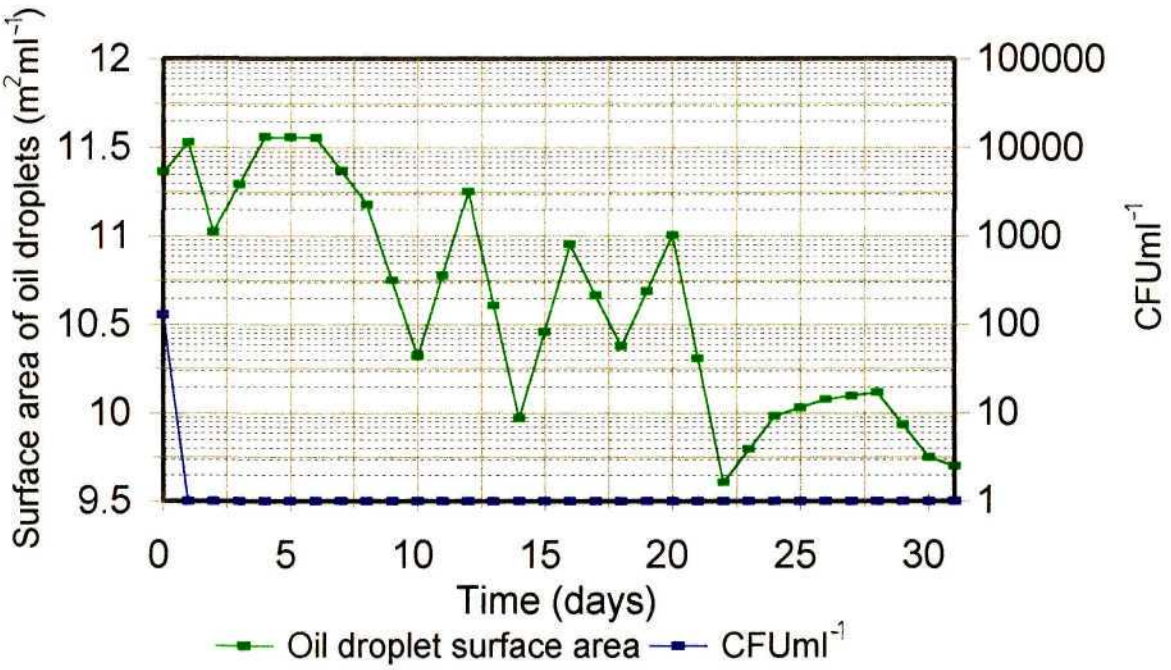


Figure 9b: Relationship between CFUml $^{-1}$ and surface area of oil droplets over time in emulsion containing 5000mgl $^{-1}$ biocide

Figure 9b shows the relationship between the surface area of the oil droplets and the number of colony forming units per milliliter of biocide-containing (5000mgl$^{-1}$) emulsion over time. The surface area of the oil droplets fluctuated from 11.36m$^2$ml$^{-1}$ on day 0 to 9.75m$^2$ml$^{-1}$ on day 30. CFUs were only detected immediately after inoculation. Further sampling showed no CFUs to be present in the emulsion throughout the duration of the experiment. Although minimal 'loosening' of the emulsion occurred, total 'breakdown' was not observed.

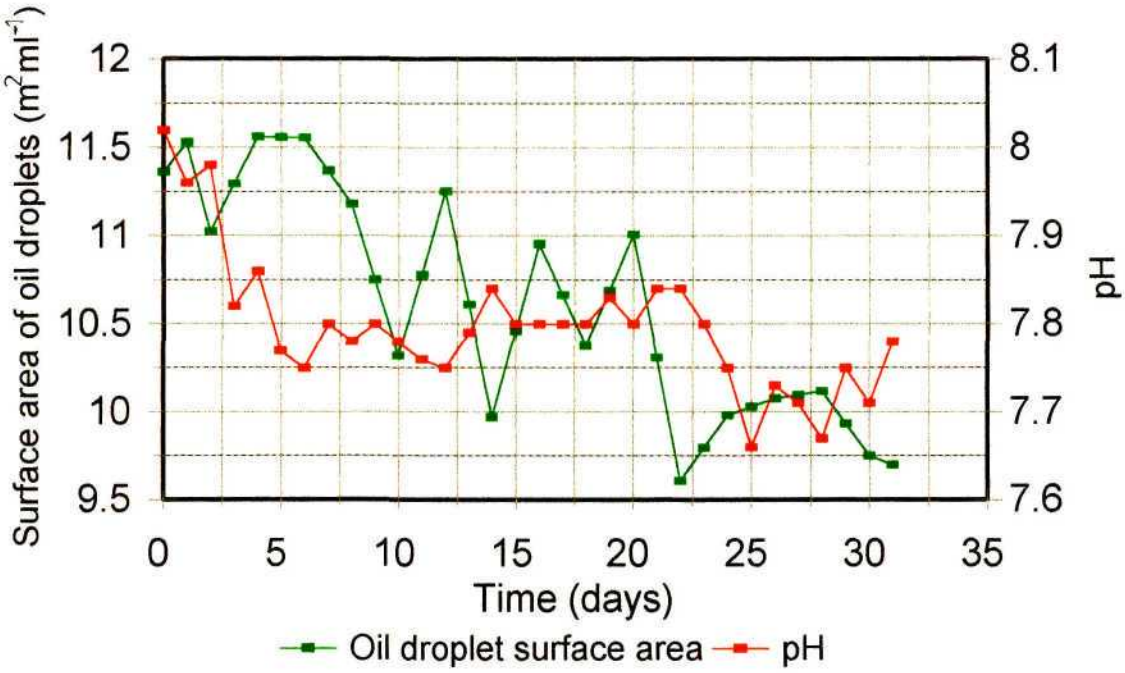


Figure 9c: Relationship between pH and surface area of oil droplets over time in emulsion containing 5000mgl $^{-1}$ biocide

Figure 9c shows that a gradual but variable reduction in the surface area of the oil droplets occurred over the course of the experiment. This coincided with a similar erratic decrease in the pH. The pH declined from an initial value of 8.02 to a final value of 7.78 on day 31. Over the same period the oil droplet surface area decreased from 11.36 to 9.75$m^2ml^{-1}$.

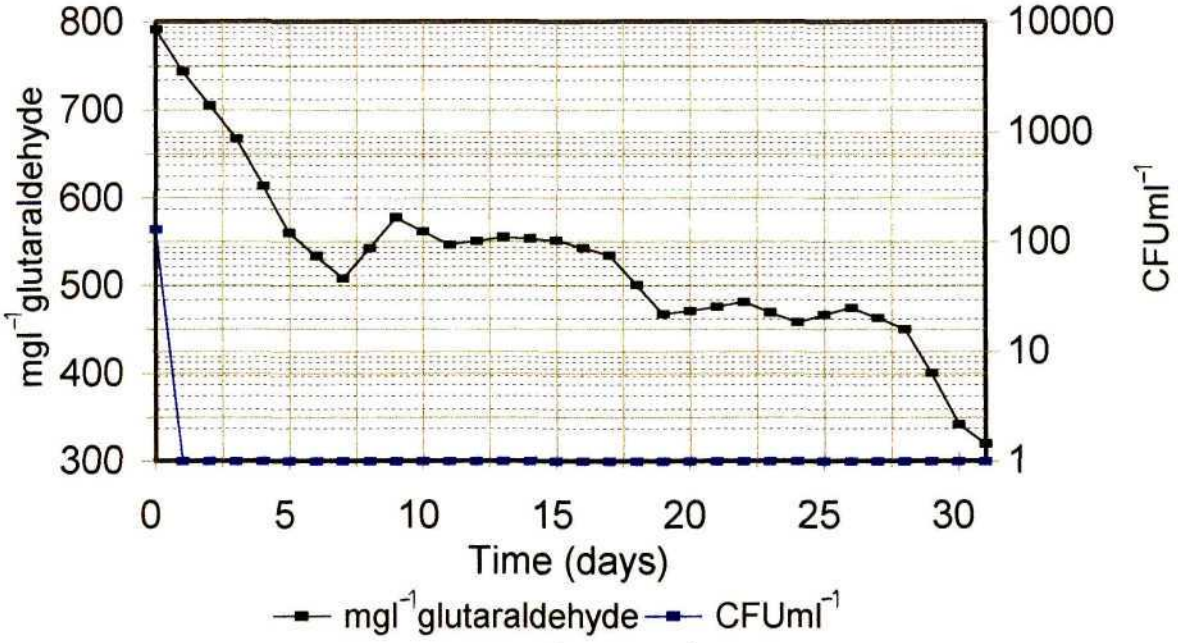


Figure 9d: Relationship between CFUml $^{-1}$ and mgl$^{-1}$ glutaraldehyde over time in emulsion containing 5000mgl $^{-1}$ biocide

The concentration of glutaraldehyde at the start of the experiment was 792mgl$^{-1}$ (Figure 9d). This concentration was far below the expected value of 1800mgl$^{-1}$ (10 times the concentration of glutaraldehyde detected in emulsion containing 500mgl$^{-1}$ Busan). This glutaraldehyde concentration gradually decreased to 350mgl$^{-1}$ on day 30 when the experiment was terminated. The glutaraldehyde concentration decreased rapidly over the first 7 days to 578mgl$^{-1}$, representing a loss of approximately 220mgl$^{-1}$ glutaraldehyde. Following this initial rapid decline, the concentration decreased gradually to 342mgl$^{-1}$ on day 30. The number of CFUs dropped significantly from the initial concentration of $1.3x10^2$CFUml$^{-1}$ to zero CFUml$^{-1}$ on day 1. Even though no CFUs were detected for the remainder of the experiment, the concentration of glutaraldehyde continued to diminish.

### 4.3.5 Experiment 5: Emulsion containing 500mgl$^{-1}$ Busan and inoculated with biocide-susceptible microbes

Graphical representation of the data obtained is presented in Figures 10a, 10b, 10c and 10d.

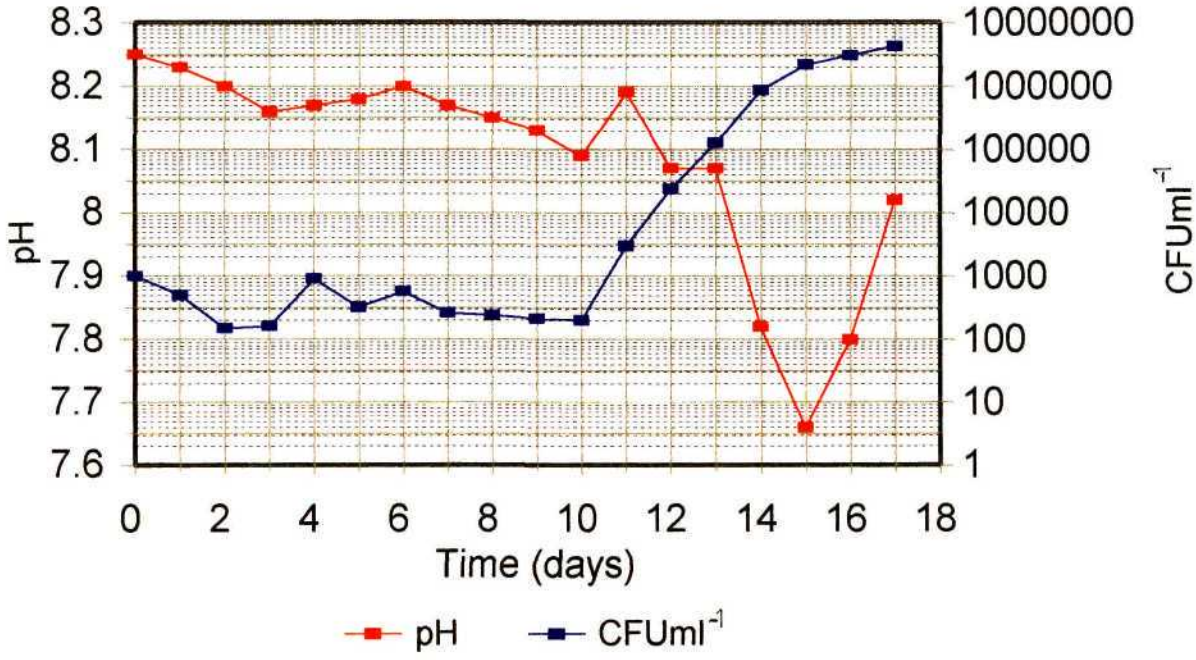


Figure 10a: Relationship between CFUml $^{-1}$ and pH over time in emulsion containing 500mgl $^{-1}$ biocide and inoculated with biocide-susceptible microorganisms

Figure 10a shows the relationship between pH and microbial population over time in emulsion containing 500mgl$^{-1}$ biocide and biocide-susceptible microbes as inoculum. Initial microbial counts of 1x10$^{3}$ CFUml$^{-1}$ of emulsion were obtained. Growth was inhibited until day 10 when a marked increase in microbial numbers occurred. By day 17 the microbial population had reached 1x10$^{6}$ CFUml$^{-1}$. The pH decreased from 8.25 on day 0 to 7.66 on day 15. This decrease in pH corresponded with the increase in microbial population. After day 15, the pH increased and attained a value of 8.02 on day 17 when the experiment was terminated.

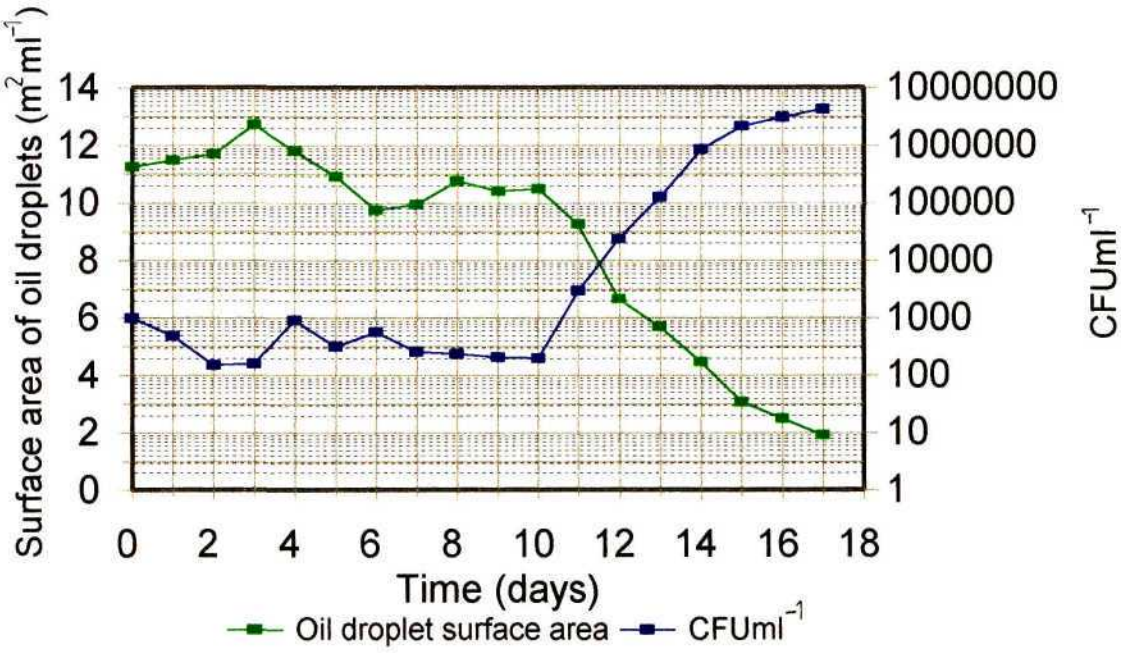


Figure 10b: Relationship between CFUml$^{-1}$ and surface area of oil droplets over time in emulsion containing 500mgl$^{1}$ biocide and inoculated with biocide-susceptible microorganisms

Figure 10b shows the relationship between the surface area of the oil droplets and the number of colony forming units per milliliter of emulsion containing 500mgl$^{-1}$ biocide over time, with susceptible microflora as inoculum. The emulsion remained 'tight' for 10 days with the oil droplet surface area ranging from 12.73 to 9.76 $m^2ml^{-1}$. It finally 'broke' over days 10-11, decreasing from 10.50 to 9.27$m^2ml^{-1}$. 'Loosening' continued over the remaining period of the experiment resulting in a final specific oil droplet surface area of 1.92$m^2ml^{-1}$ on day 17.

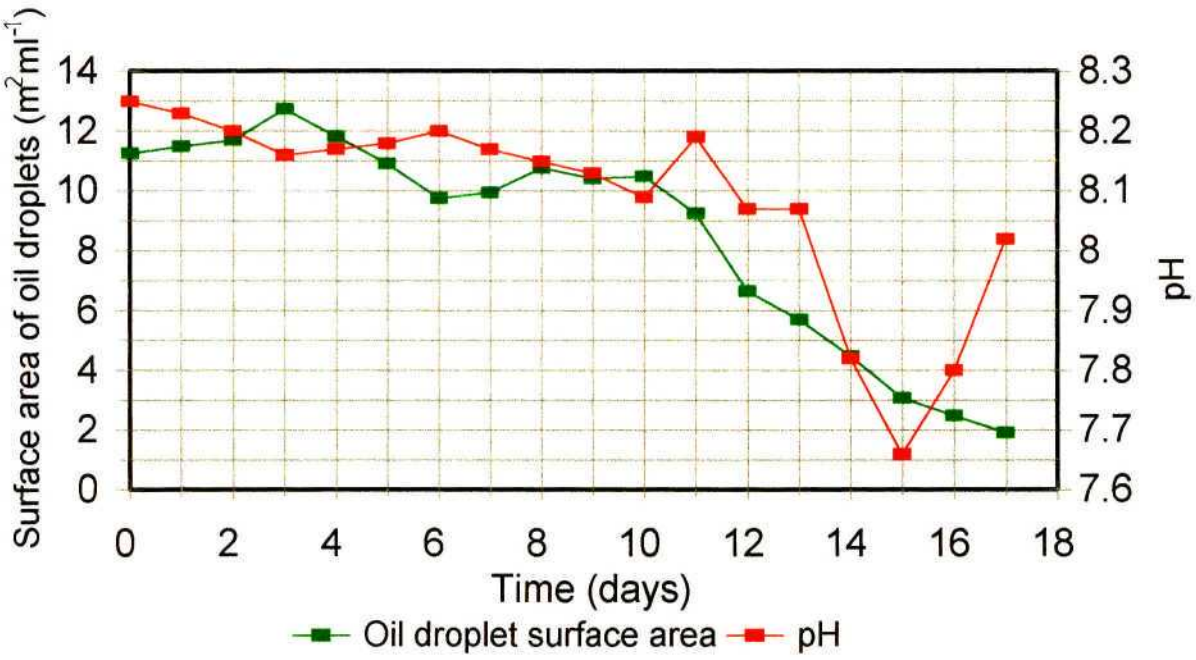


Figure 10c: Relationship between pH and surface area of oil droplets over time in emulsion containing 500mgl $^{-1}$ biocide and inoculated with biocide-susceptible microorganisms

Figure 10c shows emulsion pH and specific surface area of oil droplets over time at 500mgl$^{-1}$ biocide with susceptible microbes. The pH decreased slowly from 8.25 on day 0 to 7.66 on day 15 and then increased slightly to 8.02 on day 17 when the experiment was terminated. The specific surface area of the emulsion remained relatively constant until day 10-11 when the emulsion 'broke'. The pH at the time of the emulsion breaking was 8.14.

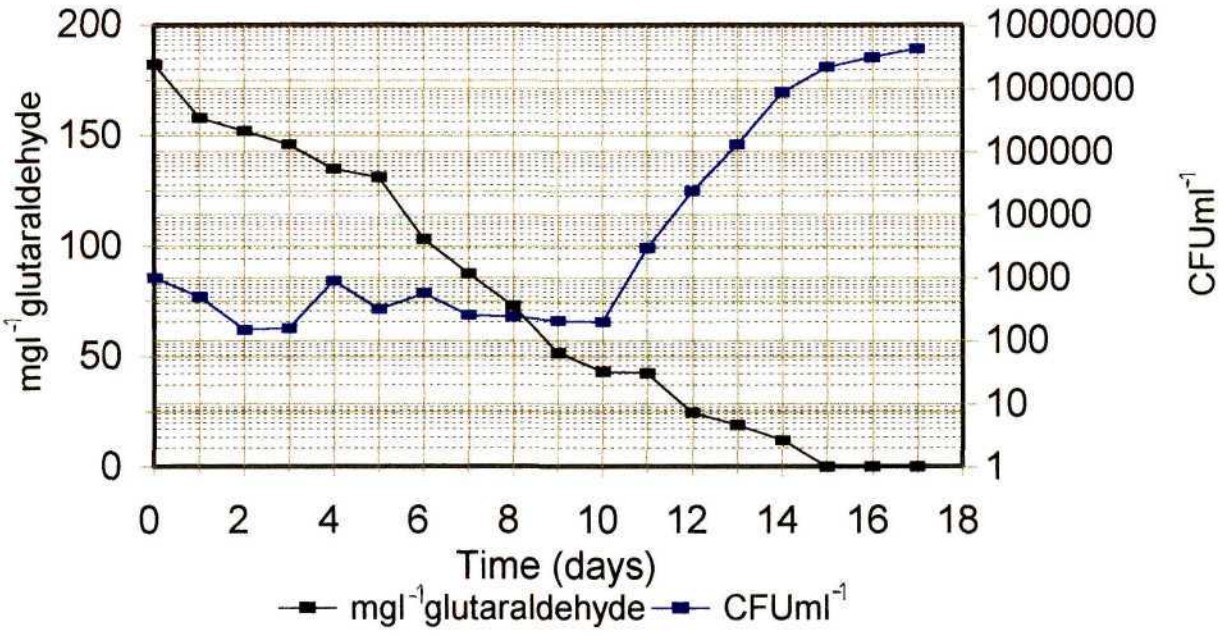


Figure 10d: Relationship between CFUml $^{-1}$ and mgl$^{-1}$ glutaraldehyde over time in emulsion containing 500mgl $^{-1}$ biocide and inoculated with biocide-susceptible microorganisms

Figure 10d shows the relationship between glutaraldehyde concentration and size of susceptible microbial populations over time. Initially the glutaraldehyde concentration was 182mgl$^{-1}$. The decrease in glutaraldehyde concentration was gradual but constant. Rapid increases in microbial numbers occurred over days 10-11 when the glutaraldehyde concentration had decreased to 43mgl$^{-1}$. A small but significant increase in microbial numbers also occurred from days 3-4 during which time the population increased by one order of magnitude. The glutaraldehyde concentration at this stage was approximately 130mgl$^{-1}$. No glutaraldehyde remained in the system by day 16.

### 4.3.6 General Trends

For ease of discussion, the results of the five experiments described above have been summarized in Figures 11-14.

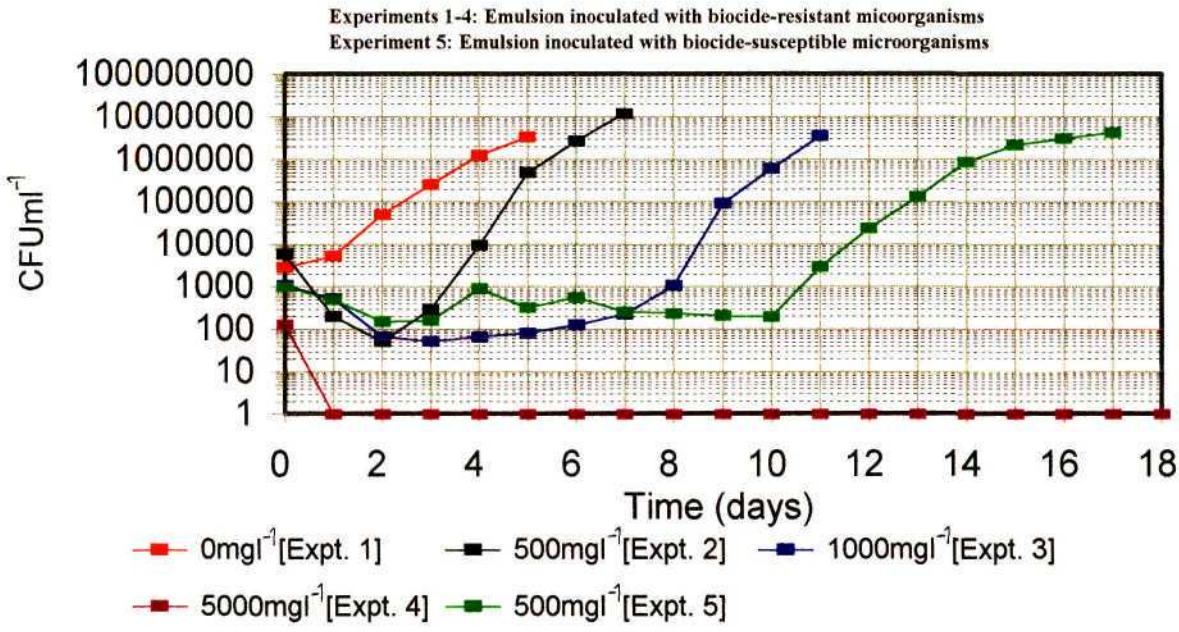


Figure 11:Changes in $CFUml^{-1}$ emulsion over time at various biocide concentrations

Figure 11 shows the change in $CFUml^{-1}$ of emulsion with time at the different concentrations of biocide tested. In Experiment 1 the microorganisms displayed rapid growth (with no lag phase evident) in the absence of biocide. Microbial growth was retarded for a longer period of time in Experiment 2, where a lag phase occurred over the first two days. In this experiment, the inoculum was cultured in emulsion containing $500mgl^{-1}$ Busan prior to introduction into the model system. In Experiment 3, where the microflora were exposed to emulsion containing $1000mgl^{-1}$ Busan, growth was restricted for a protracted period (indicated by a lag phase of roughly three days). This lag phase was followed by a gentle increase in the growth rate to day seven at which time the growth rate increased rapidly until the experiment was terminated on day 11. No microbial growth was detected in Experiment 4 where $5000mgl^{-1}$ biocide was incorporated into the emulsion. When susceptible microbes were

introduced into the model system containing 500mgl$^{-1}$ Busan (Experiment 5), a long lag phase was evident. This lag phase was longer than those observed in Experiments 2 and 3 in which biocide resistant microbial populations were used.

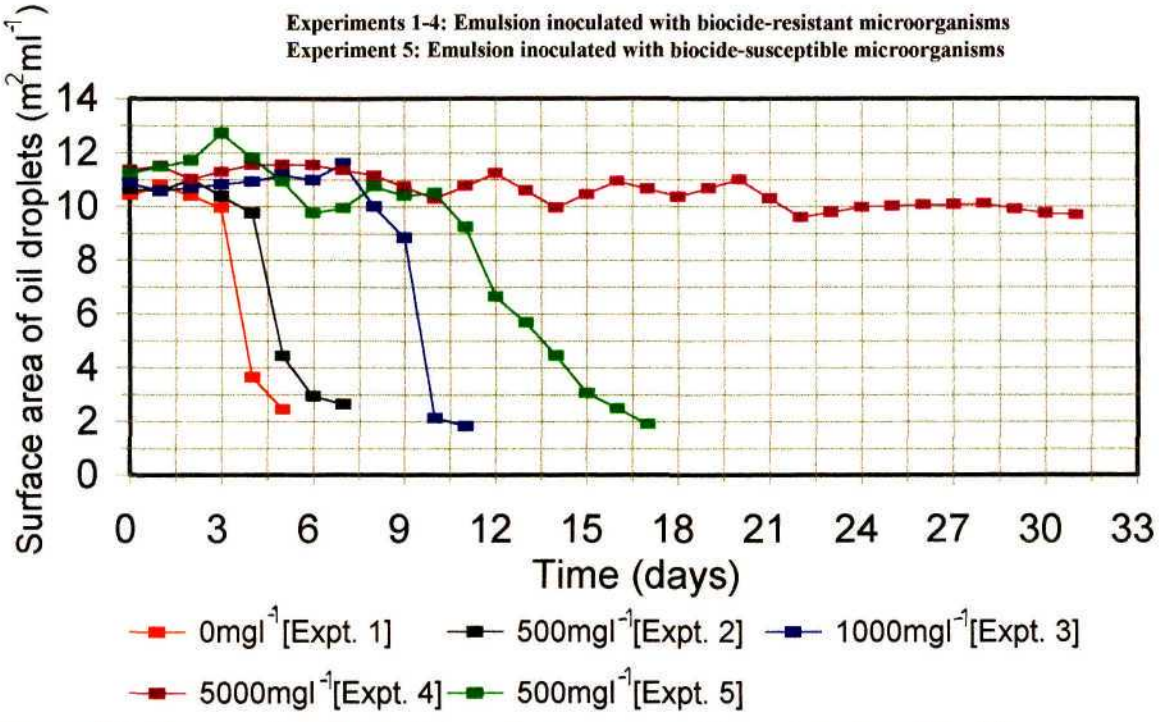


Figure 12: Changes in oil droplet surface area over time at various biocide concentrations

The biodeterioration of the emulsion over time, reflected as a decrease in specific surface area of the oil droplets, is shown in Figure 12. When 5000mgl$^{-1}$ biocide was present, the emulsion showed no sign of biodeterioration, even after 30 days (Expt. 4). The most rapid biodeterioration occurred in the biocide-free emulsion (Expt. 1). The next most rapid rate of biodeterioration occurred in emulsion containing 500mgl$^{-1}$ Busan (Expt. 2) followed by that containing 1000mgl$^{-1}$ Busan (Expt. 3). In Experiment 5, where biocide-susceptible microorganisms were inoculated into the system in the presence of 500mgl$^{-1}$ Busan, the growth of the microorganisms was inhibited for 10-11 days, thereby extending the period of emulsion stability.

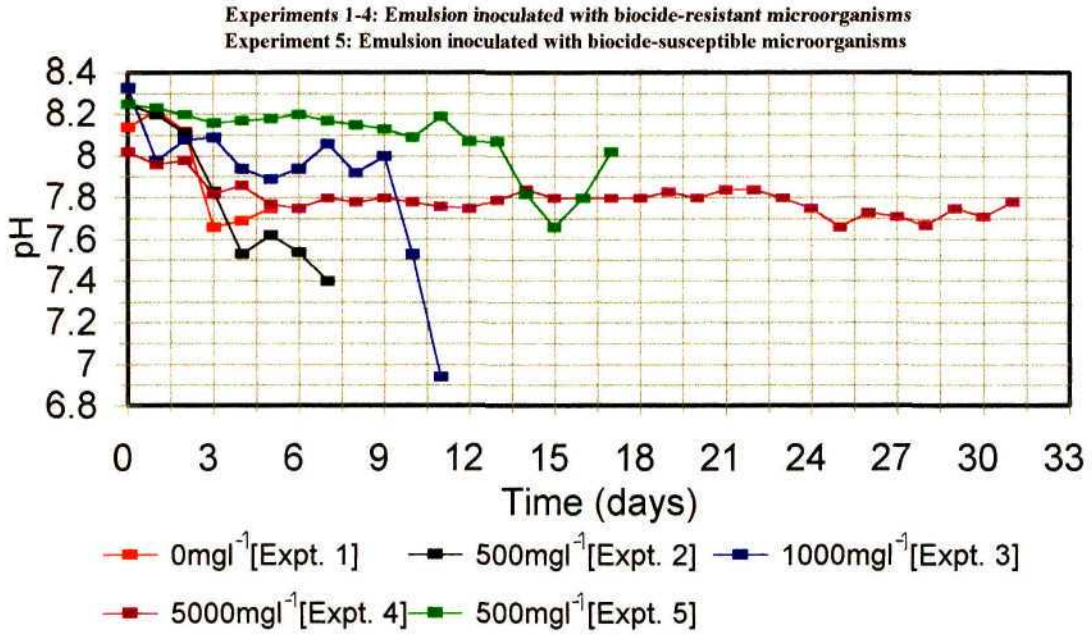


Figure 13: Changes in pH over time in emulsion containing different biocide concentrations

Figure 13 shows the changes in pH during each of the experiments. Significant and comparatively rapid decreases in pH were detected in emulsion containing 500mgl$^{-1}$ biocide and a resistant microbial population (Expt. 2) and in the emulsion containing 1000mgl$^{-1}$ biocide with resistant microbes (Expt. 3). Decreases in pH were also observed in the control where no biocide was added (Expt. 1) and in the emulsion containing 500mgl$^{-1}$ biocide and susceptible microbes (Expt. 5), except that in each of these latter cases the decrease was followed by a small yet significant increase in pH. At 5000mgl$^{-1}$ biocide the pH of the emulsion remained relatively constant (Expt. 4).

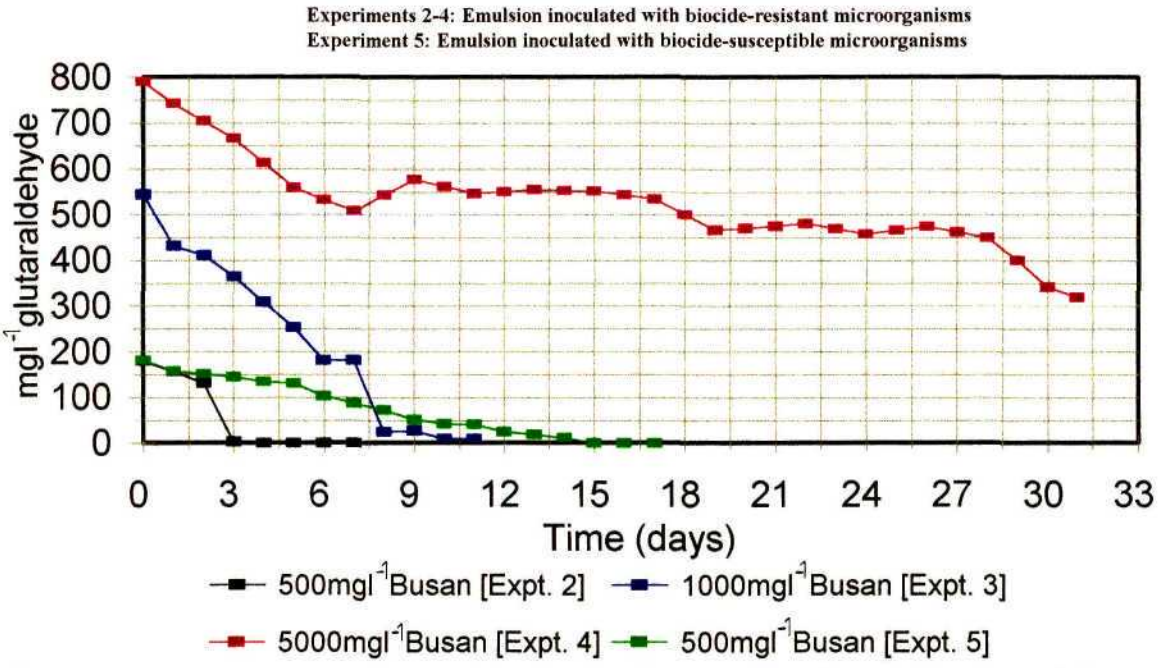


Figure 14: Changes in glutaraldehyde concentration over time in emulsion containing different biocide concentrations

Changes in glutaraldehyde concentration in the emulsion over time for each of the five experiments is shown in Figure 14. In emulsions containing 500mgl$^{-1}$ biocide, with both resistant and susceptible microbes (Experiments 2 and 5 respectively), initial glutaraldehyde concentration were 180 and 182mgl$^{-1}$ respectively. Disappearance of glutaraldehyde from the system occurred more rapidly (within 3 days) in Experiment 2 where the microbial population was biocide resistant than in Experiment 5 where the microbial population was biocide susceptible and glutaraldehyde levels were reduced to zero only after 15 days. This is evident from the respective graphs in Figure 14. In emulsion containing 1000mgl$^{-1}$ Busan (Experiment 3), initial glutaraldehyde concentration was 450mgl$^{-1}$ with near complete disappearance of the glutaraldehyde from the system occurring by day 11. In the emulsion initially containing 5000mgl$^{-1}$ Busan (Experiment 4), a gradual reduction in glutaraldehyde concentration occurred. This continued until day 30 when the experiment was terminated by which time the glutaraldehyde concentration had been reduced from 800mgl$^{-1}$ to 340mgl$^{-1}$.

## 4.4 Discussion

### 4.4.1 Model system

Despite the problems encountered during the construction and operation of the model system useful results were, nonetheless, obtained. Although an extensive search of the literature was made, to the present author's knowledge this was the first semi-continuous model system built specifically to study the effects of microbial populations on an aluminium hot roll mill emulsion. Several explanations can be offered to explain the scarcity of literature on this subject. Firstly, since many of the emulsions used in aluminium hot roll mills are of considerable economic importance to both the manufacturer and the user of the emulsion and because of competition among the manufacturers, much of the research carried out is highly confidential and, therefore, not widely published. Secondly; despite being less appropriate batch culture experiments are much easier to design and implement than are semi-continuous model systems. Thus most of the workers in this field have used batch culture experiments (Sabina and Pivnick, 1956; Ellis *et al.*, 1957; Bennett, 1962; Holtzman *et al.*, 1982; Leder and Russo, 1989). It is therefore difficult to compare the results obtained from the present model system experiments with those of other workers.

Unfortunately, due to the generally long generation times of anaerobic bacteria which grow in hot roll mill systems, and because of the aeration of the emulsion in the model system caused by the impellers, a suitable environment specific for anaerobic growth could not be incorporated into the system. Temperature ranges within the model system were established to parallel as closely as possible the conditions prevailing in the different sections of the hot roll mill, and designed to encourage the growth of both mesophilic (40-42°C) and thermophilic (58-60°C) microorganisms. Temperatures in excess of these limits would have increased generation times and limited the types of fungi and bacteria capable of growing in the model system. Samples were taken from Tank 3 because mesophiles, which numerically constitute the most important group of microorganisms, grew fastest in this tank hence facilitating their enumeration. Microbial quantification experiments showed that Tank 1 (58-60°C) supported

growth of fewer microorganisms (less than $1x10^4 CFUml^{-1}$ emulsion) than Tank 3 (40-42°C) which supported over $1x10^7 CFUml^{-1}$ emulsion.

Tank 1 was topped-up daily with emulsion containing the appropriate biocide concentration. This was done to restore losses arising from evaporation, and simulated the procedure used at the hot roll mill. Since evaporation was moderately high (especially in Tank 1), aluminium foil coverings were placed over all three tanks. Biocide determinations by High Performance Liquid Chromatography showed that no increase in glutaraldehyde concentration occurred as a result of loss of emulsion by evaporation. The rate of glutaraldehyde loss paralleled that of emulsion evaporative loss and occurred at a similar rate to that found at the hot roll mill. This showed that the biocide was subject to greater evaporative loss at temperatures above ambient temperature.

Unfortunately, samples which were collected for HPLC analysis had to be stored at 4°C for up to six months before the appropriate HPLC column arrived from overseas and the chemical 2,4-dinitrophenylhydrazine could be synthesized in the University of Natal Chemistry Department. As a result, some deterioration of the samples occurred, particularly in those stored for the longest time (Experiments 1 and 5) which were the emulsions containing $500mgl^{-1}$. In some of these samples, low-level fungal contamination was evident on the surface of the emulsion. This could obviously effect the glutaraldehyde assay as the glutaraldehyde may have combined with the fungal mass. It is unclear, however, to what extent this fungal growth affected the glutaraldehyde concentration of the emulsion.

Spray nozzles like those which spray the emulsion onto the ingot at the hot roll mill were not incorporated into the model system. This was considered unnecessary as the emulsion in the model system tanks was constantly circulated by means of impellers which ensured aeration of the system and hence availability of oxygen for aerobic microbial respiration. However, as stated previously, this did create conditions not suited to anaerobic microbial growth. The increased emulsion temperatures which arise from emulsion contact with the heated aluminium ingot could also not be simulated in the laboratory model. It was thought,

however, that under the conditions prevailing at the hot roll mill any microorganisms sprayed directly onto the heated ingot would be rapidly killed. It was conceded that the protoplasmic contents of these killed cells would be released back into the system and could serve as possible nutrients for the surviving emulsion-inhabiting microbial population.

The author decided to use a combination of cultures comprising a mixed population of cells freshly isolated from the hot roll mill and a laboratory-grown mixed culture. Fresh microbes isolated from the hot roll mill would probably be more vigorous than their lab-grown counterparts. However, since many of the isolates from the lab-grown cultures had been identified, it was useful for monitoring purposes to co-inoculate the system with known microbes. Mixed cultures were used instead of pure cultures since the results obtained from batch experiments (Chapters 2 and 3) suggested that the use of the former would be more representative of the *in situ* situation at the Hulett plant.

The hot roll mill microflora consisted of a diverse population of many different fungi and bacteria. Although many of these were identified, their relative ratios within the mill population could not be established. The use of pure cultures combined in various specific ratios could, therefore, not be justified. Also, laboratory-grown fungi and bacteria tended to lose their capacity to grow under the harsh conditions prevailing at the mill. The sensitivity of microorganisms grown on a highly nutritious medium (such as nutrient agar) to stresses caused by changes in temperature, biocide concentrations, aeration, pH and other factors, will not be the same as that of the same organisms once they have adapted to the conditions prevailing in the mill emulsion. To reduce the chances of the microorganisms losing their ability to effectively degrade the emulsion, high nutrient media were not used for the maintenance of the lab-grown cultures. Inoculum was added in relatively small doses at the start of each experiment to simulate conditions at the hot roll mill immediately after a system cleanout, thereby making the experimental results more meaningful.

### 4.4.2 Experiment 1: Emulsion containing 0mgl$^{-1}$ Busan and inoculated with biocide-resistant microbes

As indicated in Figure 6a microbial numbers started to increase immediately after inoculation. No lag period was observed. Although cell numbers were small initially the pH started to decrease after only one day, indicating that the microflora were affecting the emulsion. The lack of a lag phase indicated the high metabolic activity of the microbes inoculated into the system. The pH decrease occurred when the CFUs reached 1x10$^{4}$ml$^{-1}$ of emulsion. These microbial numbers were similar to those found at the Huletts roll mill just before the emulsion begins to deteriorate. The decrease in pH (resulting either from production of acidic compounds by the microorganisms or release of acidic substances due to the breakdown of the emulsion) did not cause noticeable inhibition of the microbial cells.

Figure 6b clearly shows that there was a 2 to 3 day period during which the emulsion remained 'tight', despite the presence of large numbers of microorganisms. Only when the microbial numbers reached 1x10$^{4}$ CFUml$^{-1}$ of emulsion did the emulsion show signs of 'loosening'. However, once these large microbial populations had been attained and 'loosening' had been initiated, the emulsion 'broke' rapidly. These findings suggest the existence of a 'window period' during which even high microbial numbers have only a limited effect on the stability of the emulsion. Therefore, if the microflora could be destroyed during this 'window period', it should be possible to maintain the emulsion in a 'tight' form for longer periods, thus increasing its useful life. However, conditions may vary when biocides are brought into the equation (refer to Experiments 2-5 below).

An obvious trend is discernable in Figure 6c; a decrease in pH was associated with a 'loosening' of the emulsion. Whether this drop in pH was brought about by products produced by the resident microbes or by compounds released from the emulsion remains unclear. A diversity of microflora was isolated from the emulsion, including many different acid producers. Both Ellis *et al.* (1957) and Passman (1988) reported that the breaking of an emulsion can result from either a lowering of the pH, or it can occur without a pH change. The reasons for the lowered

pH may vary depending on the emulsion or the conditions prevailing at the roll mill. Organic acid tests would be required to determine whether or not such acids play a major role in the biodeterioration of the emulsion. The results of these tests, however, could prove exceedingly difficult to interpret as the emulsion contains many organic acids each of which may interact uniquely with the other emulsion components and the microflora inhabiting the emulsion.

### 4.4.3 Experiment 2: Emulsion containing 500mgl$^{-1}$ Busan and inoculated with biocide-resistant microbes

Unlike in Experiment 1, a small decrease in microbial numbers occurred immediately after inoculation of the emulsion (Figure 7a). It would seem that the presence of 500mgl$^{-1}$ biocide in the system retarded microbial growth, but only for 2-3 days. As in Experiment 1 the pH decreased concomitantly with an increase in microbial numbers. However, unlike in Experiment 1, this decrease occurred immediately after inoculation, before the microbial population had a chance to increase.

The emulsion (Figure 7b) remained 'tight' for 2-3 days longer than did the inoculated, biocide-free emulsion (Figure 6b) thereby showing the protective effect of the biocide on the emulsion. Emulsion stability started to decrease when microbial numbers exceeded 1x10$^4$ CFUml$^{-1}$ on day 4. This microbial population was similar in size to that observed in the non-protected emulsion (Figure 6b). Between days 2-3 cell numbers increased, indicating either a resistance to glutaraldehyde on the part of the microbes or a reduction in the concentration of glutaraldehyde to levels which were less inhibitory to microbial growth. This reduction may have resulted from either combination of the glutaraldehyde with the microbial cell membrane and other cell components (Munton and Russell, 1973) or from volatilization from the system. A similar trend to that seen in Experiment 1 was evident.

Figure 7c shows the relationship between pH and oil droplet surface area over time. The pH decrease was slightly sharper than that observed in Experiment 1 with emulsion containing no biocide (Figure 6c). This may have been purely the result of the acidity of the biocide.

Concomitant with this decrease in pH, the emulsion started to 'loosen' and finally 'broke' on day 4. However, the rate of emulsion breakage did not seem to be greatly affected, since after the initial 'loosening', complete breakdown took 2.5 days compared with the 2 days required for this to occur in emulsion containing no protective biocide (refer to Figure 6c). This indicated that the presence of biocide did little to slow the rate of biodeterioration of the emulsion once the 'loosening' process had commenced.

Figure 7d shows that the initial glutaraldehyde concentration was 180mgl$^{-1}$, indicating that the active component comprises about 36% of the biocide formulation as compared to the control in which 40% active component was determined. The remainder is comprised of diluents and other compounds which are not necessarily antimicrobial. The glutaraldehyde concentration in the system decreased much more rapidly than was anticipated, possibly as a result of volatilization. Microbial growth started on day 2 when glutaraldehyde concentrations were still quite high (108mgl$^{-1}$). This would seem to indicate that glutaraldehyde resistance developed fairly rapidly among the microflora. The results of Experiment 1, where no biocide was added, showed that the microbial population increased exponentially immediately following inoculation (refer to Figure 6a). Yet in Experiment 2 microbial growth was delayed with death of some of the cells occurring over the first two days (Figure 7a). During this initial period, selection of biocide resistant microbial cells within the population was possibly occurring. By day 2 the majority of susceptible cells had been killed and the biocide resistant microbes began to multiply exponentially as indicated by the growth curve (Figure 7a). This exponential increase in cell numbers continued through day 7 when the experiment was terminated. At this time the microbial population was in excess of 1x10$^{7}$CFUml$^{-1}$ emulsion.

### 4.4.4 Experiment 3: Emulsion containing 1000mgl$^{-1}$ Busan and inoculated with biocide-resistant microbes

Microbial numbers decreased slightly over the first few days (Figure 8a). However, by day 3 microbial growth rates started to increase indicating acclimatization of the cells to the increased biocide levels. In this case also, the pH started to decrease when microbial numbers exceeded

$1x10^4$ $CFUml^{-1}$ of emulsion. This parallels the results obtained in Experiments 1 and 2. The emulsion thus appeared to have a 'holding capacity' which enabled it to withstand a certain number of microbes, but only until the pH began to fall, ie. a threshold of $1x10^4$ $CFUml^{-1}$ of emulsion in the case of Prosol. Here also a 'window period' was observed, during which the emulsion remained 'tight' despite the presence of high microbial numbers.

From Figure 8b it is apparent that the period of 'tightness' was further extended when higher levels of biocide were applied to the emulsion. In this experiment, the emulsion remained 'tight' for 7 days compared to 4 days in Experiment 2 (refer to Figure 7b) and 2 days in Experiment 1 (Figure 6b). Microbial numbers were of the order of $1x10^3$ $CFUml^{-1}$ of emulsion when the emulsion started to break on day 8. This cell population is one order of magnitude lower than that observed in Figures 6b and 7b (ie. $1x10^4$ $CFUml^{-1}$ of emulsion). It is possible that the various species in the mixed population that was initially inoculated into the system in Experiment 3 were somewhat more metabolically active than those comprising the inoculum used in Experiments 1 and 2. Also selection for biocide resistant microorganisms from among the population may have occurred allowing emulsion degradation to occur despite the presence of biocide. This seems a reasonable hypothesis since the microbial population used in Experiment 3 was exposed to $500mgl^{-1}$ biocide for a prolonged period during cultivation in the laboratory.

A similar trend to that observed in Figures 6c and 7c is evident in Figure 8c. The graphs showing changes in pH and surface area of oil droplets follow a similar pattern except that in this case the pH dropped only after 'loosening' of the emulsion had commenced. This shows that pH changes may not necessarily be a good sole indicator of the condition of an emulsion.

Initial glutaraldehyde concentrations were higher than anticipated (Figure 8d). Replicated analyses indicated that glutaraldehyde, the active component of Busan, comprised approximately 36% of the total biocide volume (ie. approximately $180mgl^{-1}$ glutaraldehyde in Experiments 2 and 5 in which $500mgl^{-1}$ Busan was applied). Based on 36% active component, $1000mgl^{-1}$ Busan should therefore have yielded approximately $360mgl^{-1}$ glutaraldehyde,

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however a concentration of 544mgl$^{-1}$ was detected. A number of possible reasons can be offered to explain this anomaly. Firstly, although every effort was made to effectively store the experimental samples, those collected for use in Experiments 2 and 5 (the first experiments conducted) were stored for longer than those collected during Experiment 3. Although the samples were stored at 4°C, a small amount of fungal growth was evident in some of the sample tubes. However, since contamination occurred only in a few of the samples, it could not explain the marked difference in concentration of glutaraldehyde in samples from Experiments 2 and 5 compared to those from Experiment 3. This is especially true since in both Experiments (*viz.* 2 and 5) the starting concentrations of glutaraldehyde were nearly identical (182 and 180mgl$^{-1}$ respectively at 500mgl$^{-1}$ Busan). The higher than anticipated glutaraldehyde concentration in Experiment 3 could also have been caused by seasonal fluctuations in the laboratory temperature in which the model system was located. Lower ambient temperatures in the laboratory during Experiment 3 compared to those experienced during the course of Experiments 2 and 5 could have resulted in reduced volatilization and hence a higher than anticipated glutaraldehyde level.

Even at 1000mgl$^{-1}$ Busan, which was twice the recommended biocide concentration, microbial growth was still evident. Microbial numbers increased from day 3.5 (84hrs after inoculation) onwards (Figure 8d). This increase occurred at glutaraldehyde concentrations in excess of 300mgl$^{-1}$ (±2.5 times that in Experiment 2 [see Figure 7d]). Resistance to, or tolerance of, glutaraldehyde was therefore present in the microbial population. Even though the inoculum used in this experiment originated from the same stock culture as that used to produce the inoculum for Experiment 2 it is clear that in this case the population was much more resilient to the effects of glutaraldehyde than was the population present during Experiment 2. This resistance probably arose through a selection process which occurred in the biocide-containing, liquid stock culture. Microbes resistant to the glutaraldehyde in the biocide survived and multiplied, whereas those that were less resistant died-off generally or were out-competed.

### 4.4.5 Experiment 4: Emulsion containing 5000mgl$^{-1}$ Busan and inoculated with biocide-resistant microbes

There was no microbial growth at 5000mgl$^{-1}$ biocide (Figure 9a). Even the initial viable cell counts taken immediately after inoculation ($10^3$ to $10^2$ CFUml$^{-1}$ of emulsion), were lower than those in Experiments 1,2 and 3, indicating an immediate anti-microbial effect on the inoculum. The pH of the emulsion immediately after inoculation was likewise lower (8.02 compared to an average of 8.24 in Experiments 1,2 and 3) probably because of the large amounts of acidic biocide present.

The emulsion remained 'tight' with no microbial growth detectable over the 30 day duration of the experiment (Figure 9b). The high biocide concentration had presumably eliminated all the microorganisms from the system in one day. These high concentrations (10 times the recommended dosage level) were too toxic for the microorganisms to survive.

The emulsion remained 'tight' even under the added stress of a lowered pH (Figure 9c). The oil droplet surface area in the emulsion remained between 11.8 and 9.8 m$^2$ml$^{-1}$ over the duration of the experiment. The drop in pH to below 7.80 (Figure 9c) was not a sign of emulsion breakage as was the case in Experiments 1 and 2 (see Figures 6c and 7c respectively).

At initial concentrations of glutaraldehyde in excess of 750mgl$^{-1}$ no microbial activity was detected (Figure 9d). Although this concentration was well below the expected 1800mgl$^{-1}$ glutaraldehyde (ie. 10x the 180mgl$^{-1}$ detected in Experiments 2 and 5 where 500mgl$^{-1}$ Busan was added to the emulsion), it should be remembered that at 5000mgl$^{-1}$ Busan, the normal dosing level was exceeded 10 times and therefore the saturation level of the emulsion with respect to glutaraldehyde was possibly exceeded, and the excess glutaraldehyde volatilized. No microbial growth was detected even after the concentration of glutaraldehyde had dropped to below 350mgl$^{-1}$. This suggested that all vegetative microbial cells as well as any resistant resting structures in the emulsion, had been killed very soon after inoculation.

### 4.4.6 Experiment 5: Emulsion containing 500mgl$^{-1}$ Busan and inoculated with biocide-susceptible microbes

The so-called biocide susceptible inoculum had been laboratory-grown under identical conditions to the resistant microbes, except that the cells were never in contact with the biocide. Initially microbial growth was retarded resulting in an extended lag phase (Figure 10a). Population growth commenced from day 10. This 10 day lag period was considerably longer than the 3 day lag observed in Experiment 2 in which the same biocide concentration was tested against resistant microorganisms (Figure 7a). The pH remained relatively constant until day 11 when the characteristic pH decrease occurred. The sharp increase in pH after the emulsion had completely 'broken' (day 15) is difficult to explain. It could perhaps have resulted from the release of some basic components of the emulsion once the emulsion had 'broken'. With the separation of the emulsion into its oil and water phases, these basic components may have been distributed more effectively in the aqueous phase. With this separation and redistribution, these 'free' alkaline components would have been more 'available' and hence would have given a high pH reading. This hypothesis is supported by Pitchford (1995, pers. comm.[14])

As stated above, microbial counts remained low for 10 days when the 'biocide susceptible' inoculum was used. After day 10 the microbial population increased exponentially (Figure 10b), presumably after 'selection' for resistant individuals had occurred.

Both pH and emulsion stability were maintained until day 10 (Figure 10c). Unlike in Experiments 1 and 2, but similar to Experiment 3, the pH decreased only after the emulsion had started to break, again indicating that a drop in pH alone may not necessarily bring about breakage of the emulsion.

At the start of microbial growth on day 10 the glutaraldehyde concentration had fallen to just

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[14]Dr. A. Pitchford, 1995. Hulett Aluminium hot roll mill Research and Development department, Hulett Aluminium, Camps Drift, Pietermaritzburg.

over 40mgl$^{-1}$ (Figure 10d). This biocide concentration was much lower than in the corresponding experiment involving resistant microbes (Experiment 2) where exponential growth occurred at the much higher glutaraldehyde concentration of 108mgl$^{-1}$. This difference could be attributed to the microbial population having partially 'lost' their resistance to glutaraldehyde, possibly because of the absence of biocide since their initial isolation from the hot mill emulsion. This loss of biocide resistance could potentially be exploited as an improved biocide management regime by using high concentrations of biocide to kill all the microbes present in the hot roll mill emulsion at the time of application. Microorganisms subsequently entering the system would have no biocide resistance and most would thus be rapidly killed. In this way the useful lifespan of the emulsion might be extended.

### 4.4.7 Trends

Similar microbial growth curves were evident at all the biocide concentrations tested (Figure 11). Initially microbial numbers were reduced due to lethal action of the biocide on the susceptible microbes in the inoculum (except in Experiment 1 where no biocide was used). Death of these susceptible microbes, was responsible for the observed downward trend in the growth curves (Experiments 2,3,4 and 5). The period with low cell numbers became longer as the concentration of the biocide increased, and hence the lifespan of the emulsion was prolonged. With the emergence of biocide-tolerant or -resistant microbes in the population, the colony counts increased dramatically. The initial death phase was followed by a short lag phase and subsequently an exponential growth phase. When microbial numbers reached in excess of $1x10^3$-$1x10^4$ CFUml$^{-1}$ of emulsion, the emulsion was seen to 'loosen' and breakdown quickly followed. Control measures should therefore be aimed at keeping microbial numbers below this threshold level in the mill emulsion. Reducing microbial numbers to a minimum is of considerable economic importance since it would increase the useful life of the emulsion dramatically by extending the time elapsed before the population reaches the levels required to adversely affect the emulsion. With the emergence of biocide tolerant/resistant strains, an increase in cell numbers of one order of magnitude will have much less effect on the emulsion when the initial population is low than it would were the resistant

microbial population initially high. The ideal situation obviously would be the complete removal of the microflora from the system. The 'pioneer' microorganisms which enter a microbe-free emulsion start a chain of reactions which eventually result in the 'loosening' and breakage of the emulsion. Targeting these few 'pioneers' with effective biocides (at appropriate concentrations) is therefore of prime importance.

Also worth noting is that microbes which had not been exposed to biocide for long periods were more susceptible to the effects of glutaraldehyde than those that had been more recently exposed to sub-lethal concentrations of this chemical (Refer to Experiments 5 and 2 respectively). This increasing tolerance and subsequent resistance to biocides developed as a result of contact with sub-lethal concentrations of antimicrobial agent is well documented (Leder and Russo, 1989).

Earliest possible removal of as much bacterial and fungal contamination as possible from the system helps to reduce the chances of biocide resistance developing within the population and, therefore, increases the lifespan of the emulsion.

Emulsion stability, reflected by oil droplet surface area, as determined by Malvern Laser technology, was shown to be directly related to the number of microorganisms growing in the emulsion at any given time (Figure 12). Emulsion 'tightness' may vary greatly in terms of the specific oil droplet surface area (a measurement of the total oil to water surface area of the oil droplets in suspension) depending on the nature of the emulsion; on what the emulsion is being used for; and on how the emulsion is being used. At the Hulett Aluminium roll mill Prosol is currently the emulsion of choice. Prosol is generally considered to be 'tight' when the specific oil droplet surface area is in the region of $10m^2ml^{-1}$ of emulsion. However, an oil droplet surface area within the range $9.5\text{-}12m^2ml^{-1}$ of emulsion is considered acceptable. Obviously for ideal rolling conditions it is important for the emulsion to remain at a fixed specific oil droplet surface area to ensure even oil distribution onto the rollers and aluminium ingot. Addition of $5000mgl^{-1}$ biocide caused the pH to drop but no signs of 'loosening' were detected. With increasing concentrations of biocide, emulsion stability was maintained for

extended periods of time.

pH was only slightly affected by the addition of biocides except at excessive concentrations (Figure 13). A pH decrease occurred in those experiments in which large microbial populations (in excess of $1x10^4$ CFU$ml^{-1}$ emulsion) developed. This effect on pH could arise from one of two possibilities. Firstly, the microflora could produce acidic metabolites which are released into the emulsion causing a reduction in the pH. Secondly, initial action of the microflora could result in the release of acidic emulsion components. However, with further breakdown and separation into water and oil phases, alkaline compounds may also be released. This two-phase pH response was observed in Experiments 1 and 5 where an initial drop in pH was followed by a subsequent pH increase. Results from the present investigation suggest that pH alone should not be relied upon as an accurate indicator of emulsion stability. However a 'loosening' of the emulsion may sometimes be associated with a reduction in the pH (refer to Experiments 2, 3 and 4). pH may therefore be used as a possible indicator of emulsion stability, but it should be used in conjunction with other tests.

The results presented in Figure 14 show that glutaraldehyde vaporized rapidly from the emulsion system in the laboratory model. Vaporization would not be as problematical at the roll mill because of the very large volumes contained in the emulsion holding tanks. The emulsion which is subjected to excessive temperatures at the point of rolling and the emulsion which is atomized into a fine spray just before application to the rollers and ingot, make up only a small part of the total volume of emulsion in the system at any one time. Nonetheless loss of biocide would be exceedingly high at these locations. Moving the spray nozzles closer to the rollers would possibly reduce the loss of biocide by evaporation.

Due to the large volume of emulsion held in the emulsion storage tanks at the hot roll mill, the time available for biocide concentration analysis and subsequent dosing if necessary would be greater than in the laboratory model system; thus a more effective biocide management system could be maintained. Resistant microorganisms were shown to survive glutaraldehyde concentrations in excess of 300$mgl^{-1}$ and to grow in concentrations of 100-180$mgl^{-1}$ depending

on the level of adaptation of the microbial population to the biocide (Figures 7d and 8d). Microbes which had not been previously exposed to the biocide showed decreased tolerance to glutaraldehyde.

To improve management of the biocide dosing regime at the Hulett Aluminium roll mill, high doses should be applied at regular intervals. Maintaining the biocide at sub-lethal concentrations was shown to increase microbial tolerance and induce resistance to the biocide over time leading ultimately to the establishment of large microbial populations. This agrees with Leder and Russo's finding (1989) that microbial resistance plays an important part in the management of any emulsion. A reduction in the percentage of biocide tolerant microorganisms within the microbial population which inhabits the Hulett aluminium roll mill emulsion system is essential if the lifespan of Prosol is to be extended. In order to minimize the incidence of biocide resistance, microbial populations should be kept as small as possible. This can be achieved by thorough cleaning of the roll mill during down-time periods. This cleaning should be carried out with the use of high pressure steam to sterilize not only the inner surfaces of the emulsion tanks and system pipes, but also the areas surrounding the tanks. It is particularly important to sterilize (and if possible remove) the stagnant pools of emulsion under the main and sump tanks. High concentrations of biocide should be added immediately after any downtime period in order to eliminate the surviving microorganisms. Emulsion circulating within the tanks during the downtime period should be maintained at as high a temperature as possible to minimize microbial growth. Regular additions of high concentrations of biocide should be made during the operational phase of the rolling process. Addition of large quantities of biocide was shown to have only a limited affect on the pH of the emulsion and would, therefore, not markedly affect the stability of Prosol. However, care is advocated when using biocides as the health of workers in contact with the emulsion could be compromised if extremely large quantities are added to the emulsion.

# CHAPTER 5. BIODETERIORATION OF IMPORTED EMULSIONS

## 5.1 Introduction

Efficient management of microbial contamination of roll mill emulsions is essential for extended emulsion life and improved mill productivity. Up until 1994 the problems associated with the microbiology of the Hulett Aluminium roll mill emulsion had been the focus of much research (Naidoo, 1991; Snell, 1992; Sundram, 1993). The present author hoped to take this research a step further by producing results which would aid in the development of more effective emulsion management practices.

To develop a new and effective management practice, several changes to the system in operation at the time were considered. One of these changes included the replacement of the emulsion in use at Hulett Aluminium namely, Prosol, with an emulsion less sensitive to microbial degradation. Rossmore (1993) reported that some biostable emulsions had been developed. Three foreign emulsions, supposedly in this category, were imported for testing in the model system. According to Pitchford (1995, pers. comm.[15]) these emulsions reportedly showed little or no signs of biodeterioration even in the presence of high microbial numbers.

The formulation of an emulsion will vary depending on the aluminium rolling process for which it is designed, and slight changes in rolling criteria may result in a major change in the formulation and thus the type of emulsion used. The three emulsions imported were similar in their basic compositions to Prosol but differed with respect to some of the components as they had been formulated to function under different working conditions. It was thought that these chemical differences might endow the emulsions with quite different properties from a microbiological standpoint. The three imported emulsions were: HRF3; a 1:1 mix of B207HS:B216HS; and Houghton Biostable. Each of these emulsions is used in a specific plant: HRF3 is a Canadian emulsion, B207HS:B216HS a Welsh emulsion and Houghton

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[15]Dr. A. Pitchford, 1995. Hulett Aluminium hot roll mill Research and Development department, Hulett Aluminium, Camps Drift, Pietermaritzburg.

Biostable an Australian emulsion. All are patented for use in aluminium roll mills. Since each of these emulsion concentrates is manufactured by a different company, their chemical compositions are unfortunately well guarded trade secrets.

The emulsions were tested using the model system described in Chapter 4. The following aims were identified:

- To determine if any of the three emulsions possessed so-called 'biostability' properties. To achieve this the emulsions would be tested in a similar manner to Prosol. Microbial growth and emulsion stability over time would be plotted and direct comparisons with Prosol made. Should these comparisons show the imported emulsions to be more biostable that Prosol, then;

- Recommendations for further and more detailed studies of various characteristics of the emulsions would be made. The results of these investigations may suggest some advantage to the Hulett plant switching from Prosol to one of the imported emulsions. Following this it might be necessary to recommend possible change of the current emulsion.

## 5.2 Materials and Methods

### 5.2.1 Experiment 6: Emulsion HRF3

This experiment was conducted in accordance with the specifications set out in Section 4.2.2 except that Prosol was replaced with emulsion HRF3 at a concentration of 3% v/v (concentrate/water). No biocide was added. The model system was inoculated with the biocide-resistant microbial population derived from the culture used in earlier experiments on Prosol, at an initial concentration (determined immediately after inoculation on day 0) in the range $1x10^3$-$1x10^4$ $CFUml^{-1}$ emulsion (refer to Chapter 4, Section 4.2.2).

### 5.2.2 Experiment 7: Emulsion B207HS:B216HS

This experiment was conducted in accordance with the specifications set out in 4.2.2 except that Prosol was replaced with a 1:1 mixture of the two emulsions B207HS and B216HS at a final combined concentration of 3% v/v (concentrate/water). No biocide was added. The model system was inoculated with a subculture of the same biocide-resistant microbial population at the same inoculum density as used in Experiment 6 (refer to Chapter 4, subsection 4.2.2).

### 5.2.3 Experiment 8: Emulsion Houghton Biostable

This experiment was carried out using the same procedures as those discussed in Section 4.2.2 except that Prosol was replaced with the emulsion Houghton Biostable, also at a concentration of 3% v/v (concentrate/water). No biocide was added. The model system was inoculated with microorganisms obtained from the same biocide-resistant stock culture and at the same inoculum density as used in Experiments 6 and 7.

## 5.3 Results

Graphical representation of the results obtained in Experiments 6-8 appears in Figures 15, 16 and 17 (See Appendix F for raw data). The results from Experiment 1, in which Prosol (inoculated with biocide-resistant microorganisms and containing no biocide) was tested, are included for comparison.

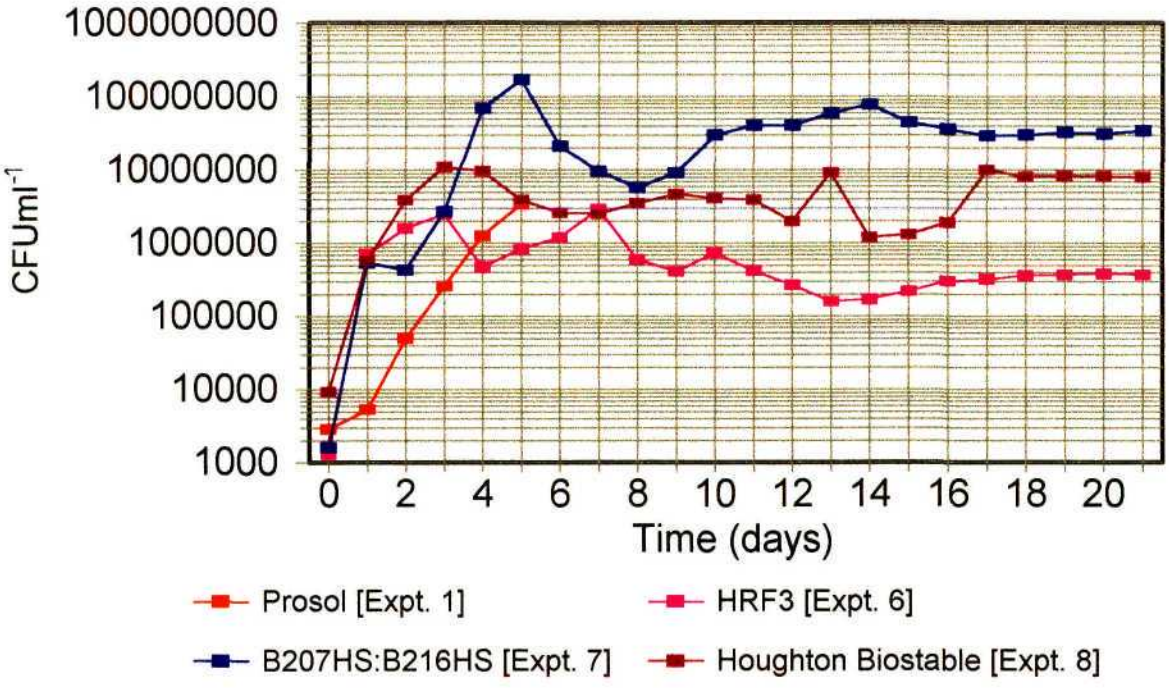


Figure 15: Changes in $CFUml^{-1}$ in different emulsions over time

Figure 15 shows the relationship between the $CFUml^{-1}$ of emulsion over time for each of the four emulsions. All of the emulsions were shown to support large microbial populations (in excess of $1x10^5$ $CFUml^{-1}$ of emulsion) following inoculation with $1x10^3$-$1x10^4$ microbial cells per ml. Depending on the emulsion, the microbial populations reached maximum size after three to five days. In Experiment 1, in which Prosol was tested, microbial numbers reached a maximum of $2x10^6$ $CFUml^{-1}$ emulsion on day 5. Experiment 1 was terminated on day 5 due to complete biodeterioration and breakage of the Prosol. In Experiments 6 and 8 (in which emulsions HRF3 and Houghton Biostable were tested respectively) largest microbial numbers in the emulsion ($2.5x10^6$ $CFUml^{-1}$ emulsion of HRF3 and $1.1x10^7$ $CFUml^{-1}$ emulsion of Houghton Biostable) occurred by day 3. In Experiment 7 in which emulsion B207HS:B216HS was tested, highest microbial numbers ($1.5x10^8$ $CFUml^{-1}$ emulsion) were detected on day 5. The initial rate of increase in microbial numbers, illustrated by the gradients of the graphs in Figure 15, was similar for all of the emulsions tested. No microbial inhibition occurred following inoculation of the various biocide free emulsions. After 21 days there were no visible signs of biodeterioration of the emulsion or further increase in microbial populations and, therefore, Experiments 6,7 and 8 were terminated.

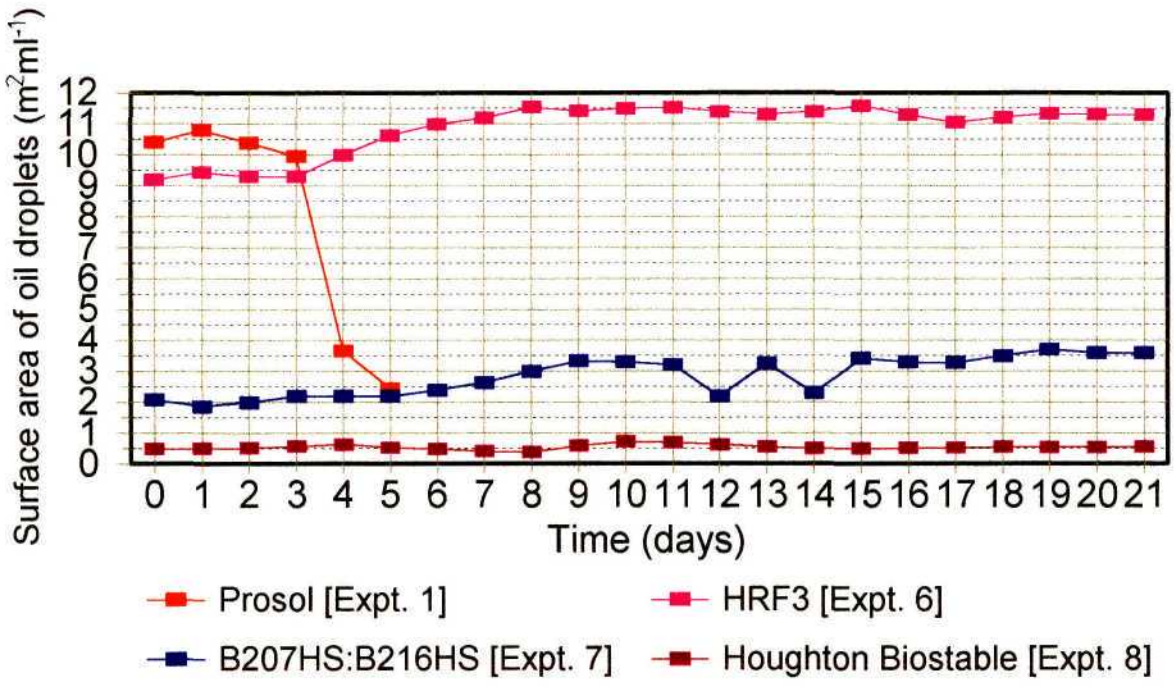


Figure 16: Changes in oil droplet surface area of different emulsions over time

Specific surface area of the oil droplets in suspension for each emulsion over time is depicted in Figure 16. The surface area of each emulsion was shown to remain relatively constant except for Prosol which underwent complete biodeterioration after 5 days. Emulsion HRF3 showed the highest specific oil droplet surface area, ranging from 9.19 to 11.58 $m^2ml^{-1}$ of emulsion over the course of the experiment. The emulsion mix, B207HS:B216HS had a low specific oil droplet surface area ranging from 2.07 $m^2ml^{-1}$ of emulsion on day 0 to 3.60 $m^2ml^{-1}$ of emulsion on the final day of the experiment (day 21). Of the four emulsions tested, Houghton Biostable had the lowest specific oil droplet surface area, ie. 0.39-0.73$m^2ml^{-1}$ of emulsion.

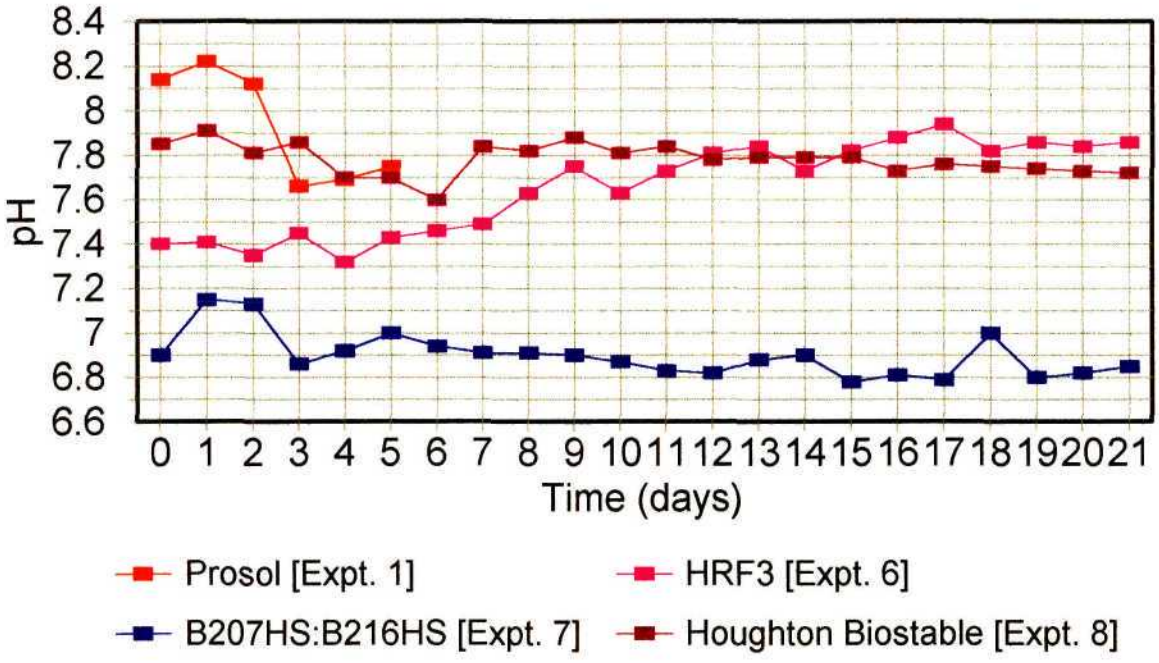


Figure 17: Changes in pH of different emulsions over time

Changes in pH with time for the four emulsions are shown in Figure 17. The pH of Prosol remained high, ranging from 8.0-8.2 for the first two days, after which it decreased to a low of 7.66 on day 3. Following this decrease, the pH increased once again to 7.75 on day 5. Houghton biostable was the most stable of the emulsions tested, the pH ranging between 7.91 and 7.60 over the 21 day period of the experiment. The pH of emulsion HRF3 remained virtually unchanged over the first 6 days (ranging from 7.32-7.46) after which it increased gradually to approximately 7.90 between days 17-18. The B207HS:B216HS mix of emulsions showed the lowest initial pH (pH 6.9) and an overall variation from 6.78-7.15 over the course of the experiment.

### 5.4 Discussion

The graphs in Figure 15 indicate that the Prosol-grown microflora showed no signs of growth inhibition in any of the emulsions when tested in the model system. This was interesting as it was the first time that the microflora had been exposed to any of the three imported emulsions. Although detailed information regarding the chemical composition of commercial

emulsion concentrates is not normally divulged, the results obtained in Experiments 1, 6, 7 and 8 suggest that most emulsions, although dissimilar in some respects, have components in common. Microbial numbers increased rapidly in all cases and all of the emulsions were heavily contaminated after 5 days. Rossmore (1993) stated that the potential does exist for 'friendly' microorganisms to proliferate in emulsions and he cites a case (Holtzman *et al.*, 1982) where high microbial loads have been reported in various emulsions without causing any evident deterioration. According to Rossmore (1993) biostability of an emulsion at any given time may be dependent on the pH and he describes a case where an increased pH supported the growth of large microbial populations without adversely affecting the functionality of the emulsion. With a decrease in the pH however, the microbes caused significant biodeterioration of the emulsion. Rossmore (1993) also showed that a similar effect can occur when emulsions are diluted. For example, a certain emulsion supported high microbial numbers with no biodeterioration evident when tested at a dilution of 1 in 40, but when the dilution was increased to 1 in 48, biodeterioration occurred.

Only Prosol showed signs of biodeterioration (ie. a significant decrease in the specific surface area of the oil droplets, together with separation of the oil and water phases) following high levels of microbial contamination (Figure 16). Houghton Biostable had the lowest specific oil droplet surface area of the four emulsions tested. However this emulsion showed signs of abiotic deterioration before introduction to the system, possibly because the emulsion concentrate had been standing for a long time during shipment from overseas. Some of the concentrate had gelled in the containers and problems were experienced in trying to emulsify the concentrate with water. There is a high probability that the low specific oil droplet surface area readings obtained for this emulsion were due to the resistance of the oils to emulsify in water, possibly the result of long term storage-induced deterioration of the emulsifying agent in the concentrate. The specific surface area of B207HS:B216HS was also low compared to Prosol. It is possible that the emulsion was designed for use under different aluminium roll conditions to those in operation at the Pietermaritzburg roll mill. Unfortunately details with respect to the conditions under which aluminium is rolled overseas, and under which the imported emulsions are used, could not be obtained due to patent rights issued on the emulsions. Likewise, information on the chemical components of the emulsions could not be

accessed as each is patented and the formulations are thus secret. Full chemical analysis of the products was outside the scope of the present investigation. It is therefore difficult to assess what degree of 'tightness' is optimal for each of the tested emulsions. Emulsion HRF3 was most similar to Prosol and showed no decrease or even 'loosening' of the specific oil droplet surface area over the experimental period.

Only Prosol showed biodeterioration following reduction in pH. Even though the other emulsions contained large microbial populations, pH changes did not seem to affect their stability. The potential exists for the microbial population to produce fatty acids which could accumulate over a period of time thereby lowering the pH and eventually causing 'loosening' of the emulsion. Although this was not evident in any of the emulsions tested, perhaps with a longer experimental period, the pH may have fallen sufficiently to cause a 'loosening' of the emulsion. However, it was clearly shown that no 'loosening' of the emulsion occurred over a 21 day test period (four times the test period used for Prosol) for the three imported emulsions.

Both B207HS:B216HS and HRF3 supported high microbial numbers while retaining 'tightness'. These emulsions showed superior biostability to Prosol at 3% concentration. Even in the absence of biocide, two of the three emulsions *viz.* HRF3 and B207HS:B216HS, were far superior to Prosol as far as their biodeterioration rates were concerned, even when the latter emulsion was protected by the addition of $1000mgl^{-1}$ Busan. Prosol only became fully bioresistant when 10 times the recommended dose of biocide was added. However, adding large quantities of biocide is both expensive and dangerous. Although it cannot be conclusively stated that replacement of Prosol should be implemented immediately at the Hulett's plant, it would be worthwhile to study in more detail the economic feasibility of replacing Prosol with an alternative emulsion. Of the three alternative emulsions tested, HRF3 was most similar to Prosol with respect to the operational specific surface oil droplet area and might, therefore, be the most likely candidate for further evaluation.

It is therefore recommended that these emulsions be tested further (with emphasis on the

imported emulsion HRF3) for their relative compatibility to the aluminium rolling process currently in operation at the Hulett Aluminium plant outside Pietermaritzburg. Such testing should include determination of the effects of varying the concentration and pH of the emulsions on the rolling process itself and on how these changes affect abiotic and biotic emulsion biodeterioration. Potential biodeterioration of the emulsions associated with a change in either the pH value or the dilution factor of the oil concentrate (as reported by Rossmore, 1993) could therefore be ruled out. Depending on the outcome of such tests, it might be advisable to replace Prosol as the emulsion of choice at the Huletts Aluminium hot roll mill. Such replacement could markedly reduce the amount spent annually on protecting the emulsion currently in use (Prosol) with biocides.

## CHAPTER 6. GENERAL DISCUSSION

Hot-rolling of aluminium is a simple process by which aluminium ingots are repeatedly squeezed through steel rollers to produce a sheet of rolled aluminium. This process is assisted by using a cooling and lubricating emulsion which is sprayed on and around the point of contact between the roller and ingot. This emulsion consists of a rich mixture of complex components. These components may include: emulsifiers, (Ellis *et al.*, 1957), mineral waxes, fatty oils, anti-rust agents, coupling agents, anti-foam agents, dyes, extreme pressure additives, synthetic and phosphate esters, amines and biocides (Sabina and Pivnick, 1956, Genner and Hill, 1981, Passman, 1988). A range of inorganic anions (sulphates, chloride and phosphates) and cations (calcium, sodium, magnesium, manganese and iron) (Passman, 1988) may also be incorporated. This research project has shown that a complex series of interactions exist between the microflora which inhabit the emulsion and the emulsion itself. In order to maximize the production rate and quality of rolled aluminium products, a number of physical and chemical constraints are imposed upon the emulsion to ensure its efficiency. In addition to these physical and chemical constraints, various microbial and chemical interactions can also occur. These interactions have been summarized in Figure 18 and are the basis for the discussion presented in this chapter.

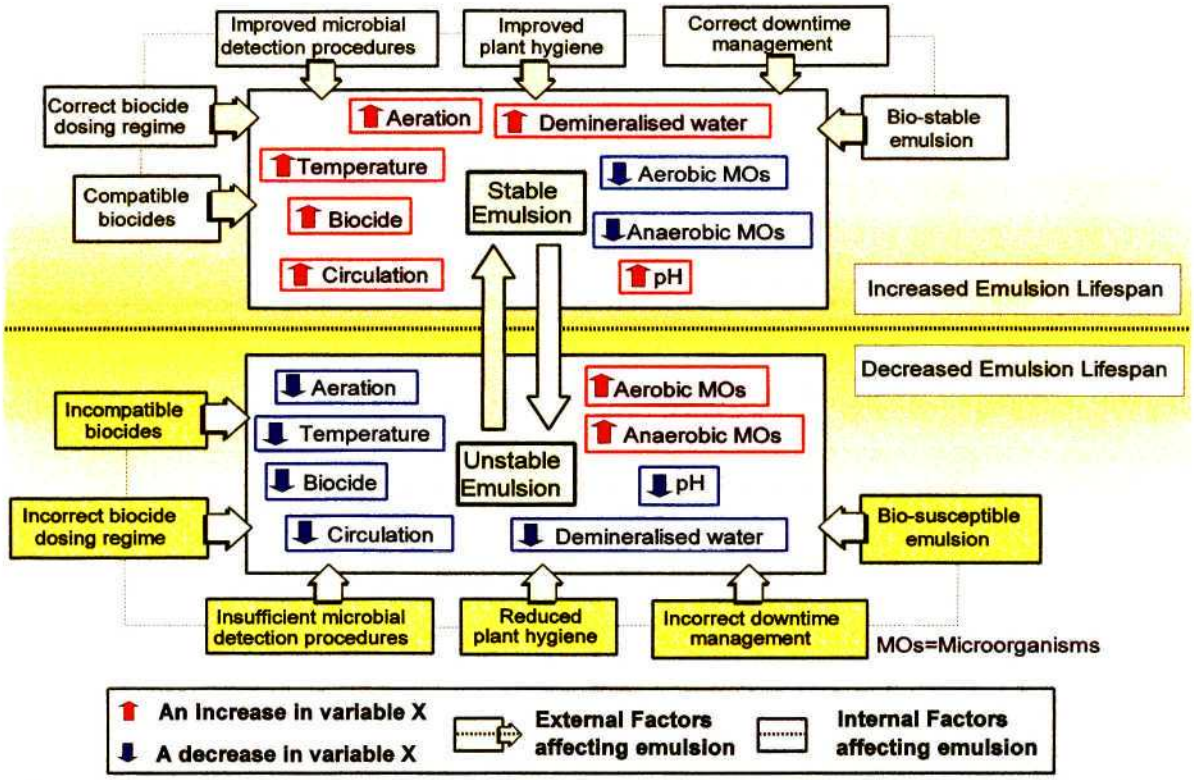


**Figure 18: Summary of the Physical, Chemical and Microbial Interactions affecting an emulsion**

The goal, in achieving an effective emulsion management strategy, is to increase the lifespan of the emulsion whilst ensuring that the cooling and lubricating qualities of the emulsion remain intact.

### 6.1 Experimental results: Conclusions and Recommendations

The following conclusions and recommendations have been made based on results obtained from experiments conducted during this research project:

- The Hulett Aluminium emulsion, Prosol, supported the growth of a wide variety of microorganisms. These included a range of aerobic bacteria previously isolated by other researchers in other hot roll mill emulsions (Duffett *et al.*, 1943; Bennett and Wheeler, 1954; and Sabina and Pivnick, 1956) including bacteria of the genera *Bacillus*, *Pseudomonas* and

*Escherichia.* Bacteria of the genera *Enterobacter*, *Sporosarcina*, *Micrococcus*, *Aeromonas* and *Chromobacterium* were also detected and have not, to the present author's knowledge, been previously isolated from any other emulsion system. A species of anaerobic bacterium of the genus *Desulfovibrio* (probably *Desulfovibrio desulfuricans*) was also detected, which concurs with the findings of Guynes and Bennett (1959). Various fungi were also isolated from contaminated emulsion samples. These included yeasts, supporting Rossmore and Holtzman (1974) and Passman's (1988) discovery of these organisms in other emulsions, and filamentous fungi of the genera *Aspergillus*, *Penicillium* and *Cladosporium*. Members of all of these genera are common contaminants of petroleum products (Alexopoulos and Mims, 1979).

- Growth rate tests, using various fungal and bacterial isolates, showed that particular components of the emulsion Prosol were more susceptible to biodegradation than others. A rudimentary visual assessment scale was developed to assess the rate of growth of fungal and bacterial isolates on various specific solid media in which selected components of the oil concentrate, Prosol, were separately incorporated. Mixed cultures were shown to have greater biodeteriorative effects on Prosol than did pure cultures. This supported the results of pioneering work in the field by Ellis *et al.* (1957) while working on different emulsions.

- A laboratory-scale, model emulsion reticulation system was constructed to carry out a series of emulsion degradation and biocide studies. The model system proved to be effective in simulating the conditions to which the emulsion is subjected at the Hulett Aluminium hot roll mill in Pietermaritzburg. These included temperature, aeration, circulation, pH and ionic effects. A range of biocide concentrations was tested to assess the effects on microbial growth.

- Breakdown of the emulsion was shown to be directly related to high microbial numbers. Experiments demonstrated that when microbial populations in the emulsion exceeded $1x10^3$ $CFUml^{-1}$, biodeterioration of the emulsion was imminent. However, this did not necessarily mean that high microbial numbers led to an immediate breakdown of the emulsion since in some cases there existed a 'window period', during which the emulsion supported high microbial numbers yet remained 'tight' (refer to Experiment 3). Colony counts as a 'stand-

alone' test, should therefore not be used as an indication of emulsion stability.

• Emulsion degradation was shown to be rapid after the onset of 'loosening', complete breakdown of the emulsion usually taking no longer than 2½ days. In larger systems (ie. the Hulett roll mill), the 'loosening' stage could take longer, due to the increased volume of emulsion in the storage tanks. Immediate curative measures should therefore be taken following the onset of 'loosening'.

• Measurement of pH was shown to be unsatisfactory as a stand-alone indicator of emulsion biodeterioration. In general, increases in microbial populations resulted in a decrease in pH. This occurred in experiments 2, 3 and 4. However, sometimes this reduction is insignificant and the decrease in pH may not be detected until microbial numbers have already increased to such a degree that control is made very difficult. A marked decrease in pH, occurring at the roll mill, would be a good indication that anaerobic sulphate reducers were present. In this case, rapid eradication of these organisms is essential. It was shown during the course of this study that it is imperative that anaerobic sulphate reducers not be allowed to proliferate in the emulsion reticulation system. These organisms not only lower the pH of the emulsion but also cause corrosion of iron surfaces. Corrosion can lead to pipes having to be replaced, and may cause the emulsion to 'loosen' and break. As a result of the work conducted during this study, the trickle-back, spill-over pipe at the Hulett mill, was re-routed directly to waste management, instead of leading to the oil sump tank. This was a major step in ensuring that the sulphate-reducing bacteria (SRB) were not returned into the working system. SRB have seldom been found in the system since this step was taken.

• Measurement of the specific surface area of the oil droplets in suspension, by means of Malvern laser analysis, was shown to be the most reliable method of directly assessing the stability of the emulsion. This measurement is critical for achieving an effective emulsion management system. However, Malvern laser analysis of the emulsion does not show the numbers or composition of the microbial population at any given time. Therefore, should the emulsion 'loosen', this analysis would not be of use in identifying the cause. Potential causes

of emulsion 'loosening' include microbial, chemical and physical stresses. A plate count test or other microbial quantification procedure is needed in conjunction with the Malvern Laser analysis, in order to assess the number of microorganisms and the condition of the emulsion at any given time.

• Results from Experiments 1-4 showed that microbes pre-exposed to the biocide Busan showed greater tolerance to its active component glutaraldehyde than did those microorganisms not previously exposed (Experiment 5). It is imperative, therefore, that microbial numbers in the emulsion be reduced to the lowest feasible levels. Complete eradication of the microflora would be the ideal situation. It is important to note that control methods should be initiated before the microflora exceed of $1x10^3$ CFUs per ml of emulsion. It was shown that when microbial numbers reached this level, breakdown of the emulsion was imminent, and once this stage had been reached, it was often too late to effectively introduce control measures.

• The detection of biocide tolerant microorganisms was a major step towards the development of a more effective biocide management system. Undoubtedly, unless a second effective biocide (which could be alternated with Busan) is introduced into the emulsion, microbial tolerance and resistance is likely to continue to be a problem. Rotation of two or more biocides with different active components or different modes of action is essential in order to minimize the development of tolerant/resistant strains of microbes. Any two or more biocides chosen for alternate application should contain different active components, thus making it highly unlikely that tolerance, and potentially resistance, would develop to any one of them. The research presented in this project clearly shows that microbial tolerance and resistance to a specific anti-microbial compound does develop with prolonged exposure to only that biocide. Repeated additions of low concentrations of biocide are not recommended, since sub-lethal doses of biocide will encourage development of microbial tolerance and resistance. Shock treatments of high biocide concentrations at calculated intervals are more effective in that they: reduce microbial numbers to zero or near zero, which effectively increases the time taken for the few remaining microorganisms in the emulsion to reestablish large populations; improve

biocide efficiency (less biocide is needed over time); and reduce the chances of development of tolerant and resistant microbes. Any microbial contaminants entering the system following a system-wide shock treatment would not have been exposed to the biocide before and, therefore, would be more susceptible to the active component. Therefore less biocide would be required for effective subsequent treatment of the emulsion.

• Random gaseous testing for the presence of harmful compounds such as glutaraldehyde and formaldehyde (especially around the sprayers) should be carried out after biocide dosing to ensure a safe working environment.

• In order to maintain low microbial populations in the emulsion, it is imperative to eliminate as many sources of external contamination as possible. Plant hygiene needs to be dramatically improved at the Hulett roll mill. This would entail thorough cleaning of the environment surrounding the emulsion reticulation system. Tanks and joints which spill and drip emulsion onto the floor need to be correctly sealed. Stagnant pools of emulsion on the floor beneath and around the main and sump tanks must be removed and the area steam-sanitized. Although this may be expensive in the short term, the inoculum entering the system would be drastically reduced, resulting in savings from decreased subsequent biocide usage. It is also important to instruct workers not to throw foreign objects into the mill system. Refuse bins should be placed throughout the mill and emptied on a regular basis. Foreign objects, especially if organic in nature, add nutrients to the emulsion which may be utilized by microorganisms thereby increasing the microbial load and decreasing emulsion lifespan.

• The temperature of the emulsion in the main and sump tanks should be maintained at 60°C in order to prevent proliferation of mesophilic species. Ideally, new heating elements should be installed where necessary, and these would have to be left on during down-time periods to ensure that microbial blooms do not develop. The tank impellers should also be left on, to avoid the development of anaerobic conditions thereby excluding anaerobic microorganisms from the system.

• Demineralized water is essential for the exclusion of inorganic anionic and cationic minerals from the water supply. The presence of such minerals has been shown (Bennett, 1962 and Holtzman *et al.,* 1982) to increase the growth rate of various emulsion degrading microorganisms. It is imperative, therefore, that the demineralizer is checked and serviced regularly to ensure that only properly demineralized water enters the premix tank for mixing with the oil concentrate.

• Of the four emulsions tested (Prosol, HRF3, Houghton Biostable and B207HS:B216HS), Prosol, which is currently in use at the Hulett hot roll mill, was shown to be the most biosusceptible. Prosol showed signs of deterioration when the contaminating microbial populations exceeded $1x10^3$ - $1x10^4$ $CFUml^{-1}$ over a 3-4 day period without protective biocides (refer to Experiment 1). Of the three other emulsions investigated, all of which were imported, HRF3 was shown to be the most promising with respect to biostability followed by Houghton Biostable and then the emulsion mix B207HS:B216HS. Unfortunately, long-term storage during shipment caused the emulsion B207HS:B216HS to gel and therefore the biostability results obtained during the testing of this emulsion were not conclusive. During the course of this preliminary study, it was found that two of the three imported emulsions (HRF3 and Houghton Biostable) performed effectively over a considerably longer period of time than did Prosol. The use of one of these two emulsions as a replacement for Prosol might, therefore, be considered.

• A rapid detection procedure for quantification of the microbial cell populations, although very useful, is not mandatory for effective emulsion management. The volumes of emulsion in the reticulation system are large enough to allow a standard plate count over a 24 hr period to be sufficient to thwart potential microbial blooms, provided an effective emulsion management system is in place. Preferably, an in-house assessment of microbial growth and biocide levels within the emulsion tanks is needed to ensure non-biased data is acquired.

## 6.2 Future plant design

Although the new roll mill at Hulett Aluminium is already under construction, the following recommendations (from a microbiological viewpoint) can be made:

- At present, one of the main problems experienced at the existing roll mill is the development of microbial blooms in the oil sump tank during downtime. The elimination of the oil sump tank from a new system would avoid this problem. If the emulsion was pumped directly into the main tank, or the main tank was placed below the roll system (allowing the emulsion to gravity-feed into the main tank) then the oil sump could be bypassed, thus reducing the opportunity for microbial growth. However, with the installation of effective heating elements within the oil sump tank, microbial blooms should not develop.

- Effective, consistent, heating equipment should be incorporated into the system. Maintaining temperatures at 60°C or greater would be ideal. If possible the roll neck emulsion system should also be maintained at high temperature.

- Preferably the emulsion tanks should be sealed to prevent as much airborne contamination as possible from entering the system, and also to reduce biocide volatilization. However, the tanks should also be easily accessible for sampling and cleaning purposes. Efficient temperature, redox potential and pH sensors should also be installed so that data can be collected and evaluated expediently.

- Biocide dosing jets should be placed throughout the system in order to provide rapid and effective biocidal treatments. These biocide dosing jets would eliminate potential biocide concentration gradients thus ensuring effective shock dosing of the emulsion.

- The new tanks, pumps and pipework should be made inspection friendly so that they can be checked for leaks on a regular basis and, if necessary, repaired immediately.

• The system should be designed and constructed with no dead-end or slow spill-over trickle-back pipes, (such as those present in the current Hulett hot roll mill). This would ensure that all of the emulsion in the system is in constant circulation. Circulation maintains oxygen levels and ensures the emulsion remains 'tight'. Such oxygenation reduces the chances of anaerobic regions developing. These anaerobic areas are potentially conducive to SRB growth and proliferation. Wherever possible, gravity fed slopes (such as those below the roll mechanism and aluminium ingot conveyor system) should be steeply angled to allow rapid flow-back of emulsion into the system.

• Wherever possible the floor should be constructed of industrial grating so that emulsion from leaks, which cannot be immediately attended to, can flow out of the reticulation system area to waste treatment. This would reduce the number of stagnant pools of emulsion which often collect under the main and oil sump tanks. This would also help to reduce the chances of airborne contamination.

• Where possible, steam points should be built into the system to ensure that all areas of the new mill can be steam sterilized when necessary. At least twice a year, the main emulsion storage tanks and emulsion reticulation piping should be steam cleaned to prevent a build-up of emulsion residues.

### 6.3 Future research

As a result of the research carried out for this thesis, the following areas have been identified for additional study :

• Further biocide studies are required in order to optimize biocide application regimes at the mill. These would include *in situ* studies at the roll mill in which laboratory generated data could be extrapolated and applied with greater confidence. These investigations should include appropriate shock treatment studies and minimum lethal biocide concentration determinations. These tests should include investigation of biocides for their antimicrobial

effectiveness and emulsion compatibility.

- Although the model system used was shown to be relatively effective for emulsion studies, further refinements are necessary. These include the incorporation of effective biocide dosing pumps, air-tight seals, tanks with greater emulsion holding capacities, improved circulators and impellers and on-line measuring equipment. Measuring devices would include pH, temperature and redox potential probes. Such a model should be established in the Research and Development laboratory at Hulett Aluminium.

- More detailed work should be undertaken to identify components of the oil emulsion that are susceptible to biodeterioration. The oil components need to be fractionated and each resulting compound analyzed before and after microbial biodeterioration, using gas chromatography and other analytical techniques. This could be done as a collaborative project involving microbiologists, chemists and biochemists.

- Construction of a laboratory-scale model system, representative of the new roll mill is needed to compare the old and the new mills in terms of their potential to resist microbial contamination. Research and development on the use of microbially resistant materials such as plastics, ceramics, stainless steels and aluminiums for the construction of the new roll mill should also be conducted.

- A rapid, user-friendly procedure, for microbial detection and enumeration is required. This could be done in one of two ways. Firstly, via the quantification of microbial populations over a period of 1-2 hrs instead of the 24-48 hrs presently required. This would entail the development of a microbial quantification procedure that is less tedious than plate counts, possibly a dipslide method which targets the more destructive species (eg. *Pseudomonas* spp.). Secondly, through detection of some microbially-induced change in the emulsion, which could be some specific emulsion breakdown product or a by-product of microbial metabolism.

- Of utmost importance is the ongoing testing of various, alternative emulsions. Initial experiments (refer to experiments 6-8), have identified emulsions that are far more biostable than Prosol.

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## APPENDIX A

1. Nutrient Agar (NA).

31g Nutrient Agar powder (Merck)

1000ml Distilled water

The ingredients were placed into a 2l Erlenmeyer flask, mixed and heated until the agar powder was fully dissolved in the distilled water. The medium was then autoclaved at 121°C for 15 minutes and subsequently poured into 90mm Petri dishes on a laminar flow bench.

2. Rose Bengal Agar (RB Agar).

10g Glucose (Saarchem)

5g Peptone (Oxoid)

1g $KH_2PO_4$ (Saarchem)

0.5g $MgSO_4.7H_2O$ (Saarchem)

0.03g Rose Bengal (BDH)

15g Agar (Merck)

1000ml Distilled water

The ingredients were place into a 2l Erlenmeyer flask, heated and mixed. The medium was then autoclaved at 121°C for 15 minutes. When the mixture had cooled to 45°C, 0.3g of streptomycin was aseptically added (after dissolving in 10ml of sterile distilled water) using a sterile filter syringe. The medium was then poured into 90mm Petri dishes on a laminar flow bench.

3. Eosin Methylene Blue Agar (EMB Agar).

10g Peptone from casein (Oxoid)

2g $KH_2PO_4$ (Saarchem)

5g Lactose (Saarchem)

5g Sucrose (Saarchem)

0.4g Eosin yellow (Saarchem)

0.065g Methylene blue (Protea)

15g Agar (Merck)

1000ml Distilled water

The ingredients were placed into a 2l Erlenmeyer flask, mixed and the pH adjusted to ±7.1. The medium was then autoclaved at 121°C for 15 minutes and subsequently poured into 90mm Petri dishes on a laminar flow bench.

4. Bromothymol blue lactose cysteine agar (BBLC Agar).

4g Peptone from casein (Oxoid)

4g Universal peptone (Oxoid)

3g Meat extract (Oxoid)

0.128g L-Cysteine (Saarchem)

10g Lactose (Saarchem)

0.02g Bromothymol blue (Saarchem)

15g Agar (Merck)

1000ml Distilled water

The ingredients were placed into a 2l Erlenmeyer flask, mixed and the pH adjusted to ±7.1. The medium was then autoclaved at 121°C for 15 minutes and subsequently poured into 90mm Petri dishes on a laminar flow bench.

5. Cetrimide Agar (Cet. Agar).

46g Cetrimide agar powder (Merck)

10ml Glycerol (Saarchem)

1000ml Distilled water

The ingredients were placed into a 2l Erlenmeyer flask, mixed, heated to dissolve, and the pH adjusted to ±7.1. The medium was then autoclaved at 121°C for 15 minutes and subsequently poured into 90mm Petri dishes on a laminar flow bench.

6. Hugh Liefson's Medium (HL Medium).

2g Peptone (Oxoid)

5g NaCl (Saarchem)

0.3g $KH_2PO_4$ (Saarchem)

0.03g Bromothymol blue (Saarchem)

10g Glucose (Saarchem)

1000ml Distilled water

The ingredients were placed into a 2l Erlenmeyer flask, mixed, heated to dissolve, the pH adjusted to ±7.1 and then dispensed into test tubes (20ml per test tube) containing a Durham tube for gas collection. The test tubes were then autoclaved at 121°C for 15 minutes. A deep overlay of sterile mineral oil was aseptically added after inoculation.

7. MacConkey Agar (Mac. Agar).

52g MacConkey agar powder (Oxoid)
1000ml Distilled water

The ingredients were placed into a 2l Erlenmeyer flask, mixed and heated until the powder was fully dissolved in the distilled water. The medium was then autoclaved at 121°C for 15 minutes and subsequently poured into 90mm Petri dishes in a laminar flow bench.

8. Iron Sulphite Agar (IS Agar).

23g Iron Sulphite agar powder (Oxoid)
1000ml Distilled water

The ingredients were placed into a 2l Erlenmeyer flask, mixed and heated until the powder was fully dissolved in the distilled water. The medium was then autoclaved at 121°C for 15 minutes and subsequently poured into 90mm Petri dishes in a laminar flow bench.

9. Modified V8 Agar (MV8 Agar).

180ml V8 juice (Campbells)
15g Agar (Merck)
2g $CaCO_3$ (Saarchem)
770ml Distilled water

The ingredients were placed into a 2l Erlenmeyer flask, mixed and heated until the powder was fully dissolved in the distilled water. The mixture was then autoclaved at 121°C for 15 minutes. When the mixture had cooled-down enough to touch the following were added:

*0.025g Streptomycin (Boehringer Mannheim)

*0.01g Penicillin (Boehringer Mannheim)

*0.5g Chloramphenicol (Boehringer Mannheim)

*Each antibiotic was dissolved in 50ml of sterile distilled water and the resultant solutions then sterilized by passage through a membrane filter.

The final volume of medium was therefore approximately 1000ml. The medium was then poured into 90mm Petri dishes in a laminar flow bench.

## APPENDIX B

Hucker's Modified Gram Stain Test:

1. A drop of sample was placed onto a clean, dry microscope slide and spread over the surface to form a thin film.
2. The film was heat fixed by passing the slide through a Bunsen burner flame.
3. The film was covered with crystal violet and allowed to stain for 1 minute. The slide was then washed with water.
4. The film was stained with iodine for 1 minute. The slide was then washed with water.
5. The film was decolorized with 95% ethanol for 30 seconds. The slide was then washed with water.
6. The film was counterstained with safranin for 1-2 minutes. The slide was then washed with water and gently blotted dry.
7. The slide preparation was then viewed using a light microscope (Zeiss Axiophot) under oil immersion.

Gram positive cells appeared violet whilst Gram negative cells appeared red or pink.

(Bartholomew and Mittwer, 1952 and Denger and Schink, 1995).

Gregersen's test:

1. Ten drops of 3% KOH were placed onto a clean dry microscope slide.
2. An inoculation loopful of bacterial cells was placed into the KOH solution, and stirred.
3. The inoculation loop was slowly pulled away from the slide.

If the tested organism was Gram positive, no thread between slide and loop formed when the loop was pulled away from the slide. If the tested organism was Gram negative, a thread formed between the loop and slide when the loop was pulled away from the slide.

## APPENDIX C

1. Neutral base oil selective medium (NBO) 46.47% (m/m)

Neutral base oil solution:

46.47g Neutral Base Oils

53.53g Distilled water

The mixture was shaken for 10 minutes and subsequently placed in a sonicator for 5 minutes.

From the above emulsion;

30ml Neutral base oil emulsion was added to

970ml Distilled water and

15g Agar

in a 2l Erlenmeyer flask to make a 3% concentration of neutral base oils. The contents were mixed and heated until the agar powder was fully dissolved. The medium was then autoclaved at 121°C for 15 minutes and subsequently poured into 90mm Petri dishes on a laminar flow bench. Whilst being poured, the flask containing the medium was constantly shaken to ensure even distribution of the oils.

2. Naphthenic base oil selective medium (NapBO) 30.15% (m/m)

Naphthenic base oil solution:

30.15g Naphthenic Base Oils
69.85g Distilled water

The mixture was shaken for 10 minutes and subsequently placed in a sonicator for 5 minutes.

From the above solution;

30ml Naphthenic base oil solution was added to
970ml Distilled water and
15g Agar

in a 2l Erlenmeyer flask to make a 3% concentration of naphthenic base oils. The contents were mixed and heated until the agar powder were fully dissolved. The medium was then autoclaved at 121°C for 15 minutes and subsequently poured into 90mm Petri dishes on a laminar flow bench. Whilst being poured the flask containing the medium was constantly shaken to ensure even distribution of the oils.

3. Oleic acid selective medium (OA) 12.90% (m/m)

Oleic acid solution:

12.9g Oleic acid
87.1g Distilled water

The mixture was shaken for 2 minutes.

From the above solution;

30ml Oleic acid solution was added to
970ml Distilled water and
15g Agar

in a 2l Erlenmeyer flask to make a 3% concentration of oleic acid. The contents were mixed and heated until the agar powder was fully dissolved. The medium was then autoclaved at 121°C for 15 minutes and subsequently poured into 90mm Petri dishes on a laminar flow bench. Whilst being poured the flask containing the medium was constantly shaken to ensure even distribution of the oils.

4. Phenolic selective medium (PH) 0.30% (m/m)

As per oleic acid selective medium except that oleic acid was replaced with phenol at the following concentrations:

Phenol solution:

0.3g Phenol
99.7g Distilled water

Method follows as per oleic acid medium.

5. Hexylene glycol selective medium (HG) 1.80% (m/m)

As per oleic acid selective medium except that oleic acid solution was replaced with hexylene glycol solution at the following concentration:

Hexylene glycol solution:

1.8g Hexylene glycol
98.2g Distilled water

Method follows as per oleic acid medium.

6. Tricresol phosphate selective medium (TCrP) 3.00% (m/m)

As per oleic acid selective medium except that oleic acid solution was replaced with tricresol phosphate solution at the following concentration:

Tricresol phosphate solution:

3g Tricresol phosphate
97g Distilled water

Method follows as per oleic acid medium.

7. Diethanolamine selective medium (DEA) 1.39% (m/m)

As per oleic acid selective medium except that oleic acid solution was replaced with diethanolamine solution at the following concentration:

Diethanolamine solution:

1.39g Diethanolamine

98.61g Distilled water

Method follows as per oleic acid medium.

8. Triethanolamine selective medium (TEA) - 3.49% (m/m)

As per oleic acid selective medium except that oleic acid solution was replaced with triethanolamine solution at the following concentration:

Triethanolamine solution:

3.49g Triethanolamine
96.51g Distilled water

Method follows as per oleic acid medium.

9. Prosol selective medium (PS) 100% (m/m)

30ml Prosol emulsion was added to
970ml Distilled water and
15g Agar

in a 2l Erlenmeyer flask to make a 3% concentration of Prosol emulsion (which contains all of the components of the other selective media at the concentration at which they would be present in the emulsion as used at the Hulett Aluminium hot roll mill, Pietermaritzburg plant). The contents were mixed and heated until the agar powder was fully dissolved in the distilled water. The medium was then autoclaved at 121°C for 15 minutes and subsequently poured into 90mm Petri dishes on a laminar flow bench. Whilst being poured the flask containing the medium was constantly shaken to ensure even distribution of the oils.

## APPENDIX D

1. Malvern Laser analysis.

The Malvern laser is an instrument which measures the size of oil droplets in an emulsion. This is done by shining a high intensity laser beam through two glass plates, between which the emulsion is being pumped. The absorbence is measured as the amount of light passing through the plates and, using a computer, the relative sizes of the oil droplets in the emulsion are calculated. The results are presented in the form of a table and graph. Of primary importance, however, is the **specific surface area** of the oil droplets measured in $\mathbf{m^2cc^{-1}}$ or $\mathbf{m^2ml^{-1}}$ of the sample. The specific surface area is a measure of the total surface area of all the oil droplets in solution. If the specific surface area is high, then the emulsion is relatively 'tight' and in good condition, conversely, if the specific surface area is low the oil droplets are not being held in suspension and the emulsion is 'loose'. The graphical representation gives the user a quick and easy visual assessment of the emulsion's condition. Prosol (the emulsion currently in use at Hulett Aluminium hot roll mill) is considered to be 'tight' when the specific surface area is approximately $10m^2ml^{-1}$. However, an acceptable surface area may range from $9.5\text{-}12m^2ml^{-1}$.

Approximately 30-50ml of sample is required for Malvern analysis. All samples were vigorously shaken before injection into the Malvern Master Sizer M520 laser system.

2. Glutaraldehyde analysis.

Glutaraldehyde analysis was carried out according to Heenan and Burrell's method (1991).

A standard calibration curve comprising 6 concentrations of glutaraldehyde ($0mgl^{-1}$, $100mgl^{-1}$, $200mgl^{-1}$, $300mgl^{-1}$, $400mgl^{-1}$ and $500mgl^{-1}$) was established as follows:

i) A one milliliter sample of each dilution were placed in separate Eppendorf microfuge tubes

and centrifuged at 13000rpm - 11400g centrifugal force for 5 minutes to separate the aqueous and oil phases.

ii) 50$\mu$l of the dissolved aqueous phase was removed and mixed with 200$\mu$l of 10mM 2,4-dinitrophenylhydrazine (DNPH) in 2N hydrochloric acid and left to stand at room temperature for 2 minutes.

iii) 1ml of High Performance Liquid Chromatography (HPLC) grade acetonitrile was added and 10$\mu$l aliquots of the mixtures injected into a Waters 486 Absorbence detector C and analyzed with the aid of a Waters 600E system controller.

HPLC set-up:

Column: Cat.

Column temperature: 30°C

Mobile Phase: 75% acetonitrile, 25% water.

Flow rate: 1ml.min$^{-1}$.

Sample loop: 10$\mu$l.

Detection wavelength: 360nm.

The resultant standard calibration curve was used to calculate the glutaraldehyde concentrations in identically treated emulsion samples by comparing peak areas on the respective chromatographs.

## APPENDIX E

Raw Data

Table A: Experiment 1. Changes in numbers of resistant microbes present, mean pH and mean oil droplet surface area with time in emulsion containing 0mgl$^{-1}$ biocide

| Day | Mean CFUml$^{-1}$ | Mean pH | Malvern ($m^2ml^{-1}$) |
|---|---|---|---|
| 0 | $2.9x10^3$ | 8.14 | 10.4122 |
| 1 | $5.4x10^3$ | 8.22 | 10.8008 |
| 2 | $5.0x10^4$ | 8.12 | 10.4013 |
| 3 | $2.6x10^5$ | 7.66 | 9.9625 |
| 4 | $1.3x10^6$ | 7.69 | 3.6625 |
| 5 | $3.4x10^6$ | 7.75 | 2.4496 |

Table B: Experiment 2. Changes in numbers of resistant microbes present, mean pH, mean oil droplet surface area and mean glutaraldehyde concentration in emulsion containing 500mgl$^{-1}$ Busan

| Day | Mean CFUml$^{-1}$ | Mean pH | Malvern ($m^2ml^{-1}$) | mgl$^{-1}$ Glutaraldehyde |
|---|---|---|---|---|
| 0 | $5.8x10^3$ | 8.25 | 10.6446 | 180.79 |
| 1 | $2.0x10^2$ | 8.20 | 10.5813 | 157.24 |
| 2 | 5.0x10 | 8.11 | 11.0328 | 131.46 |
| 3 | $3.0x10^2$ | 7.83 | 10.389 | 3.97 |
| 4 | $9.6x10^3$ | 7.53 | 9.7554 | 0 |
| 5 | $4.9x10^5$ | 7.62 | 4.4455 | 0 |
| 6 | $2.7x10^6$ | 7.54 | 2.9421 | 0 |
| 7 | $1.2x10^7$ | 7.40 | 2.6425 | 0 |

Table C: Experiment 3. Changes in numbers of resistant microbes present, mean pH, mean oil droplet surface area and mean glutaraldehyde concentration in emulsion containing 1000mgl$^{-1}$ Busan

| Day | Mean CFUml$^{-1}$ | Mean pH | Malvern ($m^2ml^{-1}$) | mgl$^{-1}$ Glutaraldehyde |
|---|---|---|---|---|
| 0 | $1.1x10^3$ | 8.33 | 10.8587 | 544 |
| 1 | $5.3x10^2$ | 7.98 | 10.6031 | 432 |
| 2 | 6.7x10 | 8.08 | 10.7065 | 412 |
| 3 | 5.3x10 | 8.09 | 10.8307 | 365 |
| 4 | 6.8x10 | 7.94 | 10.9356 | 310 |
| 5 | 8.2x10 | 7.89 | 11.1540 | 254 |
| 6 | $1.3x10^2$ | 7.94 | 10.9973 | 182 |
| 7 | $2.3x10^2$ | 8.06 | 11.6226 | 182 |
| 8 | $1.1x10^3$ | 7.92 | 10.0254 | 26 |
| 9 | $9.3x10^4$ | 8.00 | 8.8583 | 28 |
| 10 | $6.1x10^5$ | 7.53 | 2.1445 | 10 |
| 11 | $3.6x10^6$ | 6.94 | 1.8395 | 10.6 |

Table D: Experiment 4. Changes in numbers of resistant microbes present, mean pH, mean oil droplet surface area and mean glutaraldehyde concentration in emulsion containing 5000mgl$^{-1}$ Busan

| Day | Mean CFUml$^{-1}$ | Mean pH | Malvern ($m^2ml^{-1}$) | mgl$^{-1}$ Glutaraldehyde |
|---|---|---|---|---|
| 0 | $1.3x10^2$ | 8.02 | 11.3633 | 791.8 |
| 1 | 0 | 7.96 | 11.5298 | 743.9 |
| 2 | 0 | 7.98 | 11.0257 | - |
| 3 | 0 | 7.82 | - | 667.8 |
| 4 | 0 | 7.86 | 11.5611 | - |
| 5 | 0 | 7.77 | - | 560.2 |
| 6 | 0 | 7.75 | 11.5580 | - |
| 7 | 0 | 7.80 | - | 508.1 |

| | | | | |
|---|---|---|---|---|
| 8 | 0 | 7.78 | 11.1800 | - |
| 9 | 0 | 7.80 | - | 578.2 |
| 10 | 0 | 7.78 | 10.3211 | - |
| 11 | 0 | 7.76 | - | 546.5 |
| 12 | 0 | 7.75 | 11.2529 | - |
| 13 | 0 | 7.79 | - | 555.7 |
| 14 | 0 | 7.84 | 9.9694 | - |
| 15 | 0 | 7.80 | - | 551.6 |
| 16 | 0 | 7.80 | 10.9551 | - |
| 17 | 0 | 7.80 | - | 634.9 |
| 18 | 0 | 7.80 | 10.3778 | - |
| 19 | 0 | 7.83 | - | 467.5 |
| 20 | 0 | 7.80 | 11.0065 | - |
| 21 | 0 | 7.84 | - | - |
| 22 | 0 | 7.84 | 9.6103 | 481.7 |
| 23 | 0 | 7.80 | - | - |
| 24 | 0 | 7.75 | 9.9821 | 458.2 |
| 25 | 0 | 7.66 | - | - |
| 26 | 0 | 7.73 | 10.0783 | 475.1 |
| 27 | 0 | 7.71 | - | - |
| 28 | 0 | 7.67 | 10.1169 | 450.5 |
| 29 | 0 | 7.75 | - | - |
| 30 | 0 | 7.71 | 9.7497 | 342.3 |
| 31 | 0 | 7.78 | - | - |

Table E: Experiment 5. Changes in numbers of susceptible microbes present, mean pH, mean oil droplet surface area and mean glutaraldehyde concentration in emulsion containing 500mgl$^{-1}$ Busan

| Day | Mean CFUml$^{-1}$ | Mean pH | Malvern ($m^2ml^{-1}$) | mgl$^{-1}$ Glutaraldehyde |
|---|---|---|---|---|
| 0 | $1.0x10^3$ | 8.25 | 11.2545 | 182.25 |
| 1 | $5.0x10^2$ | 8.23 | - | 158.1 |
| 2 | $1.5x10^2$ | 8.20 | 11.7210 | - |
| 3 | $1.6x10^2$ | 8.16 | 12.7303 | - |
| 4 | $9.1x10^2$ | 8.17 | - | - |
| 5 | $3.2x10^2$ | 8.18 | 10.9340 | 131.2 |
| 6 | $5.7x10^2$ | 8.20 | 9.7601 | 103.1 |
| 7 | $2.6x10^2$ | 8.17 | 9.9602 | 87.6 |
| 8 | $2.4x10^2$ | 8.15 | 10.7795 | 73 |
| 9 | $2.1x10^2$ | 8.13 | 10.4186 | 51.6 |
| 10 | $2.0x10^2$ | 8.09 | 10.5021 | 43.1 |
| 11 | $3.0x10^3$ | 8.19 | 9.2663 | 42.5 |
| 12 | $2.0x10^4$ | 8.07 | 6.6647 | 24.6 |
| 13 | $1.3x10^5$ | 8.07 | - | - |
| 14 | $8.7x10^5$ | 7.82 | 4.4776 | 12.1 |
| 15 | $2.2x10^6$ | 7.66 | 3.0748 | 0 |
| 16 | $3.1x10^6$ | 7.80 | - | - |
| 17 | $4.3x10^6$ | 8.02 | 1.9226 | 0 |

## APPENDIX F

Raw Data

Table F: Experiment 6. Changes in numbers of microbes present, mean pH and mean oil droplet surface area in emulsion HRF3

| Day | Mean CFUml$^{-1}$ | Mean pH | Malvern ($m^2ml^{-1}$) |
|---|---|---|---|
| 0 | $1.3x10^5$ | 7.40 | 9.1960 |
| 1 | $7.1x10^5$ | 7.41 | 9.4316 |
| 2 | $1.6x10^6$ | 7.35 | |
| 3 | $2.5x10^6$ | 7.45 | 9.2937 |
| 4 | $4.7x10^5$ | 7.32 | |
| 5 | $8.3x10^5$ | 7.43 | 10.6409 |
| 6 | $1.2x10^6$ | 7.46 | |
| 7 | $2.9x10^6$ | 7.49 | |
| 8 | $6.0x10^5$ | 7.63 | 11.5436 |
| 9 | $4.1x10^5$ | 7.75 | 11.4239 |
| 10 | $7.3x10^5$ | 7.63 | |
| 11 | $4.2x10^5$ | 7.73 | 11.5425 |
| 12 | $2.7x10^5$ | 7.81 | |
| 13 | $1.6x10^5$ | 7.84 | 11.3167 |
| 14 | $1.7x10^5$ | 7.73 | |
| 15 | $2.2x10^5$ | 7.82 | 11.5826 |
| 16 | $3.0x10^5$ | 7.88 | |
| 17 | $3.2x10^5$ | 7.94 | 11.0619 |
| 18 | $3.6x10^5$ | 7.82 | 11.2263 |
| 19 | $3.7x10^5$ | 7.86 | 11.3419 |
| 20 | $3.8x10^5$ | 7.84 | 11.3210 |
| 21 | $3.7x10^5$ | 7.86 | 11.3049 |

Table G: Experiment 7. Changes in numbers of microbes present, mean pH and mean oil droplet surface area in emulsion B207HS:B216HS

| Day | Mean CFUml$^{-1}$ | Mean pH | Malvern ($m^2ml^{-1}$) |
|---|---|---|---|
| 0 | $1.6x10^5$ | 6.90 | 2.0777 |
| 1 | $5.4x10^5$ | 7.15 | 1.8674 |
| 2 | $4.3x10^5$ | 7.13 | |
| 3 | $2.7x10^6$ | 6.86 | 2.2059 |
| 4 | $6.9x10^7$ | 6.92 | |
| 5 | $1.7x10^8$ | 7.00 | 2.2054 |
| 6 | $2.1x10^7$ | 6.94 | |
| 7 | $9.6x10^6$ | 6.91 | 2.6445 |
| 8 | $5.7x10^6$ | 6.91 | |
| 9 | $9.1x10^6$ | 6.90 | 3.3364 |
| 10 | $3.0x10^7$ | 6.87 | |
| 11 | $4.0x10^7$ | 6.83 | 3.2220 |
| 12 | $4.0x10^7$ | 6.82 | |
| 13 | $5.9x10^7$ | 6.88 | 3.2711 |
| 14 | $7.7x10^7$ | 6.90 | |
| 15 | $4.4x10^7$ | 6.78 | 3.4283 |
| 16 | $3.6x10^7$ | 6.81 | |
| 17 | $2.9x10^7$ | 6.79 | 3.2955 |
| 18 | $3.0x10^7$ | 7.00 | |
| 19 | $3.2x10^7$ | 6.80 | 3.7198 |
| 20 | $3.1x10^7$ | 6.82 | |
| 21 | $3.4x10^7$ | 6.85 | 3.5998 |

Table H: Experiment 8. Changes in numbers of microbes present, mean pH and mean oil droplet surface area in emulsion Houghton Biostable

| Day | Mean CFUml$^{-1}$ | Mean pH | Malvern ($m^2ml^{-1}$) |
|---|---|---|---|
| 0 | $9.3x10^3$ | 7.85 | 0.4926 |
| 1 | $5.8x10^5$ | 7.91 | |
| 2 | $3.9x10^6$ | 7.81 | 0.5219 |
| 3 | $1.1x10^7$ | 7.86 | |
| 4 | $9.2x10^6$ | 7.70 | 0.6418 |
| 5 | $3.9x10^6$ | 7.70 | |
| 6 | $2.6x10^6$ | 7.60 | 0.4841 |
| 7 | $2.5x10^6$ | 7.84 | |
| 8 | $3.5x10^6$ | 7.82 | 0.3928 |
| 9 | $4.7x10^6$ | 7.88 | |
| 10 | $4.1x10^6$ | 7.81 | 0.7377 |
| 11 | $3.9x10^6$ | 7.84 | |
| 12 | $2.0x10^6$ | 7.78 | 0.6394 |
| 13 | $9.1x10^6$ | 7.79 | |
| 14 | $1.2x10^7$ | 7.79 | 0.5151 |
| 15 | $1.3x10^7$ | 7.79 | |
| 16 | $1.9x10^7$ | 7.73 | 0.5258 |
| 17 | $9.9x10^6$ | 7.76 | |
| 18 | $8.0x10^6$ | 7.75 | 0.5651 |
| 19 | $8.2x10^6$ | 7.74 | 0.5521 |
| 20 | $8.1x10^6$ | 7.73 | |
| 21 | $7.9x10^6$ | 7.72 | 0.5673 |