DEVELOPMENT AND MICROBIAL COMMUNITY ANALYSIS OF A BIOLOGICAL TREATMENT PROCESS FOR EDIBLE OIL EFFLUENT

FAIZAL BUX

I hereby declare that the thesis represents my own work. It has not been submitted before for any diploma/degree or examination at any other Technikon/University.

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I hereby approve the final submission of the following thesis

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DEDICATION

This thesis has been prepared in order to contribute to the existing knowledge on the application of Biological Treatment Processes for the remediation of Edible Oil Effluents.

ABSTRACT

Globally, wastewaters emanating from edible oil manufacturers contain high organic (BOD & COD) and phosphate loads and known for creating shock-loading problems for the receiving wastewater treatment installations. Discharge of poor quality final effluents also negatively impact on and cause eutrophication of natural water sources such as rivers and dams. In South Africa, a large concentration of the edible industries are localized in the Pietermaritzburg region of Kwa-Zulu Natal and have been regularly associated with discharge of poor quality final effluent that did not subscribe to municipal regulation standards. Current treatment of choice for wastewater's in the edible oil industry have been limited primarily to dissolved air flotation combined with the use of chemical coagulants or physical separation of oil and grease via a gravity fat trap and subsequent pH correction. These physico-chemical methods have achieved limited success and the emulsified grease tends to clog sewer pipes and pumps producing poor quality effluents. Therefore, the aim of the current research was to develop suitable treatment technology focussing on adapting activated sludge process to remediate edible oil effluents and determine the microbial community of the process using novel molecular techniques.

The activated sludge process design and operation was based on an MLE configuration which was preceded with optimizing appropriate pretreatment technology. Bench scale investigations using alum and ferric chloride combined with DAF was compared to the performance of a commercial coagulant C40 in a 25L vessel. Detailed chemistry of the raw, flocculated and final effluent was conducted by GCMS. The sludge age was maintained at 16d with a unit volume capacity of 28L and hydraulic retention time of approximately 24h. The organic strength of the wastewater was gradually increased and ranged from 500 to 1800 mg/L. In addition, the wastewater from industry had to be corrected to ensure the proper N & P balance prior to biological treatment. *In situ* hybridization techniques was restricted to probes EUB 338, alpha, beta. and gamma of *Proteobacteria* and HGC probe. PCR-DGGE was adapted from standard protocol and the survey of filamentous bacteria and protozoa was conducted using conventional identification techniques. A non polar solvent was used to remove the oily layer and facilitate ultrastructural investigation of the floc using electron microscopy.

The commercial coagulant C40 showed comparatively superior performance and was selected as the pretreatment technology to prepare the effluent for activated sludge treatment. GCMS analysis of the raw effluent revealed 28 organic compounds comprising valuable essential oils with high market value. The lab scale process was conducted in phases encompassing common operational parameters. Early stages of the lab scale process were unsuccessful since the wastewater was not pretreated. Subsequent to pretreatment, overall performance of the process was good recording maximum COD removal efficiencies of > 90% during steady state conditions. The process responded positively to increase in the influent COD concentration (organic strength) with the maximum concentration of COD removed being > 1500 mg/L. The OUR served as a overall good indicator of the process performance at any one time. The system perturbations that occasionally impacted on process performance included disruptions in the supply of influent from industry and variability in the organic

strength (COD) and quality, sometimes impacting on controlling the influent feed COD concentration of the process. In addition, bulking conditions at the end of phase 3 resulted in the process being temporarily terminated. Electron microscopy of the activated sludge floc revealed an intimate association of an oily layer with the floc surface and an abundance of microorganisms especially bacteria. Achieving high COD removal was indicative that a substantial portion of the edible oil wastewater was biodegradable. The latter was also substantiated with the GCMS analysis of the final effluent revealing most of the organic compounds being removed with biological treatment.

FISH analysis confirmed that members belonging to the kingdom *Bacteria* dominated both the seed inoculum and in the lab scale process. The microbial community analysis of the process was partitioned into 2 phases with both displaying relatively similar population profiles regardless of varying operational conditions. A noticeable feature of the *in situ* hybridization analysis was the decrease in the EUB/DAPI ratio and DAPI total cell counts as the process progressed without showing any correlation or impact on process performance. A population shift was evident with the beta subclass of *Proteobacteria* dominating the seed sludge and the alpha subclass dominating in the process. The total percentages of the respective subclasses of *Proteobacteria* also decreased as the process progressed. The group specific probes used during the current investigation accounted for most of the bacteria detected by the EUB 338 probe. The PCR-DGGE analysis served to verify the FISH findings emphasizing the differences between the microbial community profile of the seed sludge and process samples. The DGGE analysis also confirmed a population shift towards a lower diversity as the lab scale process progressed indicating the possible establishment of a 'specialized community' structure. A significant finding using DGGE was the similarity in the banding pattern representing the different zones in the reactor, inferring that the microbial community structure of the aerobic and anoxic zones in the reactor were identical. The findings presented by *in situ* hybridization and DGGE analysis complemented each other, enforcing the benefits of the combined application of these techniques for microbial community structure profiling.

The identification of filamentous bacteria associated with bulking conditions experienced in the process reiterated previous findings showing the predominance of *S. natans, H. hydrossis* and Type 1701. *M. parvicella* which was ranked secondary in the process has been previously associated with bulking conditions and is a specialized lipid consumer. Microscopic investigations of the floc structure showed that the flocs were generally irregular in shape and typical of bulking sludges and plants associated with the treatment of industrial effluents. A noticeable feature of the survey of protozoa in the lab scale process was the comparatively low species diversity in comparison to conventional activated sludge plants. The group of protozoa that predominated in the process were the sessile ciliates and were generally associated with periods of low organic loading as depicted by the F/M ratios. Although displaying erratic predominance patterns, *Vorticella* sp showed comparatively higher abundance levels. Another significant finding on the population dynamics was that the different groups of protozoa followed a clear pattern of succession shown by varying abundance levels during different periods.

The emphasis of the microbial community analysis of the lab scale process using novel and established techniques was to improve our understanding of microorganisms associated with system perturbations and undoubtedly served to enhance the current knowledge base on the microbial community profile of activated sludge processes treating edible oil effluents.

PREFACE

Some of this material presented in this thesis has/will be published and/or presented elsewhere:

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ABBREVIATIONS

ABR - anaerobic baffled reactor.

AFBR- - anaerobic fluidized bed reactors

BEPR - biological excess phosphorus removal

BNR - biological nutrient removal

BOD - biological oxygen demand

CEC - cation exchange capacity

CLSM - confocal laser scanning microscopy

COD - chemical oxygen demand

CP - cleaner production

DAF - dissolved air flotation

DAPI - 4',6-diamidino-2-phenylindole

DCM - dichloromethane

DGGE - denaturing gradient gel electrophoresis

DO - dissolved oxygen

DWAF - Department of Water Affairs & Forestry

ECP - extracellular polysaccharide

EDAX - energy dispersive x-ray analysis

EOP - end of pipe

EPS - extracellular polymeric substances

ESEM - environmental scanning electron microscope

F/M ratio - food to microorganism ratio

FFA - free fatty acids

FISH - fluorescent *in situ* hybridization

FOG - fats oils & grease

FSA - free and saline ammonia

F_{xt} - unaerated mass fraction

HRT - hydraulic retention time

IHI	-	in house improvements
LCFA	-	long chain fatty acids
MAR	-	microautoradiograpy
MCRT	-	mean cell residence time
MLE	-	modified Ludzak- Ettinger
MLSS	-	mixed liquor suspended solids
MLVSS	-	mixed liquor volatile suspended solids
OME	-	olive mill effluent
OUR	-	oxygen utilization rate
PAO	-	polyphosphate accumulating organisms
PCR	-	polymerase chain reaction
PHB	-	polyhydroxybutyrate
Poly-P-	-	polyphosphate
POME	-	palm oil mill effluent
PPM	-	parts per million
RAS	-	return activated sludge
RBCOD	-	readily biodegradable COD
RFLP	-	restriction fragment length polymorphism
Rs	-	sludge age
S_{bi}	-	biodegradable COD
S_{bpi}	-	particulate slowly biodegradable
S_{bsi}	-	soluble readily biodegradable
SCFA	-	short chain fatty acids
S_{ti}	-	influent COD
S_{upi}	-	particulate unbiodegradable COD
S_{usi}	-	soluble unbiodegradable COD
SVI	-	sludge volume index
TAG	-	triacylglycerides
TDS	-	total dissolved solids

TFM - total fatty material
TKN - total kjeldahl nitrogen
TP - total phosphate
TSS - total suspended solids
UASB - upflow anaerobic sludge blanket
VFA - volatile fatty acids
VOC - volatile organic carbons
VSS - volatile suspended solids
WRC - Water Research Commission

CHAPTER ONE

INTRODUCTION AND MOTIVATION FOR THE STUDY

1.1 IMPACT OF INDUSTRIAL EFFLUENTS

Management of natural resources and the environment has progressed rapidly in South Africa in recent years following a global trend of increasing knowledge about the environment and the need to protect it. Industrial activities consume a huge amount of resources and energy, and discharge enormous emissions to the environment. These emissions are causing local and global environmental pollution problems. It is needless to say that the minimization of pollutants from industrial activities is of great importance for the survival of mankind in the future. It is a known fact that organic industries world wide are experiencing increased pressure from both the public and their customers to eliminate their effluent streams. Efforts to put pressure on industry to treat their effluent to an acceptable standard are sometimes met with considerable resistance. The reason being that expenditure on treatment plants are often perceived as money invested without any return (van der Westhuizen and Pretorius,1998).

Some of the responses adopted by industry to deal with reducing environmental pollution include establish a zero-emission production process and optimizing technology associated with wastewater treatment, thereby minimizing pollutants discharged from the process. Appropriate combination of various wastewater treatment processes have been successful in producing high quality final effluent with less energy consumption. Effective removal of organic pollutants may not be achieved even after biological acclimation. Pre- or post treatment using chemical or physical methods may be required to obtain a sufficient effluent quality. Another approach focused on reducing the pollutant discharge from each unit process in the industry. This can be put into practice by replacing and /or refining the unit process in the industry and thereby increasing the yield of the product, and subsequently reducing waste from the process. In addition, the source, quality and quantity of the pollutants discharged from each production process should be clarified before considering the reduction of those pollutants

(Fujie and Hu, 1997).

1.2 EDIBLE OIL WASTEWATER

The vegetable oil processing industry involves the extraction and processing of oils and fats from vegetable sources. There are 16 operational edible oil processing plants in S. Africa which produce approximately 3×10^5 t of vegetable oil annually, concomitantly consuming nearly 2×10^6 m^3 of water (Mkhize et al.,2000). The process of edible oil refining includes neutralization of free fatty acids, removal of gummy materials, deodorization and color removal stages. The quantity and contamination of the wastewater from the refining of crude oil depends mainly on the temperature and hardness of the available cooling water, on the processing equipment used (continuous, discontinuous processes, process temperatures and pressures, on the raw material used and how often the type of oil is changed (Rohbrechtbuck and Sekoulov, 1990). Wastewater associated directly with the process of refining i.e. neutralization etc., contributes most of the pollution load in the effluent with high amount of COD, oils and greases etc., whereas non-process wastewater (cooling, boiler water) forms the major portion (96%) of the total wastewater quantity. However, the quantity and physico-chemical characteristics of the effluents produced vary considerably for different refineries (Mkhize *et al.*, 2000).

Conventional treatment of wastewater from edible oil refineries has been restricted to physical, chemical and occasionally biological processes with substantial mechanical equipment required for the dewatering of sludges. For many years the removal of separable fatty matters was achieved in so called 'fat traps'. The gravity fat traps which is usually installed as standard equipment on all process effluent streams is the simplest form of physical treatment. The latter technology had limitations since it could not remove emulsified fats under 500 mg/L, thus failing to satisfy municipal discharge standards (Eroglu *et al.*, 1990) Dissolved air flotation (DAF) techniques are , however, are much more widely applied to fatty effluents and more recently includes chemically enhanced DAF. The latter includes additives such as aluminum and ferric salts which are used to bind oil droplets together, and thus create a surface that can easily entrap air bubbles. Fatty material removal using DAF has been successful, sometimes as high as 80% but the effluent still do not satisfy municipal discharge standards (Ozturk *et al.*, 1990).

1.3 BIOLOGICAL TREATMENT TECHNOLOGY

Biological wastewater treatment processes are not as common in the edible oil industry. When applied , the processes that are used include aerated lagoons and activated sludge. Recently focus has also shifted towards developing anaerobic processes for the treatment of edible oil wastewater's (Beccari *et al.*, 2001). One of the constraints of biological treatment technology is the space required (Khan and Akhtar, 1998). However, the benefits associated with the technology tend to outweigh the disadvantages. Due to stringent municipal discharge standards, wastewater treated with DAF does not always satisfy these standards and therefore there is a need for biological treatment to serve as a final polishing step (Ozturk *et al.*, 1990). The majority of agro-industries are compatible with biological treatment, with or without a pretreatment step and the activated sludge process is the preferred treatment scheme (Orhon *et al.*, 1999). Treatment of wastes with the activated sludge process represents a component of the largest biotechnology industry in the world. One of the main appeals of the activated sludge systems is it's considerable flexibility, cost effective and ability to acclimatize to industrial effluents and meet increasingly stringent discharge criteria. The various conditions and design configurations of the activated sludge process is discussed in chapter 2.

1.4 MICROBIOLOGY OF ACTIVATED SLUDGE PROCESS

Activated sludge bioreactors contain complex microbial ecosystems which play a crucial role in the chemical transformation of organic material in wastewater. Process stability and final effluent quality largely depends upon the composition of the biomass in an activated sludge plant. Operational problems such as bulking and scum formation usually occur when 'uncharacteristic' microorganisms dominate the sludge population. Microbial community analysis of activated sludge processes is therefore a necessity for process control and stable plant operation (Eikelboom, 2000). The activated sludge treatment process has largely been the domain of engineers who viewed the plants simply as chemical reactors, and every operational problem encountered had an engineering solution. However, this approach is changing with increased efforts by microbial ecologist to understand the microbial processes and contribute to improving the system performance (Seviour, 1999).

1.4.1 Floc structure and composition

In activated sludge processes, much of the cell biomass is not freely suspended in the bulk liquid but organized into flocs. The floc is a complex heterogenous structure made up of aggregates of structures called micro-flocs containing both metabolically active and moribund bacteria and other organisms, and whose composition and activity change with floc age (Seviour, 1999). Flocs are held together by a polymeric matrix referred to as extracellular polymeric substances (EPS) whose chemical composition is probably highly variable. The EPS is also known to contain high protein levels, DNA and humic substance. Filamentous microorganisms are crucial since they contribute to maintaining the floc structure, by providing a skeletal matrix or micro-structure that facilitates adhesion to floc forming bacteria(Jenkins *et al.*, 1993). Floc morphology is critically important since it directly determines the ability of the solids to settle in the clarifiers, thereby impacting on plant performance. Detailed microbial community analysis reveals that bacteria comprise 70 to 90 % of the total cellular material in activated sludge systems. The different bacterial species and metabolic types found in activated sludge processes are diverse and complex in their metabolic capacity (Wagner and Amann, 1997).

Filamentous micro-organisms are bacteria, fungi and algae whose cells do not become detached from one another following cell division. Growth in the form of a filament is characteristic for certain bacteria species which form filaments under practically all conditions(Jenkins *et al.*, 1993)). Protozoa, and frequently also metazoa, are almost always present in activated sludge. Some species are attached to the flocs whereas others are free in the water between the flocs. These organisms mainly feed on bacterial cells which are present free in the liquid or at the edges of the flocs. In this manner they remove many bacterial cells that are not firmly bound to the flocs. These free bacterial cells cannot be separated from the treated water through settling in the final clarifier. Protozoa are also indispensable for COD reduction, viz, a clear effluent. Furthermore, protozoa/metazoa also consume sludge flocs, resulting in reduction of sludge production. The presence of certain species of protozoa is relevant for the process conditions in the treatment plant. Assessing the composition of the population is, therefore, an important aspect of microscopic sludge investigation. Protozoa also play a crucial role as indicator organisms i.e. they are sensitive to toxins in the influent and if they suddenly disappear, it is indicative of the toxicity of the influent into the plant (Eikelboom, 2000).

1.4.2 Factors determining population dynamics in activated sludge

The activated sludge plant acts essentially as a continuous culture system with biomass recycling, so organisms in the reactor are subjected to considerable selective pressures. Those organisms not suited to the prevailing conditions will probably fail to survive, and theoretically be washed out in the liquid effluent (Hamer, 1987). Some of the factors believed to affect an organisms ability to proliferate in a process include the specific growth rate (μ) of an organism which determines its ability to compete with other microbial populations in the aerobic reactors. Equally important is the organisms ability to tolerate the prevailing abiotic conditions in the reactor like temperature, pH and redox potential, all of which will affect its growth rate. The same consideration must be given to toxic chemicals entering the activated sludge process. If any of the latter exceed the organisms tolerance range, then it will fail to survive (Shuttleworth and Unz, 1991). Another important attribute is the ability to contribute to floc formation. Many freely suspended organisms in the bulk liquid, regardless of their growth rates, will leave the system with the final clarified effluent after biomass separation. Those associated with flocs will be re-inoculated into the system via the return activated sludge (RAS) leading to their eventual enrichment (Jenkins *et al.*, 1993).

Another crucial determinant of the composition of the microbial population is the quality/nature of the carbon compounds present in the wastewater. Most of the organisms present in activated sludge systems are chemoheterotrophs, responsible for the degradation of the carbonaceous organic compounds in the plant influent. These organisms obtain their cell carbon and energy from the oxidation of these substrates (Seviour, 1999). The aeration process serves primarily to supply oxygen to the aerobic microbes and keep the activated sludge flocs in constant agitation to provide adequate contact between the flocs and the incoming wastewater. An adequate dissolved oxygen concentration is necessary for the activity of heterotrophic and autotrophic microorganisms , especially nitryfying bacteria (Bitton, 1999). Due to differences in the composition of the wastewater entering domestic and industrial wastewater plants, the bacterial strains present also differ. In addition, individual differences in the bacterial population structure also occur within the different industries such as chemical, dairy, paper etc. The latter illustrates the dynamic nature of the activated sludge population whereby the supply of different nutrients in the wastewater plays a role in selection of bacterial species (Eikelboom, 2000).

1.4.3 Current approach to microbial community analysis

In order to fully understand the contribution of the microbial flora to wastewater treatment in activated sludge processes, there is a need for efficient methods to accurately determine the microbial profiles. These microbial consortia have mostly been analyzed by culture dependent methods such as viable plate count or most probable number techniques. However, these methods have inherent biases and have been shown to be extremely selective as to which organisms are allowed to grow and form visible colonies on media. The two different types of cells which remain viable yet non-culturable in environmental samples can either be : (1) known species for which the cultivation conditions are not suitable for growth or which have entered a non-culturable state, and (2)unknown species that have not yet been cultured for lack of suitable techniques (Amann *et al.*, 1995).

To overcome the biases associated with culture dependent techniques, recent approach to microbial community analysis have focused on using novel molecular methods such as fluorescent *in situ* hybridization (FISH) techniques using ribosomal RNA(rRNA) oligonucleotide probes, polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) and sequencing. Using the FISH approach, the fluorescently monolabelled rRNA targeted probes provided superb spatial resolution with instantaneous detection by epifluorescence microscopy allowing the detection of individual cells. Probes can be applied to parallel sub-samples in a 'top to bottom approach', initially using universal and domain -specific probes followed by probes of narrower specificity. Using this approach, the information gained from the higher-level probes can be used to select probe sets tailored for the next lower taxonomic levels such as genus/species level probes. This made whole-cell hybridization with rRNA- targeted probes a suitable tool for determinative, phylogenetic and environmental studies in microbiology (Amann *et al.*, 1990a). The combined application of FISH and PCR-DGGE has shown remarkable success in enhancing the validity of data obtained when conducting microbial community analysis of bioreactors (Luxmy *et al.*, 2000). PCR permits the in-vitro amplification of defined sequences of DNA. Amplification is accomplished by enzymatic synthesis using two oligonucleotide primers that hybridize to opposite strands. In DGGE, the DNA fragments of the same length but with different sequences can be separated. DNA molecules with different sequences stop migrating at different positions. The bands can then be excised and sequenced to determine their respective phylogenetic affiliations(Yoshie *et al.*, 2002). The above novel molecular techniques are regarded as invaluable to accurately determine microbial community structure of activated sludge processes.

1.5 MOTIVATION AND OBJECTIVES FOR THE STUDY

South African edible oil industries generally use two methods for effluent treatment: physical separation of oil and grease using DAF and pH control. Even after application of these methods, the remaining emulsified grease tends to clog the sewer pipes and pumps and the high COD and phosphate concentrations create shock loading problems for receiving wastewater treatment plants (Mkhize *et al.*, 2000). A preliminary survey in KwaZulu Natal, South Africa revealed that the above mentioned problem was prevalent in the Pietermariztburg region, where most of the edible oil industries were localized. Development of appropriate treatment technology was restricted to selecting a sample edible oil refinery (Company X) as a 'test case' due to the varying composition of the wastewater generated amongst the different oil refineries in the region.

Research had been conducted previously on optimizing activated technology to treat edible oil effluent (Eroglo *et al.*, 1990, Ozturk *et al.*,1990, Mkhize *et al.*, 2000). However, the latter research did not focus defining the microbial populations in the process. The success of engineered processes such as activated sludge for the biological destruction of organic pollutants is largely due to the broad distribution of microbial species capable of biochemical conversion of the components of the waste. The identification and quantification of all contributing populations is necessary to establish the link between microbial structure and function (Amann *et al.*, 1995) which would assist in understanding and further optimizing the process.

Therefore, there was a need to develop and optimize suitable technology using activated sludge process focusing on wastewater treatment in the South African edible oil industry. Optimizing the technology necessitated a clearer understanding of the microbial composition which could be accurately determined using novel molecular techniques. Hence, development and application of the technology was clearly beyond the bounds of a single discipline and required a coordinated focus among research disciplines.

The aim of the project was to design and optimize a laboratory scale activated sludge process to remediate edible oil effluents and conduct a detailed microbial community analysis. However, the pretreatment of wastewater was not within the scope of the proposed research but included in the study in order to satisfy the conditions for activated sludge treatment.

The objectives of the research were :

1. To characterize edible oil effluent with regards to polluting parameters such as fats, oils and grease content, chemical oxygen demand (COD), total nitrogen and phosphates.
2. To conduct a detailed chemical characterization of the wastewater and final effluent using mass spectroscopy (*).
3. To develop and optimize suitable pretreatment technology to prepare edible oil wastewater for biological treatment (*).
4. To design and assess the performance of a laboratory scale activated sludge process to treat

the edible oil effluent.

5. To optimize the operational conditions to facilitate maximum removal of pollutants.
6. To determine the microbial community structure of the process using novel molecular techniques to establish any links between microbial structure and function.
7. To elucidate any population shifts or changes in the dynamics of the microbial communities with progression of the treatment process.
8. To investigate the ultrastructural detail of the floc using electron microscopy and EDAX (*).
9. To investigate the population dynamics of filamentous bacteria and protozoa in the process and correlate to process conditions (*).

(*) objectives that were not in the original research proposal but added value to the investigation.

LITERATURE REVIEW

2.1 WATER - THE LIFELINE OF A NATION

Water is essential to life, to social development and to economic progress. Its value is well appreciated in undeveloped communities, where many individuals experience the tedium of bearing heavy burdens of water, possibly of dubious quality, over long distances to meet their basic needs. Yet in developed societies with a higher quality of life, the convenient and reliable availability of relatively cheap, purified water seems often to be taken for granted. Of all the water on the planet, only 0.6% is available as fresh water for use by municipalities, industry and agriculture. As the world population grows exponentially, preserving water quality will be tantamount to human survival (Falkenmark, 2001). In South Africa, the increasing demand for water arising from the growth of the population and increased industrialization has to be met from limited resources that have to be shared by competing user groups and by other states.

Whilst the water that moves through a landscape mirrors the integrated result of parallel societal activities, it also constitutes the life-sustaining bloodstream of the biosphere. The neglect in past policy making regarding the implications of the water cycle on human activity is difficult to understand. One of the greatest threats to our existence is pollution. Observations worldwide indicate that water quality degradation continues to increase in severity and scale. The major constraints to long term and stable water resource management is not a lack of knowledge but transfer of this information in a clear and understandable form to the policy and decision makers. The new paradigm must distinguish between what man can change - in effect, human behavior and what he cannot change, such as laws of nature. Awareness of such distinctions is necessary in seeking out the new strategies that will carry us over into the future (Falkenmark, 2001).

The average annual rainfall of about 497 mm for S. Africa as a whole is well below the world average of 860 mm . Sixty-five percent of the country receives less than 500 mm of rain annually. Twenty one percent of the country receives less than 200 mm annually (Department of Water Affairs, 1986). Based on the above information, S. Africa could be regarded as a water starved country. Therefore there is an urgency to conserve the limited natural resources and prevent misuse. By practicing multiple internal re-circulation of water and effluents in factory processes, the fresh water intake can be reduced by between 50 and 90%. Between 60 and 100 % of the fresh water intake to factories and residential areas reaches the sewage treatment works, and in a rather limited number of cases purified effluents are reused (Department of Water Affairs, 1986).

Water of potable quality can be used only for processes where it is essential and high quality effluents can be used for processes where water of a lesser quality is suitable e.g. factory floor washing. Valuable by-products can be recovered from concentrating effluent streams.

2.2. THE GLOBAL WATER SITUATION

2.2.1 Current trends

Fresh water resources are becoming scarce in many countries, as a result of population growth, increasing pollution, poor water management practices and climatic variations. Despite increasingly efficient water use in many developed countries, the demand for fresh water has continued to climb as the world's population and economic activity have expanded. According to some recent projections, in 2025 two thirds of the world's population will be suffering moderate to high water stress and about half of the population will face real constraints in their water supply. The water crisis has also affected some temperate regions with normally plentiful resources, such as Europe and North America, where periods of drought are becoming more frequent and are lasting longer. One approach used to evaluate water scarcity is the exploitation rate of water resources (the ratio between the volume of available renewable water resources and annual withdrawals). When the exploitation rate exceeds 20 % of existing reserves, water management becomes a vital element in a countries economy.

It is evident from FIG 2.1 that numerous countries such as Jordan, Hungary, Israel and Belgium and already reached very high exploitation rates which surpass their local water resources. The data suggests that to meet future water needs, many countries will have to manage water resources far more efficiently than they do presently (Lazarova *et al*, 2001). When comparing exploitation rates with other countries, the crisis in South Africa is not as serious but efforts will have to be targeted to reducing to below 20 %.

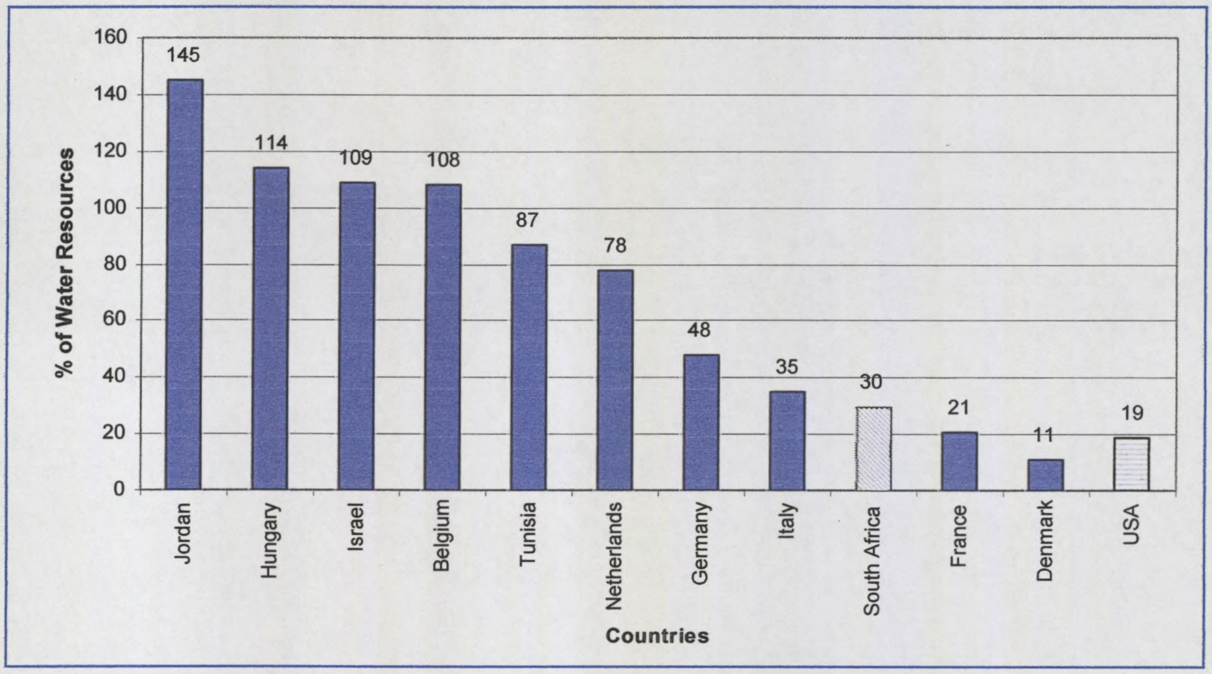


FIGURE 2.1 Renewable water resource exploitation in some European and Mediterranean countries, in comparison with the USA and South Africa (Lazarova *et al.*, 2001)

2.2.2 Water quality and waste management

Water quality management should include the use of economic incentives and penalties to reduce pollution; and the possibility of irretrievable environmental degradation as a result of pollution should be prevented. Water resource development and supply activities should be managed in a manner which is consistent with broader environmental management approaches (Asmal, 1996). The following prerequisites form the basis of water quality management policies and practices in S. Africa (Department of Water Affairs and Forestry,1995) :

- Because water is such a scarce resource, effluent has to be returned and reused in order to help balance water supply with the demand for water. Water quality at pristine , unaffected levels therefore cannot be maintained and some changes in water quality is accepted as being inevitable.
- Waste disposal to air or soil (for example, effluent irrigation, dumping mining wastes or gaseous emissions) impacts on the quality of surface and ground water. Therefore, waste disposal has to be evaluated holistically and the best practical environmental option has to be selected.
- Sustainable economic development which is in balance with environmental protection and sustainable resource use is encouraged. The weight being given to the economic and social benefits associated with an operation discharging an effluent will, however, depend on other factors such as the available assimilative capacity of the receiving water body and the hazardous nature of the effluent.

The above guidelines emphasizes the role of the authorities in attempting to minimize pollution of the scarce water resources in S. Africa. In the last decade, the sanitary engineering of the seventies has matured to environmental engineering. A particular type of waste can no longer be treated without considering all the consequences for the environment. For instance, activated sludge treatment now not only refers to the water component, but also to the bio-solids produced and the volatile organic carbons (VOC's) and odour generated. Recently environmental engineering has become integrated in a broader scheme of environmental management. Indeed the concepts of environmental management systems, environmental management and auditing systems, responsible care and the ISO 14000 standards are very well known all over the world (Verstraete, 2000).

2.2.3 The water act of South Africa

The use of water for industrial purposes together with the control of effluent production and water pollution is governed in S. Africa by the National Water Act of 1998. The Water Act is administered by the Department of Water Affairs and Forestry (DWAF). The General Authorization (GA 3.7) in terms of Section 39 of the National Water Act pertaining to :

1) Discharge of waste or water containing waste into a water resource through a pipe, canal, sewer or heated in , any industrial or power generation process, or

2) Discharging of domestic and industrial wastewater into water resources requires :

a) discharge up to 2000 cubic metres of wastewater on any given day into a water resource provided

- the discharge complies with the General Limit Values set out in Table 2.1
- the discharge does not alter the natural ambient water temperature of the receiving water resource by more than 3 degrees Celsius; and
- the discharge is not a Complex Industrial Wastewater i.e. wastewater arising from industrial activities that contains a complex mixture of substances that are difficult to chemically characterise and quantify, or one or more substances, for which a wastewater limit value has not been specified and which may be harmful or potentially harmful to human health, or to the water resource.

b) a person may discharge storm water runoff from any premises, not containing waste or wastewater emanating from industrial activities and premises, into a water resource (The National Water Act, 1998)

The industries have to formally apply to the Department of Water Affairs and Forestry for a effluent discharge permit. The objective of applying for effluent discharge is for the discharger to obtain the privilege of using some of the assimilative capacity in the receiving water for the disposal of waste. It allows the DWAF, as well as other interested and affected parties, to obtain information they require to decide whether or not the application can be approved or whether there is a need for further investigation. In the application, justification must be provided for :

- The need for the discharge in terms of the development's significance for sustainable development
- The necessity of producing an effluent containing waste.

During the screening of the application, the DWAF ensures that the applicant has taken all feasible action to firstly prevent and secondly minimise waste. In addition the applicant the following information for the preliminary investigation :

- Location of the activity
- Type and scale of the activity
- Nature of the effluent
- Water users in the catchment
- Sensitivity of the catchment
- Water quality status of the catchment
- Who the interested and affected parties are and their concerns

Table 2.1 Wastewater limit values applicable to discharge of wastewater into a water resource in terms of the South African Water Act, 1998 (section 39)

SUBSTANCE/PARAMETER	GENERAL LIMIT	SPECIAL LIMIT
Faecal Coliforms (per 100ml)	1 000	0
Chemical Oxygen Demand (mg/l)	75*	30*
pH	5,5-9,5	5,5-7,5
Ammonia (ionised and un-ionised)as Nitrogen (mg/l)	3	2
Nitrate/Nitrite as Nitrogen (mg/l)	15	1,5
Chlorine as Free Chlorine (mg/l)	0,25	0
Suspended Solids (mg/l)	25	10
Electrical Conductivity (mS/m)	70mS/mabove intake to a maximum of 150 mS/m	50 mS/m above background receiving water, to a maximum of 100 mS/m
Ortho-Phosphate as phosphorous (mg/l)	10	1 (median) and 2,5 (maximum)
Flouride (mg/l)	1	1
Soap, oil or grease (mg/l)	2,5	0
Dissolved Arsenic (mg/l)	0,02	0,01
Dissolved Chromium (Vi) (mg/l)	0,05	0,002
Dissolved Copper (mg /l)	0,01	0,002
Dissolved Cyanide (mg/ l)	0,02	0,01
Dissolved Iron (mg/l)	0,3	0,3
Dissolved Lead (mg/l)	0,01	0,006
Dissolved Manganese (mg/l)	0,1	0,1
Mercury and its compounds (mg/l)	0,005	0,001
Dissolved Selenium (mg/l)	0,02	0,02
Dissolved Zinc (mg/l)	0,1	0,04
Boron (mg/l)	1	0,5

2.2.4 Waste minimization programs

2.2.4.1 Industry based recycling and reclamation

The Department of Water Affairs relies on the co-operation of both research and industrial organizations in the development of alternative approaches which will ensure the industrial and economic future of S. Africa. It is the Department's policy to encourage industrialists to develop improved on-site wastewater treatment processes and subscribe to effluent discharge standards.

Industry- based recycling and reclamation is desirable in that pollutants may be removed at source from segregated, purer streams. In addition, heat energy, water and chemical savings may be achieved. Recycling will include the reuse of minimally contaminated process water from one process, in another subsequent process. Reclamation measures include primary treatment to enable reuse of the stream or advanced treatment to allow for the separation, recovery and direct reuse of effluent constituents (Groves and Buckley, 1990, Fujie and Hu, 1997).

2.2.4.2 Significance of environmental biotechnology

Environmental biotechnology for hazardous waste can be defined as the use of microorganisms and their processes for socio-economic benefits in environmental protection and restoration. The application of biological processes for disposal and control of waste from human activities is established technology dating back at least 4000 years (Schonborn, 1986). The success of these engineered processes for the biological destruction of organic pollutants is largely due to the nature of the pollutants and the relatively broad distribution of microbial species capable of biochemical conversion (biodegradation) of the components of the waste. Environmental biotechnology encompasses methods that use naturally occurring microorganisms to remove contaminants from the environment. Initially it was regarded as a relatively straightforward extension of the time- honored biological processes for treatment of wastewater. Degradation capacities were measured by nonspecific parameters such as Biological Oxygen Demand (BOD)or Chemical Oxygen Demand (COD) and by certain compound-specific analyses. In making this extension of classical biological treatment to new application and new matrices, and in tracking the fate of specific priority pollutants, the initial efforts began to delineate the complex mechanisms involved. Engineers and scientist began to realize that systems for dealing with environmental contamination were much more complex, and that moving from the flask to the field was fraught with potential unknowns and problems (Sayler and Fox, 1991).

The activated sludge process was recognized as a treatment system with three major removal pathways for chemicals: stripping, adsorption on biomass and bio-transformation. The removal of organics by biological processes requires an understanding of the microbial community and the changes induced by the microbiological activity. Examination of the microbial community showed that the actual number of degraders of a target contaminant in a mixed culture might only represent 5-10% of the total microbial community (Sayler *et al.,* 1984). This observation has created the need for the development of methods to measure specific microorganisms in mixed cultures. With the recent advances in molecular biology, various such techniques exist e.g. Fluorescent *in situ* hybridization (FISH) techniques, Denaturing gradient gel electrophoresis (DGGE), Quinone profiling etc. Therefore among the important parts of the science of microbiology are the conventional and new emerging techniques for identifying microorganisms and understanding the physiology associated with degradation , e.g. nutrient types and utilization. Biochemistry identifies biochemical pathways, the extent of degradation, and the enzymology involved. Molecular ecology provides insight into the interrelationship among organisms , specific genes and their environment (Sayler and Fox, 1991). Researchers globally have identified the need for integrated interdisciplinary research in order to achieve the desired objective of providing a cleaner environment. There was a clear consensus that environmental biotechnology as a research field was evolving at the interface of molecular life sciences, environmental and ecological sciences and engineering (FIG. 2.2) (Sayler and Fox, 1991). This consensus was an affirmation that the problems affecting the development and application of the technology were clearly beyond the bounds of a single discipline and required a coordinated focus among research disciplines.

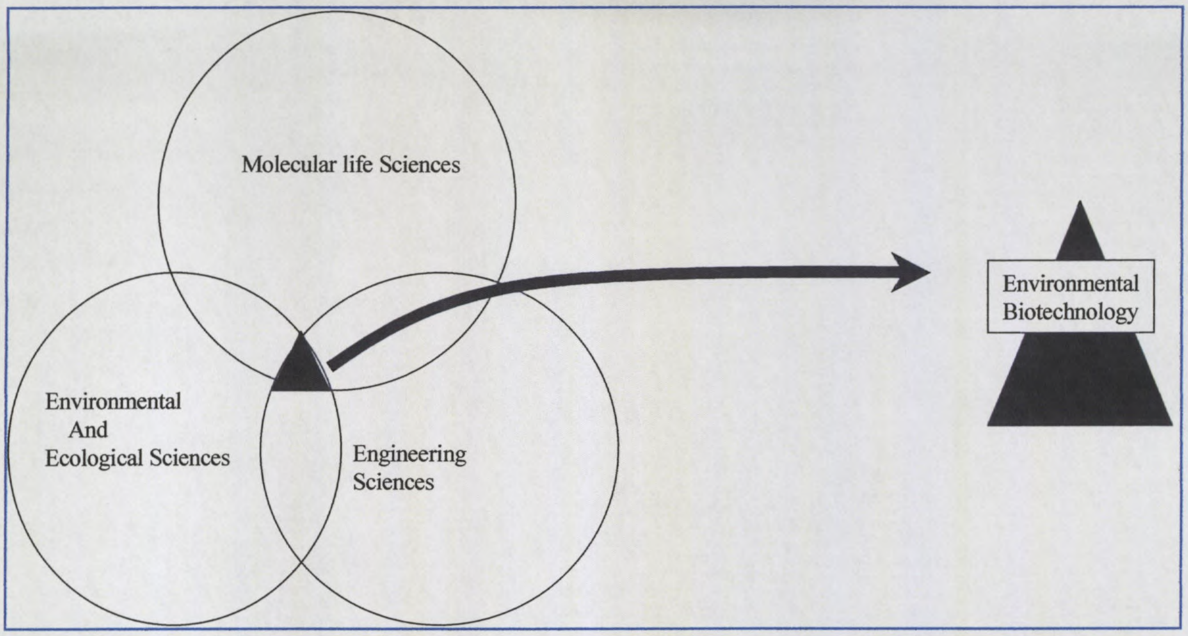


FIGURE 2.2. Integration of science and engineering discipline required to achieve practical environmental biotechnology (Sayler and Fox, 1991).

At present, the world is facing a number of formidable environmental problems such as acid rain, depletion of the ozone layer, industrial pollution of limited water resources, etc. Solving these problems not only require an integrated approach based on technology, but also a detailed knowledge of environmental biology and ecology.

2.2.4.3 Integration of biotechnology to waste minimization programs

To obtain a good perspective on waste minimization, industry needs to answer the following two questions :

- How has industry responded to the increased environmental regulations and social consciousness on waste generation ?
- What waste minimization programs are implemented and what are planned for the future?

In the USA, the chemical process industries firstly identify and quantify the sources of waste being generated by conducting extensive "audits" in each of the chemical processing plant facility. The audits pinpoint the sources of waste discharged, enable characterization of the nature of the waste and ultimately assist in quantification of these wastes. The results of these quantitative audits enable individual companies to define the waste minimization targets and the resources to meet these targets (Tong, 1991). The waste minimization programs that were implemented after the completion of these audits were classified under four categories :

a) *Source Reduction Programs*

   Aggressive preventative maintenance projects to stop leaks in pumps, flanges, valves etc., implementation of back-up systems to prevent accidental spills. Such measures not only reduce waste generated but also reduce manufacturing cost.

b) *By-Product Recovery and Utilization Programs*

   Unlike point-source reduction were the immediate action taken by processing plants, by-product recovery requires greater efforts to develop the technologies to upgrade by-products and to develop markets for these recovered chemicals (Osborne *et al.,* 1990).

c) *"End of Pipe" Treatment of Wastes*

   These "end of pipe" treatment involves either physical, thermal, chemical and/or biological treatment technologies for waste. These treatment technologies are designed to destroy waste converting it to carbon dioxide, water and/or minerals. Compare to the former two programs, end of pipe treatment is less economically attractive (Tong, 1991).

d) *"Structural" Changes in Environmentally Unsound Processes or Products*

   Excessive cost associated with treating waste from unsound processes forced industries to develop cleaner processes and get as close to "zero discharge".

The role of biotechnology in waste minimization can be examined under two categories :

- Location within the processing plant where biotechnology is applicable.
- Type of biocatalysts that can be used in these waste minimization applications

Biotechnology applications to waste minimization programs can be seen at three locations within a typical process plant (FIG 2.3) :

- In the process plant and off-site utilities,
- in by-product utilities and
- in “end of pipe” treatment

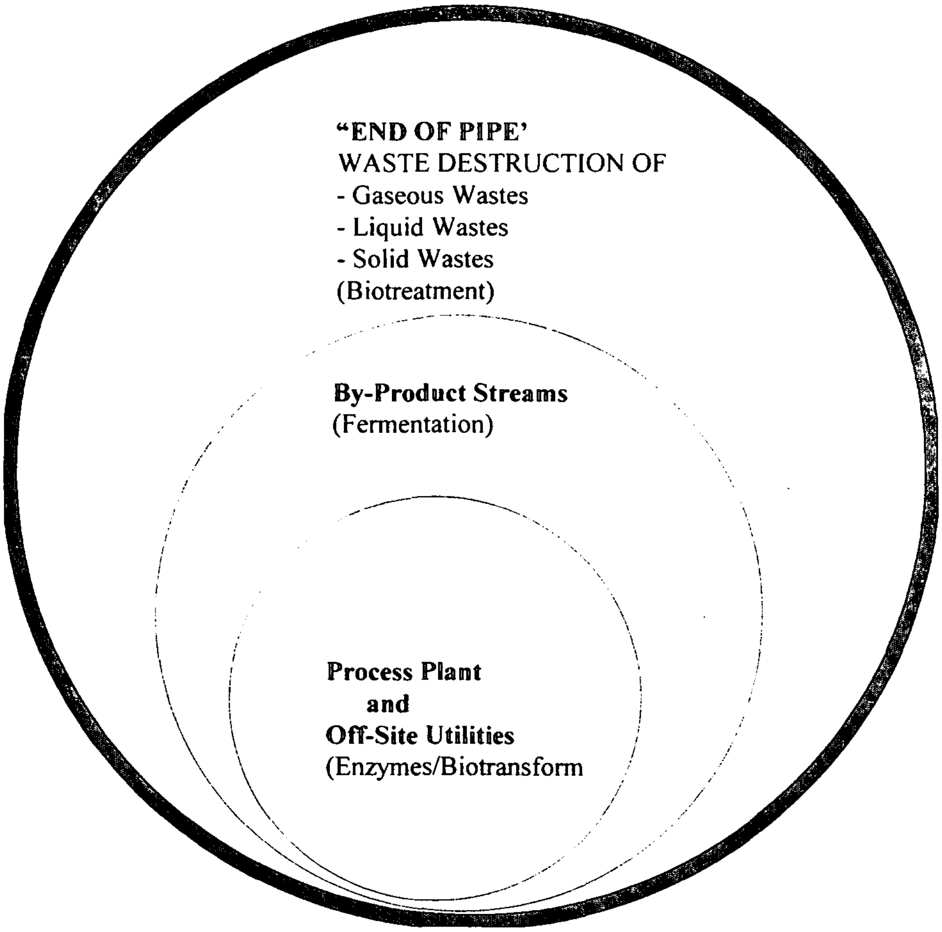


FIGURE 2.3. Biotechnology roles in waste minimization (Tong, 1991)

Some of the biotechnological applications in waste minimization include :

a) *Radical generating enzymes*

A promising new area in environmental biotechnology is the use of radical generating enzymes systems to degrade pollutants. Aitken *et al.*, (1989) have shown that, by applying ligninase together with hydrogen peroxide and manganese ions to a wastewater containing *o*- cresol, some 99% of the latter could be removed due to the produced radicals.

b) *Microbial community structure function relationship*

A battery of molecular tools exist presently that allow a census of the microbes present in activated sludge wastewater treatment processes. This creates a better understanding of the microbial- biochemical interactions thereby further optimizing these processes. In addition, molecular methods have been used to characterize the active microbial population in a range of systems, including a biofilter to purify air in a manned spacecraft.

c) *Application of photosynthetic sludges*

A biological wastewater treatment process using anoxygenic photosynthetic bacteria, termed photosynthetic sludge process has been successfully used for purifying various organic wastewater. In addition, the sludge can subsequently be used as a fertilizer(Kyoko *et al.,* 2000)

d) *Energy from waste*

Energy from waste became mainstream approach in the '90's due to the development of integrated sustainable waste management tools. Anaerobic digestion of organic municipal solid waste generates biogas that can be used as an energy source. Thermal efficiency ranges from 30 to 85%.

Also the nutrient rich residue may be used as fertilizer if it meets quality and regulatory standards (Ioannis and Osada, 2000).

e) *Treatment of palm oil mill effluent with membrane anaerobic system*

Membrane filtration has been gaining popularity in wastewater treatment processes. One of the major difficulties in anaerobic wastewater treatment processes is the need to retain a sufficient quantity of active biomass in the anaerobic reactor. The use of membranes to achieve external solid/liquid phase separation resulted in the retention of biomass at the surface of the membranes. At a influent COD concentration of 39 910 mg/L, there was a removal efficiency of between 91and 94% at a average hydraulic retention time (HRT) of 3.03 days (Fakhru'l-Razi and Noor, 1999).

Therefore the application of biotechnology in waste minimization programs has phenomenal potential, both economically and to provide a pollution free environment. Environmental biotechnology will develop provided capable people are encouraged to deal with the problems and to design concepts, systems, technologies which solve existing problems. However, even if low cost integrated solutions are developed, the people required to conceive, structure, operate and control these approaches will have to be motivated to take on the job and to build up experience with it. It therefore is essential that environmental technology becomes an established business opportunity. The prerequisite for this is that society no longer dumps its wastes and exploits the ecosystem, but handles the waste correctly at the rate that it's produced and manages the environment in a holistic approach. Only when the latter is achieved will it be possible for future generations to live properly on our planet (Verstraete and Top, 1992)

2.3 EDIBLE OIL PRODUCTION

The edible industry consumes approximately 1.75 million m^3 of water each year. The total quantity of oil refined in S. Africa has remained relatively constant, at approximately 250 000tons/annum. The production of seed is dependent on the weather. Drought conditions result in poor harvest and and to satisfy local demand, crude oil is imported and refined in S. Africa (Steffen *et al*, 1989).
Vegetable oil may be obtained from a wide variety of seeds, including cotton seed, soya bean, peanuts, sunflower seed, maize, linseed, olives and coconut. The varying climatic conditions found around the world favour the cultivation of different oil bearing seeds. North America's primary oil seed crops are cotton seed and soya bean, whilst the Far East's oil is extracted from palm and coconut sources. In S. Africa the most commonly grown oil bearing crops are sunflower, groundnut and maize.
There are two stages involved in the production of edible oil i.e. separation of the oil from the oil bearing seed and subsequent refining.

2.3.1 Crude oil production

Crude oil production involves receiving the seed at the mill and storage, seed preparation and solvent extraction.

2.3.1.1 Seed preparation

Seeds received by the mill are transferred to a screening unit to remove all foreign matter and impurities. This is usually achieved by the use of magnets, bar screens, sieves and air separators. These seeds are subsequently decorticated after which the meats are separated from the hulls using vibratory screens.

2.3.1.2 Oil extraction

The separated meats which contain the oil is rolled into flakes to make them more permeable to steam in the ensuing cooking step. The flakes are subjected to live steam and brought up to a predetermined optimum temperature and moisture content. Conditioned seed from the cookers is passed through expellers where approximately 75 % of the oil is extracted from the flakes. The remaining seed cake which consist of approximately 25 % residue oil is sent to the solvent extraction unit to remove the oil.. The cake is washed with hexane leaving about 1% oil in the final meal. The process operates using a counter current system in which the oil rich miscella is contacted with fresh oil cake, whilst fresh hexane contacts with outgoing cake. The discharged cake loses about 95 % of it's entrained hexane in a vapour desolventiser. Hexane is reused and the solvent free meal is removed and cooled. Crude oil is extracted from the miscella by a 3 stage evaporation system. The recovered oil is cooled and pumped to storage (Steffen *et al,* 1989; Abou-Elela and Zaher, 1998). The crude oil is composed largely of glycerides of various saturated and unsaturated fatty acids, notably oleic and linoleic acids. There are a number of impurities which must be removed from the oil before it can be suitable for human consumption. These impurities can be classified into four broad categories :

a) <u>Gums</u>

A collective term referring to phosphatides , sugars, resins and other proteinaceous material. The gum content of sunflower, maize and soya is approximately 2 %.

b) <u>Free fatty acids</u>

These can occur in extremely high quantities (as high as 40%) but usually range between 0,5% and 8% depending on oil type (Forster and Harper, 1983).

c) Pigment

Usually of chlorophyllic or carotenic base. Cotton oil in particular has a deep red-black colouration associated with gossypol.

d) Taste and odour

Chiefly as a result of volatile aldehydes and ketones (Forster and Harper, 1983)

The crude oil is refined to remove this objectionable content.

2.3.2. Oil refining

There are two approaches to refining and both are in wide commercial use. The classic method of refining a crude oil is chemical refining. This involves neutralization of the oil with caustic soda followed by bleaching and deodorising of the neutralised oil. Adaptation of the chemical method led to the introduction of physical refining (FIG 2.4). The constituents and properties of oil depend upon their source and therefore impact on slightly different refining techniques. The chemical refining of crude oil involves the following :

a) *Crude oil storage*

Refining is initiated in the crude oil storage tank since the oil- insoluble matter separates by gravity. Semi-solid oils are maintained at slightly elevated temperatures.

b) *Degumming*

Gums are separated into hydratable and non hydratable types. Their removal is important because :

- their emulsifying properties increase losses during chemical refining ;
- on heating to deodorising temperatures, they tend to impart a deep brown colour to finished oil; and
- the tendency to form complex compounds with certain trace metals which adversely affect product stability.

Hydratable gums (usually amounting to between 60 and 75% of total gum content) are removed by the addition of hot water and subsequent separation of the swollen, insoluble gum using a centrifuge (Forster and Harper, 1983). Removal of non-hydratable gums requires prior treatment with phosphoric or citric acid to render them hydratable, followed by centrifugation or use of an activated absorbent coupled with a filter (Editorial comment, 1986).

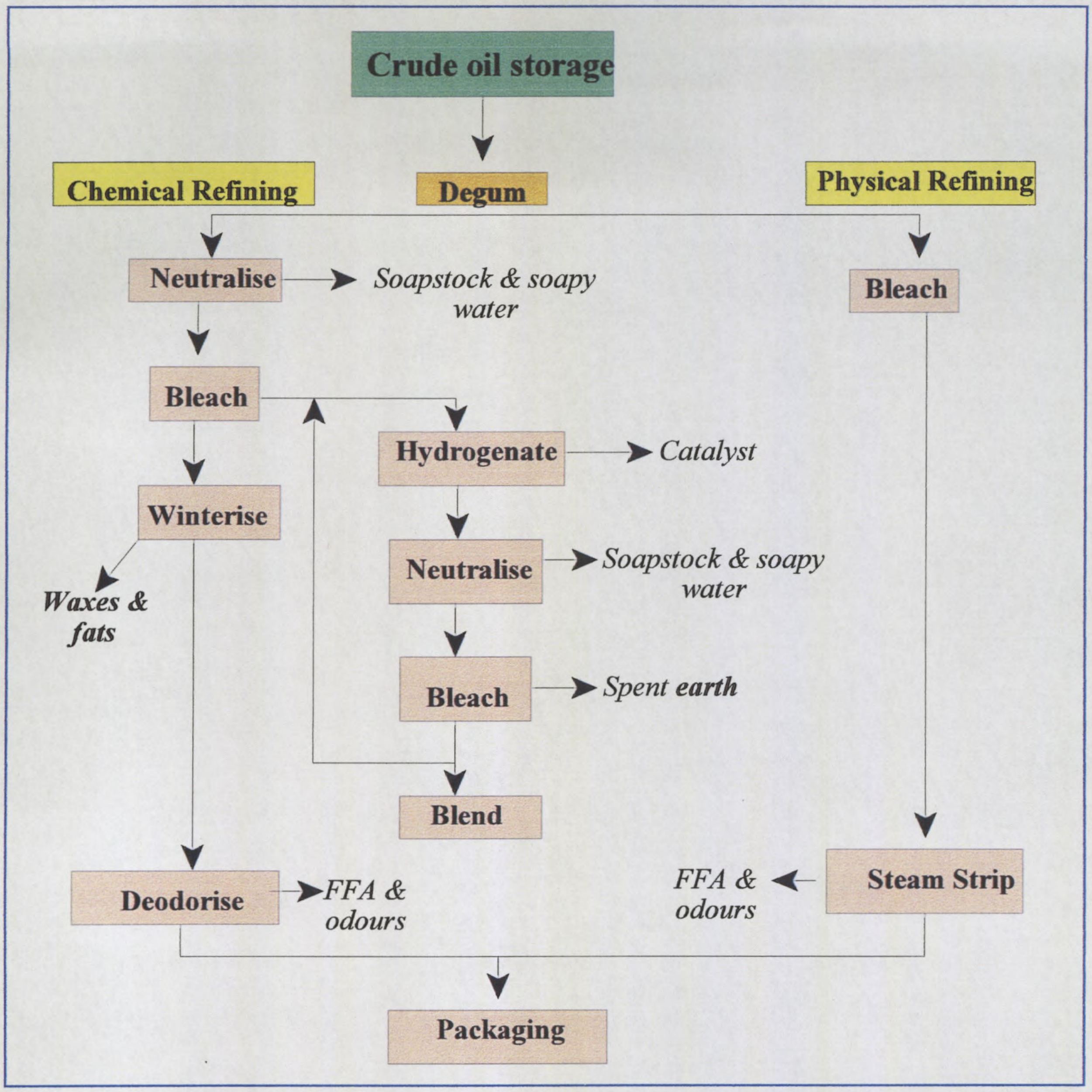


FIGURE 2.4. Edible oil refinery process and wastewater production (Steffen *et al.*, 1989)

2.3.2.1 Chemical refining

a) *Neutralisation*

In the chemical refining process the free fatty acids (FFA)- carboxylic acids are neutralised by the addition of caustic soda which is mixed with the oil at a predetermined optimum temperature in either batch, semi-continuous or continuous process. A strong caustic solution will tend to saponify the neutral triglycerides with consequent loss of neutral oil. Therefore careful control of the operating conditions is required. In continuous processes the immiscible soap termed soapstock which is produced upon neutralisation is separated from the neutralised oil by centrifugation. The soapstock is usually treated further on site to produce acid oil (Rohbrechtbuck and Sekoulov, 1990)

b) *Bleaching*

It serves primarily in the removal of color and as a further purification step. The process is conducted under vacuum at elevated temperatures using clay of up to 2% by weight. The clay -oil mix is agitated and filtered. The clay absorbs soaps, trace metals, pigments and various oxidation products which would otherwise cause severely reduced product life (Haroldsson, 1983).

c) *Hydrogenation*

The process is employed to convert liquid vegetable oil into semi-solid plastic fats, which are used in a variety of fatty products such as shortenings, margarine, frying fats and stabilizers. Hydrogenation is the process by which hydrogen is added directly to points of unsaturation (the double bonds in fatty acid chains) in the fatty acids in the presence of a catalyst. The catalyst most widely used is nickel supported on a diatomaceous earth. Naturally occurring oils contain fatty acids having up to six double bonds in the chain. Hydrogenation attempts to saturate these selectively , reducing the six double bonds to five, the five to four and so on. There are two basic hydrogenation processes : batch and continuous. Most commercial operations use the batch process where the oil is heated to the required temperature in the presence of the catalyst. The control of the temperature is crucial. When hydrogenation has proceeded to the desired end point, the oil is cooled from the reaction temperature of approximately 185^{0}C and is filtered to separate the catalyst from the product. The hardened oil is further subjected to a post neutralisation and bleach step to remove impurities and deodorised as other oils (Steffen *et al.*, 1989 ; Herbert *et al.,* 1996).

d) *Dewaxing (Winterising)*

The dewaxing process removes the waxy type of nonoily constituents that are not removed either by alkaline refining or the degumming process. These impurities causes an undesirable cloudy appearance at lower temperatures, when they crystallize. The quantity of wax in the crude oil varies from a few hundred ppm to over 2 000 ppm. To get an oil with sufficient cold stability, the wax content has to be reduced to about 10 ppm. The classical dewaxing process which is normally conducted after bleaching involves careful cooling of the oil to 5^0 C, followed by crystallization of the wax, which then is removed by filtration (Haroldsson, 1983).

e) *Deodorization*

Deodorization is the last in the processing steps used to produce edible oil. It involves steam distillation under high temperature and vacuum. The process reduces free fatty acid content, removes undesirable volatile flavor and odor compounds, removes some sterols and pigments and destroys peroxide. The flavor and odor compounds are aldehydes, ketones, alcohols and hydrocarbons, which even at low concentrations are detectable by taste and smell (Gunstone, 1996). The process can be conducted either as batch, semi-continuous or continuous, depending on operational circumstances. In a semi-continuous deodorizing unit, partially heated oil to the top tray or tank where air and other volatile materials are released from the oil under full vacuum. In a second tank an indirect heat exchange medium, such as Dowtherm, is used to heat the oil to the operating temperature of between 180^0C and 250^0C before the bulk of the total of 4% open steam is injected for deodorization. The deodorized oil is now cooled in lower tanks before being to storage (FIG 2.4). Minor variations of this process do occur (Steffen *et al.*, 1989 ; Herbert *et al.*, 1996).

2.3.2.2 Physical refining

This method of refining is used primarily on lower molecular weight oils such as palm, coconut and to some extent soybean oil. The objectives in physical or steam refining are to reduce the free fatty acid content, fully deodorize the oil, operate without substantially greater utilities consumption than standard or regular deodorization and recover the free fatty acid from the distillate. The physical refining technique has two primary advantages over conventional caustic refining : reduction in oil loss and the elimination of soapstock and it's asociated effluent treatment problems. The disadvantages of steam refining are that the oils must be thoroughly degummed and bleached prior to physical refining. In addition, the percent of bleaching clay required for decolorizing is greater than when the oil is caustic or chemically refined (Herbert *et al.*, 1996). Pretreatment of high FFA oils such as sunflower prior to physical refining comprises the addition of phosphoric or citric acids at a temperature of approximately 95^{0}C followed by high speed centrifugation to remove the hydrated gums. The centrifuged oil is dried, bleached and winterized before being sent to physical refining. Here the oil is pumped into a vacuum deaerator before entering the top heating section of the stripper. The oil is subsequently heated to a temperature of up to 270^{0}C using a thermal fluid from a vapouriser. In a continuous unit the oil is then refined by flowing over the top series of trays counter- current to the flow of stripping steam which is injected below the bottom tray. The refined oil now flows down to a holding section which provides the retention necessary for heat bleaching before a final series of trays enables steam stripping of all remaining odoriferous material. Exiting oil transfers some of it's heat to the incoming feed before being pumped to polishing filters and on to storage (Steffen *et al.*, 1989).

2.4 WASTEWATER PRODUCTION

Oilseeds processing, refining and processing of vegetable oils produces a variety of waste products. In no other industry is the proper handling of these wastes as dependent on the understanding and control of the processes themselves. The processes that contribute to effluent production are reflected in Table 2.2. The information presented is indicative of the average effluent production of the industry globally. Wastewater generation in an edible oil industry can be divided into two categories :

- Wastewater generated directly from the processes- mainly from the neutralization of oil, soap section, acid oil production and washing (FIG 2.4)
- Wastewater generated from auxiliary systems- includes cooling water, vacuum water, water from the boiler and softening plant, and sanitary water.

TABLE 2.2 Oilseeds processes and wastewater loads (Boyer, 1996).

Process	**Waste Load**		
	Flow (m^3/day)	BOD (kg/day)	FOG(kg/day)
	Average	Average	Average
Milling & extraction	95	168	11
Caustic refining	42	100	52
Further processing	19	68	34
Deodorization	19	18	9
Acidulation	72	1451	11
Tank car washing	19	113	57
Packaging	38	113	57
Sub total	**304**	**2031**	**231**
Margarine production	265	272	136
Salad dressing/mayonnaise	189	907	454
Total	**758**	**3210**	**821**

2.4.1 Source of wastewater production

The major processes that contribute to the wastewater stream include (Table 2.3) :

a) *Neutralisation*

Chemical refining with caustic soda produces a potent effluent. During the process, free fatty acids present in the crude oil are removed as highly alkaline soapstock. A quantity of neutral crude oil is also saponified in this process. This soapstock is usually converted back into fatty acids using a strong acid, either as a batch or continuous process. The product is called acid oil which is used in the manufacture of paints, varnishes etc and the process is called acidulation which consist of three basic steps :

- acidification of the highly basic soapstock, usually with sulphuric acid
- breaking the emulsion of fatty acid and foreign material in water by means of heat (steam

injection), pressure and agitation; and

- separation of the phases.

The aqueous phase from the splitting operation is approximately pH 2, and after some has been recycled to dilute incoming soapstock, the remainder must be neutralised by the addition of caustic soda. The resultant neutralised liquid, predominantly a sodium sulphate solution, is discharged to drain. After the separation of soapstock from the neutralised oil, the oil is washed to remove all traces of caustic. The resultant soapy water is discharged to drain. However, this results in the loss of valuable oil and contributes a large percentage of the pollution load in the final effluent. To minimize the latter, soapy water is mixed with soapstock already removed from the oil, forming an homogenous mixture from which acid oil is more easily recovered in the soap splitting operation (Steffen. *et al.*, 1989; Boyer, 1996).

b) *Deodorization*

This process is generally a minor contributor; however, it can produce significant waste loads if the deodorizer does not have a well operated scrub cooler. The distillate from the oil contains the volatile compounds responsible for the oils characteristic odour as well as any remaining FFA. Waste loads should be no greater than the deodorizer steam load; with about 500 mg/L FOG, the soluble fraction at steady state recycle. The deodorizer units are boiled-out annually with sodium hydroxide and citric acid during maintenance. This results in a extremely high waste load and must be stored for regulated release into the sewer system (Boyer, 1996).

c) *Cleanup and Handling Losses*

Most of the plant used in an edible oil refinery must undergo regular cleaning, which generally occurs on a weekly basis between changes in feedstock. Cleaning of vessels is usually effected using live steam and hot water. The optimum use of steam reduces effluent volume. Other cleaning, such as floor is conducted using hot water. This fat and oil bearing effluent is discharged to the sewer via fat traps, where the oil rich scum is recovered and sent for reworking (Steffen *et al*, 1989).

The bulk of the remaining effluent, in terms of volume, emanates from the boiler house. The high steam demand of the industry requires large volumes of softened water, and this produces large volumes of regeneration effluent flowing to the drain from ion exchange water softening units. Return of condensate from the various vessels in the oil processing plant is no more than 20%, since the risk of oil contamination is considered to outweigh any possible water savings. These streams combine with cooling water blow-down to form a significant volume of comparatively saline effluent (Haroldsson, 1983; Boyer,1996).

TABLE 2.3 Breakdown of water usage at an edible oil processing plant (Steffen, 1989)

	Mill %	**Refinery %**	**Total**
Process	2	13	15
Boilers	10	30	40
Cooling	25	10	35
Washdown	1	7	8
Domestic	1	1	2
	39	**61**	**100**

2.5 WASTEWATER TREATMENT PROCESSES AND TECHNOLOGIES

Wastewater generated from the edible oil industries globally vary in pollution load and concentration depending on the seed, process employed and size of the industry. When considering effluent from various oil mills, there are several points which should be considered :

- Whether finished oil is produced from seed or the factory only produces crude oil or only refines purchased oil.
- Whether the predominant refining method is chemical or physical. This will be governed both by type of feedstock and by installed plant.
- Whether other products such as margarine, peanut butter and mayonnaise are manufactured on the site.
- Whether the existing effluent drainage system allows representative sampling of individual process effluents. The tendency of free fats to float on the surface of aqueous effluents increases the difficulty of obtaining representative samples.
- Whether any form of effluent treatment is practiced prior to final discharge.

In terms of volume and load the largest proportion of effluent discharged by an edible oil plant originates from refining operations. Typically about 80% of the effluent volume is attributable to the refinery. The main types of pollutant in edible oil plant effluents are fats and oils. Following neutralisation of the crude oil and subsequent soap splitting , water is used for washing giving rise to effluents known as soapy water and acid water. Detergents used in washdown operations often emulsify oils leading to high levels of oil in the plant effluent which are difficult to remove (Steffen *et al.*, 1989).

2.5.1 Conventional treatment technology

Effective treatment of refinery effluent may be achieved by various processes such as :

2.5.1.1 Screening and gravity oil separation

Solids such as de-fatted meal and husks, which may have entered the effluent stream may be removed using a rotary, vibrating or static cleaning screen. Since fats and oils are less dense than water, the simplest way of separating these pollutants from oily effluent is flotation. All waste streams must receive oil separation prior to any treatment in order to capture floatable fats and oils resulting from both day to day losses and larger spill situations (Boyer, 1996). A flotation system consist of a tank containing a series of baffles beneath which the aqueous phase can flow freely. A manometric arm maintains the liquid level at baffle height so that fatty material is trapped. Periodically fat has to be removed manually from the surface. The limitations of this process includes :

- It can only remove gravity-separable oils and will not separate stable emulsions.
- Any emulsifier, spent caustic or detergent present will tend to reduce the efficiency of removal.
- Suspended solids attached to oils may not settle, and
- Hydraulic overloading reduces efficiency (Steffen *et al.*, 1989)

2.5.1.2 Dissolved air flotation (DAF)

This process can be used as an alternative or subsequent treatment stage to gravity separation.

Dissolved air flotation is a technique that utilizes minute air bubbles to enhance the flotation of oils and suspended materials not removable by gravity separation. The bubbles are formed by pressurizing all or part of the waste flow and introducing air to a pressurization cell. Subsequent depressurization allows the bubbles to form which then adheres to any oil, fat or other suspended solids in the effluent bringing it to the surface (Ozturk *et al.*, 1990 ; Boyer, 1996). This process is enhanced by the addition of polymers such as ferric chloride, alum, lime and other flocculents. This chemical addition provides for coagulation and flocculation of colloidal oils and solids into larger particles that can be easily removed. In fats and oils applications, this technique has been shown to be very effective FOG and associated insoluble BOD; however, significant quantities of waste sludges are generated. The selection of chemical flocculent must consider pH conditions as well as removals desired. The layer of frothy solids , fats and oils which form on the surface is swept into an inclined exit chute by a rotating arm or other skimming device (Steffen *et al.,* 1989; Boyer, 1996).

2.5.1.3 Acid hydrolysis

An alternative approach used successfully at several fats and oils handling facilities employs a low pH, high temperature hydrolysis effect to recover oils and grease.

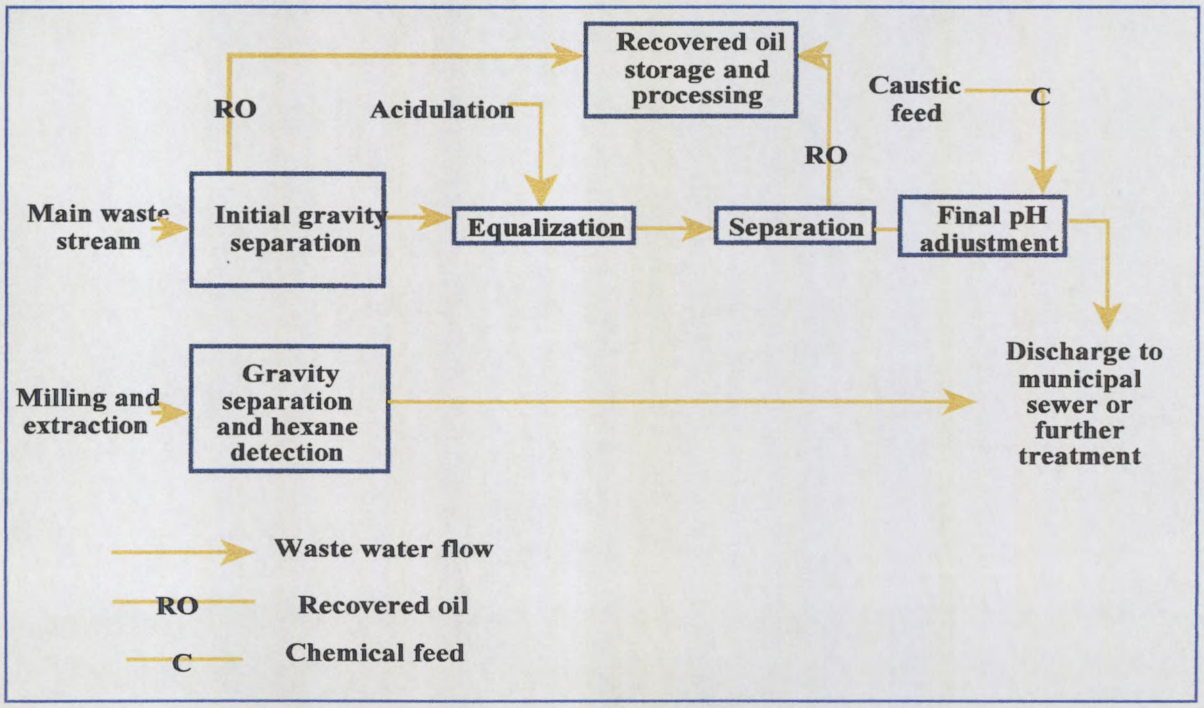


FIGURE 2.5: Acid Hydrolysis Process (Boyer, 1996)

If the plant acidulates soapstock and /or wastewater, the resulting acidulation waste will contain sufficient residual heat and acidity to effect the entire waste stream pH and temperature. This obviously assumes an alkali refining process rather than a physical refinery. This will produce an acidulation effect on residual oils. These oils can then be readily removed in a gravity separation process. Acidulation of washwater as part of the treatment process is recommended (Boyer,1996). The process is outlined in Fig. 2.5.

2.5.1.4 Neutralisation

Effluent from the first, low pH separation will need neutralising with caustic soda before being mixed with the remaining effluent and being subjected to a final flotation process. The liquor discharged from this second flotation unit must be neutralised to bring the pH value up to local statutory requirements. The final neutralisation can be effected with either caustic soda or calcium hydroxide. Addition of the latter would avoid a further increase in the sodium content of the final effluent, but would give rise to a sludge handling problem requiring the use of a filter. Although requiring an additional stage of treatment the filtration process would provide further removal of fatty material, COD and suspended solids (Steffen *et al.*, 1989).

2.5.2 Alternative treatment technology and recent developments

Global trends show progression towards developing more efficient and cost effective treatment technology as opposed to the conventional approach. Some of the developments include :

2.5.2.1 Membrane technology

Various membrane technologies have been applied to edible oil effluent waste streams. Reverse osmosis making use of ultra-thin synthetic or natural membranes may be viable for treating process effluent high in total dissolved solids (TDS) such as acid water. This is particularly true in areas where strict standards are enforced regarding, for e.g. sulphate content, where edible oil plants are presently transporting effluents high in sulphate off their premises for disposal elsewhere (Steffen *et al.*, 1989). While pollutant removals are good in some applications using membrane technology, membrane life and regeneration have presented problems. As a result, operating costs for periodic membrane replacement are quite high (Boyer, 1996).

2.5.2.2 Activated carbon and use of filters

Attempts have been made to utilize activated carbon to remove residual organics. The nature of the soluble components in the acidulation stream (the primary source of solubles) is such that they cannot be effectively removed by activated carbon. Several processes attempted to use a mixed media(sand, anthracite) filters to remove residual oils. This technology was adopted from petroleum waste applications; however, it did not function well in oilseeds applications. This was due primarily to an inability to backwash these units caused by the highly viscous nature of these oils at ambient temperature. Attempts have been made to apply inclined plate and other packaged media separator systems. These have not functioned well in edible oil applications due to the viscous oil coating over the separator media (Boyer, 1996; Herbert *et al.,* 1996).

2.5.2.3 Integration of chemico-physical and biological processes

Combining chemical and biological processes poses interesting challenges in conducting research and operating a full scale plant efficiently. It may be important that the chemical oxidant and bio-culture do not mix. Ozone, for example, is used as a disinfectant and would be detrimental to the biological culture if toxic residual amounts remained from pretreatment. High concentrations of hydrogen peroxide can also prove detrimental to the bio-culture although relatively low concentrations do not pose a serious problem for microorganisms (Carberry and Benzing, 1991). Special precautionary measures taken by scientist to prevent the introduction of chemical oxidants into subsequent biological processes included holding the chemical reaction mixture for a day to dissipate residual ozone (Barton and Drake, 1994). Application of some integrated processes for edible oil effluent treatment include :

a) *Integrated treatment of olive oil mill effluent* : *Study of ozonation coupled with anaerobic digestion.*

The possibility of coupling a chemical treatment (ozonation) with biological treatment (anaerobic digestion) was investigated for olive mill effluents (OME). Preliminary research showed that total phenols and unsaturated lipids are reduced to approximately 50% in 3 h of ozonation and that the total COD remains about unvaried. Nevertheless ozonated OME exhibit in general a longer lag phase and a lower yield in methane than OME itself. These effects are more evident at higher OME concentrations. Results also indicate that the ozonation products of oleic acid are more inhibitory than the original substrate, thereby sometimes impacting on the subsequent biological process. However there was no effect of ozonation on the acidogenic phase of fermentation (Andreozzi *et al.*, 1998)

b) *Integrated treatment of olive oil mill effluent : impact of chemical and physical pretreatment on anaerobic treatability*

Difficulties experienced in the treatment of OME suggested the use of a chemico-physical pretreatment for the removal of biorecalcitrant and/or inhibiting substances (essentially lipids and polyphenols) as selectively as possible before anaerobic digestion. Laboratory scale experiments were conducted in order to identify the ideal pretreatment technology. Ultra-filtration allowed high removal of lipids and polyphenols but produced poor selectivity (large amounts of biodegradable COD were also removed) (Beccari *et al.*, 1997). Centrifugation turned out to be preferable to sedimentation owing to smaller volumes of separated phase. Research also showed that application of a continuous process combining pretreatment (with calcium hydroxide and bentonite) and anaerobic digestion without the intermediate phase separation produced promising results (Beccari *et al.*, 1999).

2.5.2.4 Waste minimisation approach in the edible oil industry in Pakistan and Egypt

Pakistan and Egypt are amongst the largest produces of edible oil in the world. Two case studies were selected to outline their approach to waste management.

a) Sila Edible Oil Company- Egypt

A range of waste minimisation opportunities have been identified and are currently being implemented. To date, this has involved a total investment of LE 621,000 and resulting in a annual savings of LE 1,557,110. Expenditure on the wastewater treatment plant has also been reduced from LE 1.5 million to LE 549,800. The measures that were identified for implementation included conducting an audit with particular attention to improvements that could be carried out at minimal cost to the company. These were easy to implement and yielded significant savings as well as reducing final effluent strength and volume. These waste minimisation strategies included :

i) *Preventative Maintenance programs*

The measures undertaken included :

- Steam trap modifications
- Repair of leaking or broken valves
- Repair of damaged water pipes
- Repair of damaged steam pipes

ii) *Packaging unit : Collection and Recycling of Spilt Oil*

A system was installed to recover edible oil from accidental spillages in the bottling department. The spilt oil is immediately collected in troughs and pumped to a collection tank where the oil is recycled to the refinery for reprocessing. Approximately 1.16 tons of edible oil is recovered on a monthly basis.

iii) *Water and Energy Conservation*

This involved a detailed inspection and overhaul of the steam production system including rehabilitation of steam lines , recycling of steam condensates and insulation of hot water and steam pipes. As a result, steam consumption had been reduced 1,800 tons per month and one boiler was taken off line. Water consumption was reduced by 2,400 tons per month.

iv) *Wastewater Segregation*

Wastewater produced by the factory includes process effluents , domestic sewage, boiler blowdown, cooling tower blowdown and steam condensate. Of these, process effluents have the highest organic loads, the effluent emanating from the refinery being particularly strong with a BOD of 3,420 mg/L. Segregation of the remaining effluent was good enough to be used for land reclamation activities within the factory , such as watering lawns and trees.

v) *Minimising Wastewater Treatment Costs*

Prior to the audit, there was a planned expansion of the wastewater treatment plant with a design capacity of $15m^3$/month. Due to waste minimisation strategies there was no need for the latter since water consumption was reduced by $2,400m^3$ with a 3,420kg BOD/day organic load. As a result a treatment plant with a design capacity of only $5m^3$/h was required to treat the effluent.

The benefits and achievements of the waste minimisation approach included :

- Maintenance costs have been reduced by 10%
- Water consumption has been reduced by 46%
- Wastewater treatment requirements have been reduced by 66%
- Boiler fuel consumption has been reduced by 48%
- Annual recovery of oil, soapstock and meal valued at LE 692,650
- Discharge compliance achieved (Sila Edible Oil Company, 1999).

b) Pakistan's edible oil sector

Pakistan's edible oil industry was nationalized in 1972 and a public sector organization Ghee Corporation of Pakistan was created to run the industry. The environmental challenge for the edible oil industry is mainly associated with liquid waste, solid waste and inefficient use of energy. However, the biggest problem faced by an edible oil industry is wastewater, both quantitatively and qualitatively. As discussed previously, wastewater generated from processes and auxiliary systems vary in pollution load and concentration. Process wastewater contributes most of the pollution load in the effluent with high amount of BOD, COD, FOG, Total Suspended Solids (TSS), TDS, phosphates etc.. Various measures were taken to address the environmental challenges and were categorized as in-house improvements (IHI), cleaner production (CP) technologies and end of pipe(EOP) treatment. IHI and CP technologies not only resulted in the improvement of economic and environmental performance of the mill, but also reduced the cost of EOP treatment. Measures taken for IHI related to waste reduction at source and adoption of environment friendly processes. Some of the measures adopted included :

i) *Size optimization of separation chambers*

oil losses in the effluent can be reduced by increasing the size of the separation chambers beneath the pre- and post- neutralization vessels.

ii) *Recycling of washing water after neutralization*

counter current washing in pre-and post- neutralization will significantly reduce the process wastewater, hence cost of EOP treatment.

iii) *Recycling/reusing cooling and vacuum water*

water consumption as well as discharge can be reduced significantly by reusing/recycling the non-process water, such as cooling water and vacuum water after proper treatment.

iv) *Use of pressurized water for floor cleaning*
increases the efficiency of washing and also saves water.

The following CP technologies were adopted to minimise the level of pollution, as well as increase the efficiency of energy use :

- Replacement of gravity settling by centrifuges after neutralization resulting in the reduction in oil losses and washing water and improvement of oil quality.
- Segregating the water according to its use and pre-treating, recirculating and finally treating/discharging each separately. This would result in decrease in water consumption and hence a smaller wastewater treatment facility.
- Oil recovery from spent earth can be done either by injection of compressed air/steam at the plate and frame filters or by solvent extraction.
- FFA at deodorizer can be recovered more efficiently for recycling in the soap section.
- Heat should be recovered from cooling water used during hydrogenation.
- Pre-heating of incoming oil with outgoing oil at hydrogenation as well as at deodorization resulted in savings of natural gas and cooling water.

As discussed, waste minimisation strategies have been recommended to significantly reduce both the pollution and the hydraulic loads thus reducing the total of EOP treatment systems (Pakistan's Edible Oil Sector, 2000).

2.5.3 Biological treatment technology

Wastes from oilseeds operations have been successfully treated using biological treatment processes. Edible oils of animal and vegetable nature are highly biodegradable and compatible with municipal domestic wastes. However pretreatment using physical-chemical methods is mandatory to remove floatable oils prior to biological treatment (Steffen *et al.*, 1989). Various biological processes have been used with good results. Some of the technologies include:

2.5.3.1 Anaerobic digestion

Anaerobic digestion involves the breakdown of organic matter by the action of microorganisms in the absence of oxygen producing biogas, which contains mathane and carbon dioxide as well as quantities of ammonia, nitrogen, hydrogen and hydrogen sulphide (Callander and Barford, 1983). Although some fungi and protozoa can be found in anaerobic digesters, bacteria are the dominant microorganisms. The four different groups include hydrolytic bacteria, hydrogen-producing acetogenic bacteria, homoacetogenic bacteria and methanogenic bacteria (Bulock and Kristiansen, 1987). Experience from sewage sludge digestion has shown that anaerobic systems have the ability to degrade fatty material. Some of the benefits associated with technology include :

- Degradation of fats
- Low chemical requirements
- Low sludge production
- Minimal power requirements, and
- moderate COD removal efficiency

Some of the more common anaerobic processes currently in use for treatment of high strength industrial effluents include contact digesters, packed bed digester, upflow sludge blanket digester and fluidized bed digesters. The application of the anaerobic contact and packed bed processes provided efficient removal of fatty matter without the need for preliminary chemical treatment Treated effluent total fatty material(TFM) concentrations did not exceed 100mg/L and removal efficiencies ranged from 80-90 %. Carbonaceous COD removal efficiencies ranged from 76 to 92 %. However, overall COD removal was only in the range of 21 to 54 % due to the oxygen demand exerted by dissolved sulfides in the treated effluent (Saw *et al.*, 1987).

Effluents arising from palm oil production are complex waste containing residual oils and greases, polysaccharides and proteins (Cail and Barford, 1985). Although many industries that produce Palm Oil Mill Effluent (POME) in Malaysia use conventional ponding systems for treatment, the trend is now changing towards anaerobic treatment systems. Anaerobic treatability of POME using Anaerobic Fluidized Bed Reactors (AFBR) has been investigated. It was reported that AFBR can be operated at HRT of 12 hrs with a COD removal of 85 % and at HRT of 4 hrs up to 65 % COD removal (Idris and Al-Mamum, 1998). High rate semi-continuous anaerobic digestion of POME was also investigated and it was found that at HRT of 5.6 days with a space loading of 12.6 kgCOD/ m^3.d, soluble COD removals were greater than 97 %. (Cail and Barford, 1985).

Anaerobic treatability and methane potential of olive oil mill effluent has also been investigated. Findings showed that OME yielded biogas with a methane content of 77 % and 57.5 L CH_4/L wastewater. COD removal of 85-93 % was achieved (Demirer *et al* ., 2000). Laboratory and pilot-scale Upflow Anaerobic Sludge Blanket (UASB) reactors were used to investigate anaerobic treatment of OME yielding COD removal efficiencies (Demirer *et al.*, 2000). Some of the difficulties in the anaerobic treatment of OME are mainly connected with the presence of biorecalcitrant and/or inhibiting substances, essentially polyphenols and lipids (Beccari *et al.*, 1999).

Research conducted at the Centre for Water and Wastewater Research, Technikon Natal using Anaerobic Baffled Reactor (ABR) technology for the treatment of sunflower oil effluent presented the following findings. Gravitational separation of lipids was not found to be the recommended method for reducing the lipid content of oil effluent. High FOG content of the oil effluent formed a scum barrier in the compartments disrupting the normal circulation of liquid, thus causing the reactor to fail after 33 days. High sulphate content of the sunflower oil effluent proved to be toxic to the bacteria. However the use of barium chloride as a pretreatment process alleviated this problem. ABR technology has not been recommended as a treatment of choice for edible oil effluent. (Frost, 2002).

2.5.3.2 Treatment of effluent using microorganisms

The use of microorganisms to reduce, eliminate or detoxify environmental pollutants is referred to as bioremediation. Some of the microbes and processes used for the treatment of oil effluent include :

a) Fungal degradation of lipids

The degradation of lipids by fungi is predominantly an enzymatic process. Fungal lipases are considered to play a vital role in lipid degradation and are produced by a variety of fungi. Those belonging to the genus *Mucor, Rhizopus* and *Geotrichum* are considered to be extremely effective in the hydrolysis of triglycerides. Lipases belong to a group of enzymes known as hydrolases and catalyse the degradation of triglycerides by hydrolysing the ester bond between the glycerol and acyl moiety. Lipase activity is effected by pH, temperature and the availability of essential growth nutrients. It has been reported that highest lipase activity occurs at the oil- water interphase in the presence of hydrophobic substrates (Lazar and Schroder, 1992). Fungi are also known to play a role in the pretreatment of the oil effluent prior to biological treatment. Preliminary aerobic treatment with *Aspergillus Niger* or *Geotrichum candidum* turned out to make shorter the residence time required for anaerobic processes (Hamdi *et al.*, 1991; Martin *et al.*, 1993). However, aerobic pretreatment is affected several problems (need of optimizing mycelial growth conditions, disposal of large amounts of excess biomass due to high aerobic yield coefficients) that have not yet found a satisfactory solution in terms of achieving the desired full scale performances. More promising seems to be the alternative based on a chemico-physical pretreatment carried out to remove lipids and polyphenols as selectively as possible before anaerobic digestion. This way follows a general trend towards integration between chemico-physical and biological processes in the case of wastewaters characterized by difficult biotreatability (Scott and Ollis, 1995).

b) Degradation of oil using bacteria and yeast

Bacteria and yeast were isolated from contaminated environments on site at a edible oil industry and screened for their ability to degrade edible oil effluents. Laboratory scale trials showed that *Bacillus spp* removed approximately 91 % of FOG using a shake flask process at a temperature of 32^{0}C. However, the application of yeasts such as *Candida spp* and *Rhodosporidium spp* did not show the same removal capabilities. *Candida spp* removed approximately 82 % of FOG at pH 7 (21^{0}C) and *Rhodosporidium spp* only removed 42 % of the FOG (Reddy,2001). A strain of bacteria has been developed for commercial application which digested fats from edible oil effluents. However problems associated with the process and operating a full scale wastewater treatment plant in S. Africa included periodic shock loads of, for instance, soapstock spillages which caused the process to fail (Steffen *et al.*, 1989). The latter problem could be overcome by utilizing a holding tank to prevent shock loads prior to biological treatment. Wakelin and Forster, 1997 applied a range of pure and mixed cultures (activated sludge) to determine their ability to degrade vegetable oils and greases. The pure cultures used were *Acinetobacter sp, Rhodococcus rubra, Nocardia amarae* and *Microthrix parvicella* and these were compared with activated sludge. *Acinetobacter* was the most effective of the pure cultures tested typically removing 60-65 % of the fatty material. Activated sludge gave a more consistent removal which was generally better than 90 %. In addition to achieving high removal efficiencies, acclimatized activated sludge did not exhibit a long lag phase, resulting in faster growth and fat removal. The application of activated sludge, particularly when it has been acclimatized to FOG substrate, offers the best option for treatment of fat rich wastewater. This is fortunate, since a pure culture would not be ideally suited to use in any commercial, non-sterile, open bioreactor that would have to be set up in industry. Another disadvantage would be the high cost associated with the production of a pure culture and problems associated in maintaining the integrity of the culture in the reactor (Wakelin and Forster, 1997).

2.6 NUTRIENT REMOVAL ACTIVATED SLUDGE PROCESSES

Immediately after its discovery in 1914 by Ardern and Lockett, the activated sludge process found wide application as an effective means of wastewater treatment (Droste, 1997). Nevertheless, the process has been continuously challenged by the ever increasing magnitude and complexity of waste loads, and more recently, by fundamental changes in traditional concepts of wastewater treatment. The activated sludge treatment process is a suspended growth system which could be defined as a suspension of microorganisms, both dead and living in wastewater (Aitken *et al.*, 1994). The process relies on the dense growth of microorganisms in a reactor where air is continuously supplied to allow for carbonaceous oxidation (Droste, 1997)

2.6.1 General description of biological nutrient removal

All life forms require a source of energy and matter to sustain their existence (Lilley *et al.*, 1997). The most prominent elements passing through the life system are Hydrogen (H), Oxygen (O), Carbon (C), Nitrogen (N), Phosphorus (P) and Sulphur (S) plus a host of minor elements. Energy is derived principally from three sources and these form a convenient criterion to catagorise the organisms implicated :

- Solar radiation - photosynthetic autotrophs
- Organic compounds - heterotrophs
- Inorganic compounds - chemical autotrophs

In the biosphere the fundamental source of energy is solar radiation. The *photosynthetic autotrophic* cells fix a small fraction of the solar energy by forming complex high energy organic (H,C,O) compounds and producing oxygen. Of the matter requirements, the elements H and O are obtained from water; carbon from CO_2 and the minor elements from the dissolved salts of these elements. The high energy organic compounds synthesized by the autotrophs form the basic source of energy for *heterotrophic cells* to synthesize more complex molecules that constitute the cell mass, including proteins. Only a fraction of the energy utilized is incorporated in the cell mass generated, the balance is lost as heat. The mass of organisms thus generated in turn form the matter and energy sources (prey) for other organisms that live on them (predator). Each prey - predator transformation is accompanied by a substantial loss of energy. *Chemical autotrophs* derive their energy for growth from the oxidation of inorganic compounds. The nitrifying bacteria fall within this category. They are obligate aerobes and derive their energy by oxidizing saline ammonia to nitrite and nitrate (Ekama *et al.*, 1984). Presence of organic energy in the receiving body of water gives rise to fungal and other heterotrophic growths, de-oxygenation of water thereby rendering the water unsuitable for higher life forms such as fish etc. These effects reduce the aesthetic appearance, recreational use and the reuse of water (Ekama *et al.*, 1984).

In wastewater treatment, the objective of Biological Nutrient Removal (BNR) is to remove the primary nutrients which cause eutrophication namely C, N, and P from wastewater.

2.6.1.1 Carbon removal

Activated sludge systems are traditionally designed for the removal of carbonaceous organic compounds in wastewater. Almost complete removal efficiencies may attained in these systems. Carbon in wastewater streams occurs in organic and inorganic compounds. Organic compounds can be utilized by heterotrophs and inorganic compounds by autotrophs. Both forms of carbon are removed from the wastewater through a series of redox reactions, oxidising the carbon source to carbon dioxide and water . The carbon dioxide then escapes to the atmosphere, removing C from the waste stream (Orhon and Artan, 1994 ; Lilley *et al.*, 1997). Characterization of the carbonaceous material in the influent is done via the COD test. For activated sludge process design it is necessary to identify and know the magnitudes of the various fractions of the influent COD as these significantly affect the response of the process. Carbonaceous material (that which contains carbon) in wastewater (Sti) can be split into two principle forms : biodegradable(Sbi) and un-biodegradable (Sui). Each form has two fractions, viz. soluble and particulate (Fig. 2.6). The relative fractions of each constituent can vary considerably with different types of wastewater.

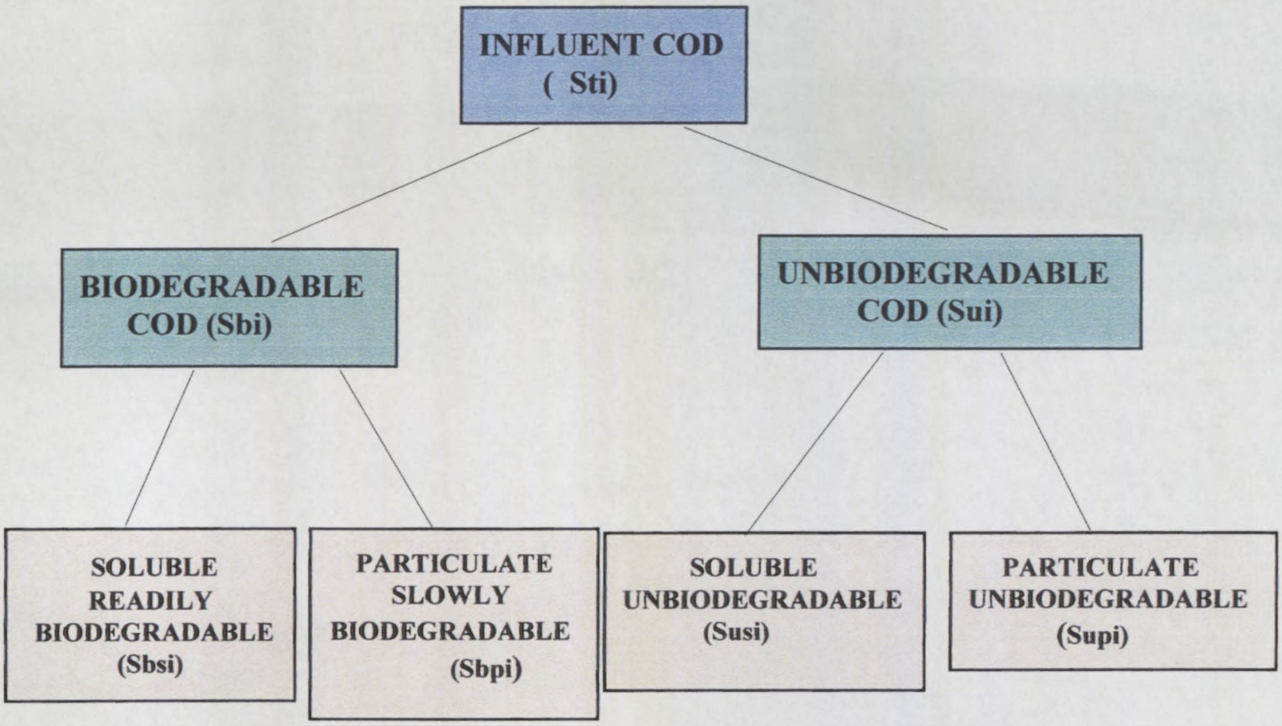


FIGURE 2.6. Division of influent COD into constituent fractions (Ekama *et al*., 1984)

The unbiodegradable material is not broken down within the treatment process. The unbiodegradable particulate material becomes enmeshed in the sludge, settles out in the sedimentation tanks and is removed from the system with the waste sludge. The unbiodegradable soluble fraction passes through the treatment process and is discharged with the effluent, sometimes giving rise to high effluent concentrations. Both fractions are hypothesized to be unaffected by biological action so that at steady state the masses that enter equal the masses that leave the treatment system (Lilley *et al.,*1997). Usually it is more convenient to express the Supi fraction not in terms of its COD but in terms of its influent volatile solids concentration (Xii), where

$Xii = S_{upi}/f_{cv}$ and

f_{cv} = COD/VSS ratio = 1.48 mgCOD/mg VSS

The measurement of S_{usi} is as follows ; a laboratory scale unit is run at two different sludge ages both greater than 3 days. The COD of the centrifuged effluent or the filtrate through a fine filter, estimates S_{usi}. The balance of the influent COD, after subtracting S_{bi} and S_{ui} from S_{ti}, is the S_{upi}, i.e.

$S_{upi} = S_{ti}-S_{bi}-S_{usi}$ (Ekama *et al.*, 1984)

The biodegradable carbonaceous material is broken down in the treatment process by heterotrophic organisms under aerobic conditions in the aeration basin. The soluble biodegradable material is rapidly used by organisms (and is thus called readily biodegradable COD) whereas the particulate fraction is rapidly adsorbed but assimilated more slowly (and therefore called slowly biodegradable COD). In a biological system, a substrate may be associated with two different major processes : it may be incorporated into a sequence of degradative reactions converting first into a number of intermediate products and then to stable end products with energy generation. These reactions are collectively called catabolism or dissimilation. Alternately, the substrate may be part of the anabolic or assimilative reactions where major biochemical components of cells are manufactured from the intermediate products by means of a variety of biosynthetic pathways (Orhon and Artan, 1994; Lilley *et al.*, 1997)

Within the chemical reactions associated with the breakdown of carbonaceous material , electrons are transferred and accepted in redox reactions via the catabolic and anabolic pathways. In the catabolic pathway, a fraction of the organic molecules is taken up by the organism and oxidised to CO_2 and water with the release of large amount of energy. Some of this energy is captured by the organism and utilized e.g. for cell growth while the remaining energy is lost to heat. The anabolic pathway is used to construct new cell mass. In the utilization of an organic compound by the organism, both the energy and the electrons must be conserved i.e. one must be able to trace the fate of both the energy and the electrons. The electrons are conserved in that, of those available in the organic compound some are retained in the organic compounds of the organism mass formed and the rest are passed on to the terminal electron acceptor. The proportion of the of the electrons (or energy) retained by the organisms defines the specific organism mass *yield coefficient* (Y_h)i.e mass of organism mass formed per mass of organic compound oxidized, all in terms of electron equivalents, i.e.

$$Y_h = \frac{e^{-} \text{ (or energy) conserved in organism mass}}{e^{-} \text{ (or energy) in substrate utilized}}$$

These two cycles together form the metabolism of the organism and in this manner energy is removed from wastewater. Under aerobic conditions, it has been established that the amount of energy released in the anabolic and catabolic pathways is proportional to the mass of oxygen utilized for cell growth, which in turn can also be related to the number of electrons donated in the oxidation of organic compounds (Ekama *et al.*, 1992; Lilley *et al.*, 1997).

The BOD and COD test is used to determine the energy content of a wastewater, and consequently the oxygen requirements for carbon removal. The BOD test is an empirical test performed under strictly specified conditions, and measures the oxygen utilized by organisms over a 5 day period. Recently, global trends have shown limited application for the BOD test as opposed to the COD test for the following reasons :

- The BOD test underestimates the actual energy within the wastewater
- The long time required to obtain test results often makes this test impractical for use as a monitoring parameter at treatment works
- Nitrifying organisms in the sample may utilize oxygen to convert ammonia to nitrates giving an inflated carbonaceous energy measurement

The COD test uses a strong oxidising agent (hot dichromate sulphuric acid solution) to oxidize organic compounds to water and CO_2. It has become a method of choice to measure the carbonaceous energy within wastewater. The advantages include :

- It does not oxidize ammonia so it gives only the electron donating capacity of the organic compounds.
- It measures the electron donating capacity which is directly related to the free energy changes in the oxidation of organics.
- It does not require sophisticated analytical equipment- it is a simple wet chemical titration procedure and such is suitable for field application.
- Provides results in a short time i.e. approximately 3 hrs as opposed to 5 days for the BOD.
- It allows an electron balance to be made over the biological system (Ekama *et al.*, 1992).

Subsequent to biological treatment, the liquid effluent which is low in COD i.e. the carbonaceous energy is removed, is then discharged from the wastewater treatment system.

2.6.1.2 Nitrogen removal

Nitrogen is one of the principal nutrients of concern in wastewater and can be subdivided into two main forms i.e. free and saline ammonia and organically bound nitrogen. The organically bound nitrogen can be subdivided further into non-biodegradable and biodegradable, both forms having soluble and particulate fractions. Generally wastewater do not contain any nitrate and nitrite in the influent. Biodegradable organic nitrogen is broken down into free and saline ammonia within a sludge age of approximately 3 days. Non-biodegradable particulate nitrogen is generally settled out in sedimentation tanks and removed with the waste sludge stream. The non-biodegradable soluble nitrogen passes through the treatment system and is discharged with the effluent (Orhon and Artan, 1994; Lilley *et al.*, 1997).

The first step in the nitrogen removal process is called nitrification. Under aerobic conditions nitrifying organisms convert ammonia, via nitrites , to nitrates in two different oxidative reactions known as *nitritation* and *nitratation.* In nitritation, ammonia is oxidised to nitrites where after nitratation commences with further oxidation of nitrites to nitrates (Mauret *et al* ., 1996).

$$2\ NH_4^+ + 3O_2 \longrightarrow 4H^+ + 2H_2O + 2\ NO_2^- \qquad \text{(Nitritation)}$$

$$2\ NO_2^- + O_2 \longrightarrow 2NO_3^- \qquad \text{(Nitratation)}$$

During nitritation (oxidation of ammonia), hydrogen ions are released which cause a decrease in the pH and alkalinity of the wastewater (Muyima *et al.*, 1997; Lilley *et al.*, 1997). In addition, nitrification in activated sludge systems require a long sludge age and retention times to achieve complete ammonia and nitrite oxidation. Among all biological reactions responsible for nitrogen removal (Fig. 2.7), ammonia oxidation is generally the rate limiting step because of slow growth and poor yield of chemoautotrophic ammonia oxidising bacteria (Suwa and Noto, 1998). The common genera of bacteria implicated in nitrification include *Nitrosomonas*, which is implicated in conversion of free and saline ammonia to nitrite and *Nitrobacter* which converts nitrite to nitrate. The oxygen requirement associated with this conversion amounts to 4.57 mg oxygen/mg N utilized (Lilley *et al.*, 1997).

With the resultant release of nitrates into receiving water bodies via nitrified wastewater, it became necessary to include denitrification into activated sludge systems. Denitrification is the reduction of nitrates, via nitrites, to nitrous oxides and gaseous nitrogen under anoxic conditions (Wu and Knowles, 1995).

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \qquad \text{(Denitrification)}$$

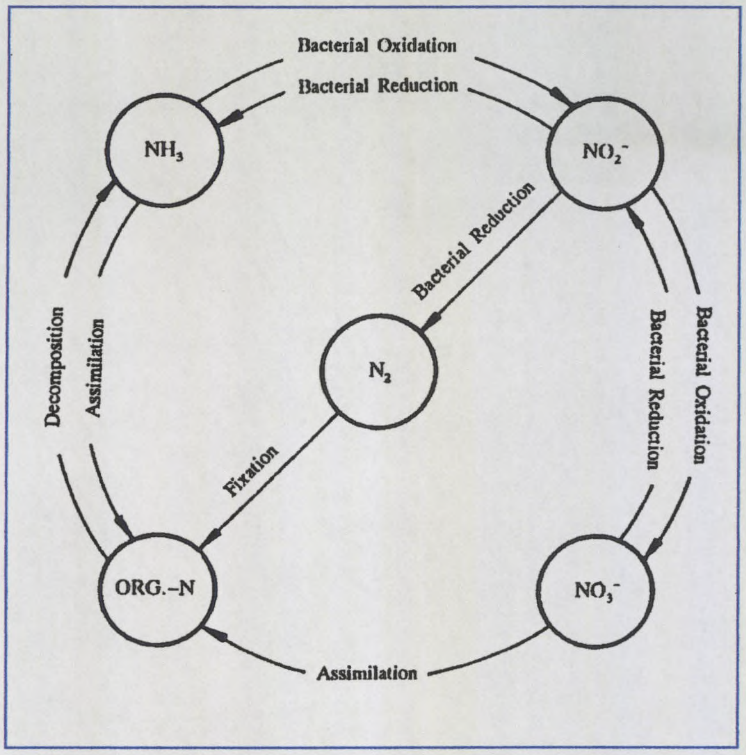


FIGURE 2.7. Simplified nitrogen cycle (Orhon and Artan, 1994).

Nitrogen naturally exist in various compounds with a valence ranging from -3 to +5. Transformations of nitrogen forms resulting in valence changes are associated with metabolic activities of different types of organisms. The various forms of nitrogen present in nature, and pathways by which these forms are changed is schematically represented in Fig. 2.7.(Orhon and Artan, 1994).

The inclusion of denitrification into activated sludge systems is achieved via the addition of anoxic zones in the process. Under anoxic conditions denitrifying microorganisms are stimulated into utilizing nitrates and nitrites as final electron acceptors for cellular respiration with the resultant production of gaseous nitrogen accompanied by concurrent COD removal (Cappucino and Sherman, 1992). The inclusion of denitrification to nitrifying activated sludge systems has resulted in reduced daily oxygen requirements and final effluent nitrate loads. From stoichiometric relationships, when nitrate acts as a electron acceptor, 1 mg NO_3^- as N is approximately equivalent to 2.86 mg oxygen (as O) if oxygen was the terminal electron acceptor. If one thus combines the nitrification and denitrification processes, up to approximately 63 % of the oxygen demand for nitrification can be recovered if complete denitrification is achieved.

2.6.1.3 Biological phosphorus removal

In wastewater, phosphorus exist in three forms : orthophosphate, polyphosphate and organically bound phosphorus. Simple orthophosphates and polyphosphates or condensed phosphate constitute the total inorganic phosphorus. While polyphosphates are easily converted to orthophosphates via acid hydrolysis, a total oxidative destruction of the organic matter is required for the liberation of organic phosphorus as orthophosphate. Orthophosphates are easily assimilated by microorganisms. Other forms of phosphorus become available to microorganisms only after hydrolysis to orthophosphates. (Orhon and Artan, 1994).

Phosphorus can be removed from wastewater by either biological means or by chemical precipitation. Traditionally, phosphorus removal has been carried out in an additional tertiary chemical treatment step, whereby dissolved orthophosphate in the secondary effluent is precipitated as calcium or metal phosphate, and separated as chemical sludge in a tertiary clarifier (Henze,1992). In the last decades, however, many full scale plants were built to achieve biological excess phosphorus removal (BEPR) and involves the removal of dissolved phosphorus in wastewater. The process refers to the biological uptake and subsequent removal of phosphorus (P) by the activated sludge in excess of the amount that is removed by completely aerobic (or conventional) activated sludge systems. This process is as the result of synergy between different groups of bacteria (Poly-P bacteria), other heterotrophs and autotrophs in the same systems (Danesh and Oleszkiewicz, 1997).

It is generally accepted that enhanced P removal occurs as a result of the ability of certain organisms having the capacity to accumulate large quantities of polyphosphate (poly-P) within the cellular mass. *Acinetobacter* spp, was first implicated by Fuhs and Chen (1975) as the bacteria that plays an important role in BEPR processes. Since then much research has been done on the contribution of *Acinetobacter* spp in biological P removal (Cloete *et al.*, 1985; Streichen *et al.*, 1990; Beacham *et al*., 1992). However there has been much debate on *Acinetobacter* as the bacteria that plays a major role in BEPR, since the carbon and phosphorus transformation patterns of the species are not consistent with those of BEPR sludge (Groenestijn *et al.*, 1989). The number of *Acinetobacter* spp evaluated by culture-independent analysis, such as fluorescent antibody method and in situ hybridization techniques is very small in BEPR processes (Wagner *et al.,* 1994b). Nakamura *et al.*, (1995) claimed that *Microlunatus phosphorus,* a Gram-positive coccus with a high G+C content, to be the candidate for the dominant poly-P accumulating bacterium in BEPR processes because it contained high amount of phosphorus and its carbon and phosphorus transformation patterns coincided with those of BEPR sludge. Until present times scientist are striving to attribute Bio-P removal to a single species or it could be that many species contribute holistically to biological phosphorus removal.

In order to design and operate activated sludge plants successfully for P removal, conditions have to be created which support the propagation and growth of poly-P organisms over organisms which do not have this propensity. Bio-P removal occurring in activated sludge is a direct result of the ability of certain microorganisms , termed polyphosphate accumulating organisms (PAO's), to accumulate large quantities of poly-P intracellularly. The term 'enhanced' and 'excess' are often incorporated to emphasize the ability of these organisms to accumulate poly-P in excess of their normal metabolic requirements. In order to encourage the growth and proliferation of these organisms, as well as to induce the bio-P mechanism, two conditions are essential i.e. sequential anaerobic and aerobic reactors and the presence of volatile fatty acids in the anaerobic reactor(Wentzel et al., 1990).

Under aerobic conditions the poly-P organisms are not able to compete non-poly-P organisms for substrates such as glucose or other saccharides. Under anaerobic conditions (no nitrate or oxygen present) and in the presence of short chain fatty acids (SCFA) the poly-P organisms break down or hydrolyse stored polyphosphate. This process releases orthophosphate to the surrounding liquid and leads to the phenomenon known as phosphorus release in the anaerobic zone. The bond energy released in hydrolysing polyphosphate is utilized by the poly-P organisms to absorb, process and store the SCFA within the organisms, thereby reserving substrate for their exclusive use when they enter an environment which contains external electron acceptors such as nitrate or oxygen. In this way they do not have to compete with non-poly-P organisms which are unable to utilize SCFA under anaerobic conditions because of the lack of a suitable electron acceptor. Therefore, the function of the anaerobic zone in BEPR processes is two-fold : (1) the reduced redox potential induces conversion of the influent readily biodegradable COD (RBCOD) to SCFA's, also known as volatile fatty acids

(VFA's) via acidogenisis by non-PAO heterotrophs, and (2) it provides an ideal environment where PAO's are able to take up the VFA's and accumulate them intracellularly as polyhydroxyalkonoates (PHA's), the most common of which is polyhydroxybutyrate (PHB) (Ekama and Wentzel, 1997; Lilley *et al.*, 1997).

When re-entering the aerobic environment the poly-P organisms utilize the reserved SCFA both for growth and to replenish their poly-P pool by abstracting orthophosphate from the surrounding medium. This gives rise to the BEPR phenomenon in the aerobic environment.

2.6.2 Operational parameters and configurations of activated sludge processes

In designing and operating an activated sludge process, a number of key parameters govern both the selection of the process and the removal of nutrients including nitrogen and phosphorus attainable in the process.

2.6.2.1 Operational control parameters

Several parameters are used to monitor the performance of the activated sludge process on a routine basis. The commonly used key parameters include :

a) *Mixed Liquor Suspended Solids (MLSS) and Mixed Liquor Volatile Suspended Solids (MLVSS)*
The content of the aeration tank in an activated sludge system is called mixed liquor. MLSS is the total amount of organic and mineral suspended solids, including microorganisms in the mixed liquor. It is determined by filtering an aliquot of mixed liquor, drying the filter at 105^0C, and determining the weight of solids in the sample. The organic portion of the MLSS is represented by the MLVSS, which comprises non- microbial organic matter as well as dead and live microorganisms and cellular debris (Nelson and Laurence, 1980). MLVSS is determined after heating of the dried filtered samples at $600\text{-}650^0$ C, and represents approximately 65-75 % of the MLSS. Both are functions of influent load , process volume and particularly sludge age and therefore of limited value unless the sludge age is accurately known (Bitton, 1999; Seviour *et al.*, 1999).

b) Sludge Loading or Food/ Microorganisms Ratio (F/M Ratio)

The FM ratio indicates the organic load into the activated sludge system and is expressed in full scale plants as kilogram BOD per kilogram MLSS per day (Nathonson,1986). It is expressed as follows:

$$\mathbf{F/M\ ratio} = \frac{\mathbf{Q \times BOD}}{\mathbf{MLSS \times V}}$$

where Q = influent flow rate (L/d)
BOD = BOD of influent (mg/L)
MLSS = mixed liquor suspended solids (mg/L)
V = volume of reactor (L)

However, the BOD test is slow and imperfect (Seviour *et al.*, 1999). Marais and Ekama (1984) described organic loading in terms of COD since it offers a more quantitative representation of the electron donor capacity and therefore the amount of energy contained within a wastewater sample. Therefore when determining the F/M ratio it is advisable to use the COD rather than the BOD. The F/M ratio is regarded as the most practical of the loading parameters as it is the only form over which the plant operator has any control through maintenance of a fixed concentration of MLSS in the aeration basin (Horan, 1990). The F/M ratio is controlled by the sludge wasting rate, and a high wasting rate will decrease the sludge age and so increase the F/M ratio. This parameter may be useful in monitoring conventional plants which usually operate with F/M ratios of 0.1-0.4, since if M is large and F is small, less substrate is available for biomass production. Low F/M processes therefore produce less biomass per unit of applied organic load and high quality effluent with good floc formation, but the O_2 requirement is greater (Seviour *et al.*, 1999). Chudoba *et al.* (1992) and Grady *et al.* (1996) support the use of the S_o/X_o ratio to characterize the influent, where S_o is the initial substrate concentration and X_o the initial biomass concentration at the start of the batch process. This ratio is not the same as the F/M ratio. It has been adopted by many modellers to measure the growth kinetics of the biomass although it's biological significance in uncertain.

c) *Hydraulic Retention Time*

The hydraulic retention time (HRT) is the average time spent by the influent liquid in the aeration tank of the activated sludge process; it is the reciprocal of the dilution rate. (Bitton,1999).

$$\mathbf{HRT} = \frac{\mathbf{1}}{\mathbf{D}} = \frac{\mathbf{V}}{\mathbf{Q}}$$

where V = volume of aeration tank (L)
Q = flow rate of influent wastewater into aeration tank (L/d)
D = dilution rate

d) *Sludge Age or Sludge Residence Time*

Sludge age(R_s) is the mean cell residence time (MCRT) of microorganisms in the system. While the HRT may be in the order of hours, the MCRT may be in the order of days (Bitton,1999). Once mixed liquor has moved to the secondary clarifiers , the sludge will settle and separate from the effluent. A portion of this settled sludge will be wasted from the system whilst the remaining fraction will be returned to the inlet of the biological reactor via the clarifier underflow. The fraction of solids which is wasted will determine the average amount of time which the biomass will occupy the reactor. Sludge age can therefore be defined as the mass of sludge in the reactors (aerated and unaerated) divided by the mass of sludge wasted per day (Lilley *et al* ., 1997)

$$R_s = \frac{V x X}{X_w}$$

where R_s = sludge age (d)
V = reactor volume (L)
X = reactor suspended solids (g/L)
X_w = daily wasted sludge suspended solids (g/L/d)

Sludge age may vary from 5 to 15 days in conventional activated sludge. It varies with season and is higher in the winter than in summer (Bitton, 1999). Although sludge age control is of prime importance in modern plant design(Henze et al., 1995), adequate methods for precisely controlling it are not always implemented.. Modelling activated sludge systems requires that the sludge age be known, since it is considered the fundamental parameter in the design (Orhon and Artan, 1994).

e) *Dissolved Oxygen Concentration*

Oxygen supply and aeration period is a major influence on plant function since any inability to meet the oxygen demands exerted by the aerobically respiring biomass will drop dissolved oxygen (DO) levels to those unable to sustain all aerobic activity including nitrification (Gray, 1990). The design aerobic mass fraction of a plant (the fraction of plant biomass which is aerobic) is usually determined to ensure effective removal of organic compounds, and complete nitrification. If zones of low or zero DO exist within the aeration basin, denitrification may occur. Therefore in most plants the DO levels are constantly measured and monitored with robust DO probes, and remedial action effected if the DO falls to unacceptable levels (Seviour *et al.*, 1999). The Oxygen Utilization Rate (OUR) is the term given to the rate at which microorganisms use oxygen. When measured it is a combination of the oxygen required to oxidize both COD and nitrogen entering a plant as well as the oxygen used by the organisms in endogenous respiration. In laboratory scale plants, it is measured by elevating the dissolved oxygen concentration to about 6 mg O_2/L and then cutting the oxygen supply. The DO concentration is then plotted against time. The slope of the linear plot obtained gives the OUR (Lilley *et al.*, 1997).

f) *Other factors affecting plant performance*

Temperature changes can also be important in determining wastewater characteristics and plant performance especially where nitrification and denitrification are desired (Sollfrank et al., 1992). Growth rates are temperature- dependent and at low temperatures, biological processes can cease or slow down substantially. Temperature also affects O_2 solubility which decreases with an increase in temperature. Therefore at higher temperatures, metabolic rates will increase, but O_2 solubility will decrease, and vice versa and so environmental conditions may vary between flocs at different timesof the day, thus impacting on reaction rates. Increases in temperature have also been associated with increased bulking and foaming problems in some plants (Seviour *et al.*, 1999).

2.6.2.2 Process configurations

A conventional activated sludge process (Fig.2.8) includes :

a)*Aeration Tank*

Aerobic oxidation of organic matter is carried out in this tank. Primary effluent is introduced and mixed with Return Activated Sludge (RAS) to form a mixed liquor, which contains 1 500-2 500 mg/l of suspended solids. Aeration is provided by an air diffuser. An important characteristic of the activated sludge process is the recycling of large proportion of the biomass. This makes the MCRT much greater than the HRT. This practice helps maintain a large number of microorganisms that effectively oxidize organic compounds in a relatively short time. The retention time in the aeration basin varies between 4 and 8 h (Sterritt and Lester, 1998).

b)*Sedimentation Tank*

This tank is used for the sedimentation of microbial flocs (sludge) produced during the oxidation phase in the aeration tank. A portion of the sludge in the clarifier is recycled back to the aeratio basin and the remainder is wasted to maintain the F/M ratio in the system (Bitton, 1999).

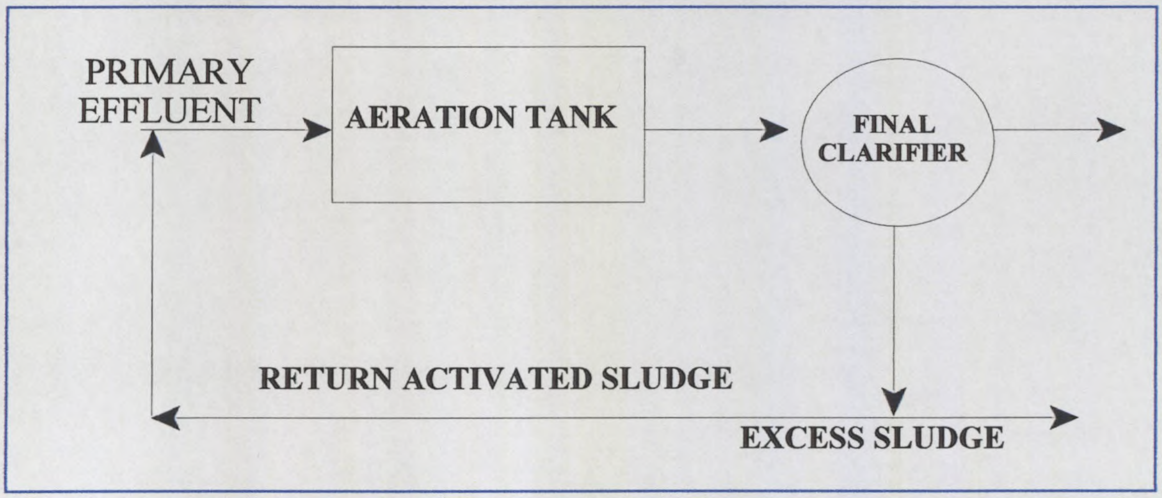


FIGURE 2.8.Conventional Activated Sludge System (Bitton, 1999)

Increasing industrial and population demands on water supplies has necessitated the activated sludge process to undergo various modifications in order to cope with the increase in carbonaceous and nutrient loadings. Single aerobic systems are no longer sufficient to treat domestic and industrial wastes and changes incorporated into the process relate to size, number and configuration of reactors, flow direction and mixed liquor recycle and flow regime within the reactors (Wentzel *et al*., 1992).

However in order to achieve biological nutrient removal of nitrogen and phosphorus, the process must incorporate an anaerobic zone, an anoxic zone and an aerobic zone. In most processes it is usual to have the anaerobic zone first, the anoxic zone second followed by the aerobic zone.

a) *Anaerobic Zone*

This is a zone within the activated sludge process which is virtually free of oxygen and nitrate. This zone is fundamental to the biological removal of phosphorus because it allows the organisms principally responsible for this phenomenon to proliferate in the system.

b) *Anoxic Zone*

This zone is free of oxygen but contains nitrite and nitrate or has substantial input of nitrate. This zone is fundamental to the biological removal of nitrogen because the absence of oxygen allows organisms to utilize nitrate as electron acceptors, reducing it to nitrogen gas, thus promoting denitrification of the mixed liquor.

c) *Aerobic Zone*

This zone is aerated by introducing either air or oxygen. In this environment the utilization of biodegradable organic matter is virtually completed while ammonium nitrogen is converted to nitrate by the nitrifiers present (Lilley *et al.*, 1997).

d) Unaerated Mass Fraction (f_{xt})

This is the fraction of the sludge mass which is in the anaerobic and anoxic zones. Its maximum value is limited by the necessity of having a sufficient aerated sludge mass to allow nitrification to occur. The fraction present in the anaerobic zone is called "anaerobic mass fraction" (f_{xa}) and it affects the overall phosphorus removal potential of the plant. The fraction present in the anoxic zone is known as the "anoxic mass fraction" (f_{xn}) and affects the overall nitrogen removal capacity of the plant. The f_{xt} of a process is the sum of the f_{xa} and f_{xn} (Wentzel *et al.*, 1992; Lilley *et al.*, 1997).

Recycle of flows in activated sludge processes are as follows :

S- recycle : This is the activated sludge recycle from the underflow of the secondary clarifiers back to :

- the anaerobic zone in the Phoredox system,
- the anoxic zone in the UCT or Modified UCT systems downstream of the anaerobic zone,
- the settler anoxic zone in the Johannesburg system.

A-recycle :This is an internal recycle of mixed liquor from the aerobic zone to the inlet of the anoxic zone. Its function is to introduce the nitrate formed in the aerobic zone into the anoxic zone for denitrification.

R- recycle : This is an internal mixed liquor recycle from the anoxic zone to the anaerobic zone and applies to the UCT and modified UCT configurations (Lilley *et al.*, 1997). For a particular configuration, the degree and success of achieving optimum nutrient removal in each zone depends on controlling the different recycle rates to each zone. A number of mainstream biological nutrient removal systems and configurations have been developed both locally and internationally in the past twenty years (Seviour *et al.*, 1999).

A) Nitrogen removal systems

The first system for nitrogen removal was developed by Wuhrman in 1957 and consisted of an aerobic zone followed by an anoxic zone (Seviour *et al.*, 1999). Consequently, substrates for denitrification were supplied mainly from the endogenous death and lysis of active biomass since most of the organic substrates had been utilized in the aerobic reactor. As the rate of substrate release was low, so was the denitrification rate (Toerien *et al.*, 1990).

Subsequently, Ludzack and Ettinger in 1962 changed the configuration by placing the anoxic reactor in front of the aerobic tank and in partial contact with it. These anoxic zones were called 'primary anoxic zones'. Much of the organic content was therefore removed in the organic zone, reducing both the active heterotrophic population in the aerobic zone and the competition for O_2, allowing increased nitrification to occur. Crude mixing of the contents of the anoxic and aerobic zones was provided by the action of aerators and allowed denitrification to occur, but the lack of mixing control gave variable plant performances (Bryan, 1993). Barnard in 1975 (as cited by Seviour *et al.*, 1999) showed that separation of the anoxic and aerobic zones and recycles between the two was necessary to improve plant function, and derived a configuration referred to as the ' Modified Ludzack-Ettinger Process' (Fig.2.9). The recycle from the aerobic zone to the anoxic zone which recycled the nitrate for stripping was introduced. Complete denitrification was not possible and nitrate was discharged with the effluent (Lilley *et al.*, 1997). The 'Bardenpho System' (Fig.2.10.) was subsequently introduced by adding a secondary anoxic reactor after the aerobic reactor, giving a four stage system. Thereby both primary and secondary anoxic zones were now incorporated into one design(Toerien *et al.*, 1990; Randall, 1992). This had a dual effect of increasing denitrification rates and producing a relatively nitrate free effluent. A re-aeration reactor was also incorporated after the secondary anoxic reactor where air stripped of N_2 gas from the sludge was achieved preventing it rising subsequently in the clarifier and permitting nitrification of any NH_3 produced in the secondary anoxic reactor (Toerien et al., 1990). This design configuration, which also often removed some additional phosphorus, can be viewed as the precursor of most of the plants which have since followed (Seviour *et al.*, 1999).

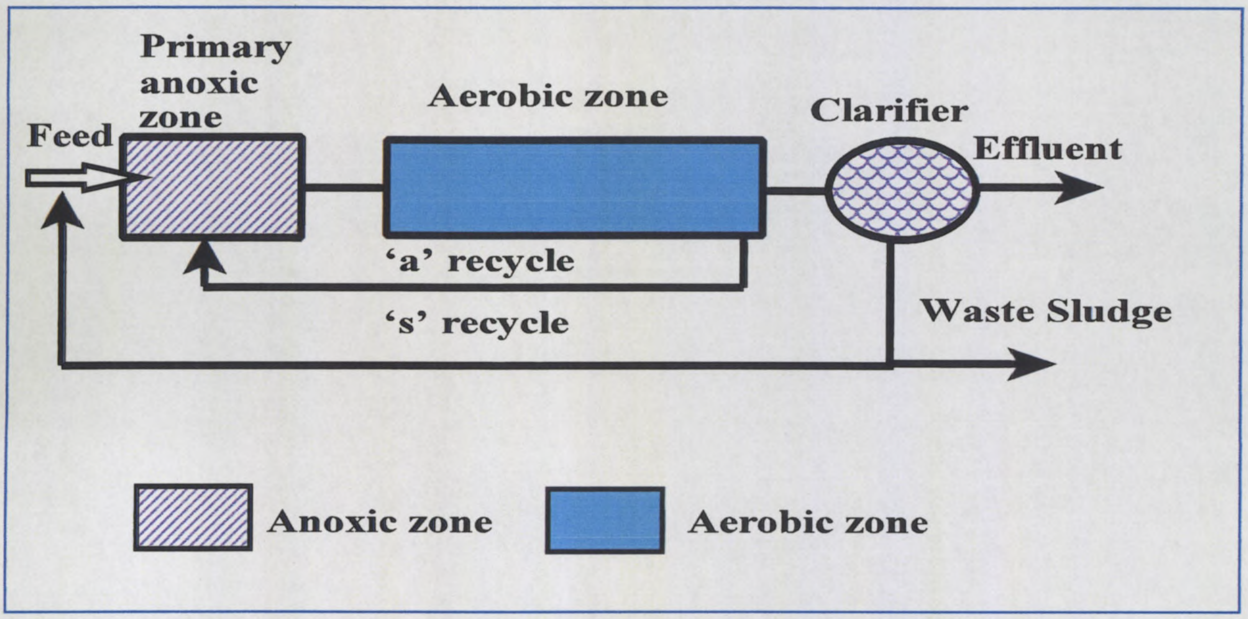


FIGURE 2.9 Modified Ludzack-Ettinger Activated Sludge Process (Adapted from Seviour *et al.*, 1999)

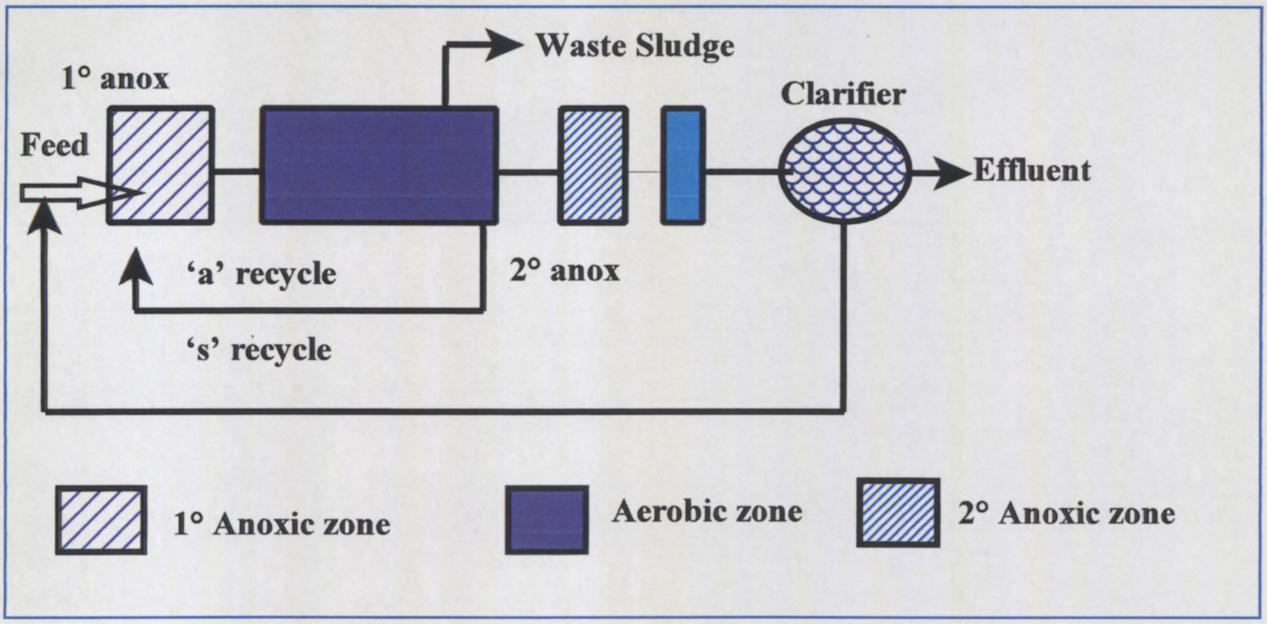


FIGURE 2.10 Bardenpho Activated Sludge Process (Adapted from Seviour *et al.*, 1999)

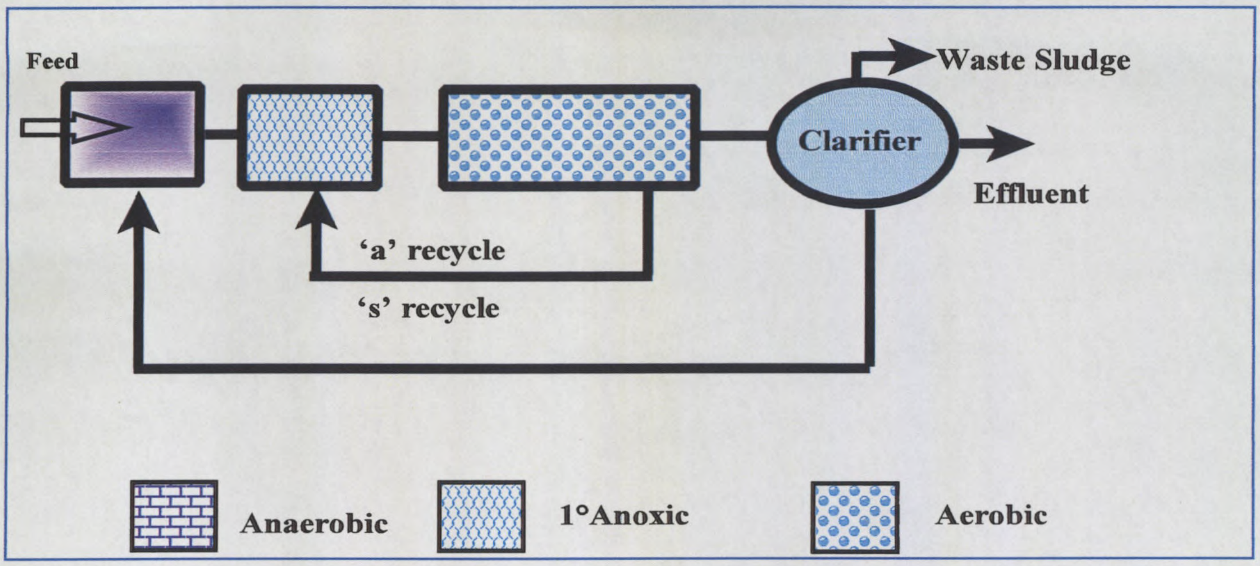


FIGURE 2.11 The Three- Stage Phoredox Process (Adapted from Seviour *et al.*, 1999)

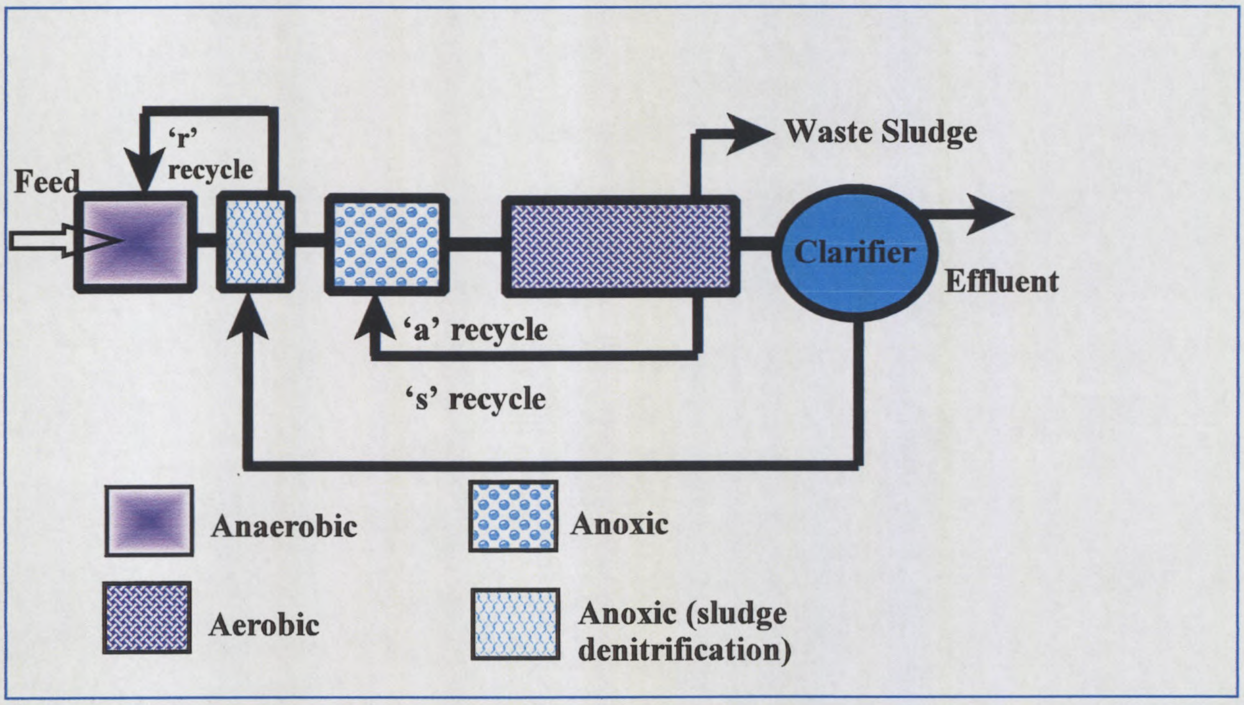


FIGURE 2.12 Modified UCT Activated Sludge Process (Adapted from Seviour *et al.*, 1999)

B) Nitrogen and phosphorus removal systems

Phosphorus removal is ultimately dependent on the creation of an anaerobic zone in the system. The pioneer work of Barnard was crucial in helping to resolve the process requirements for BEPR (Bryan,1993), and modifying the Bardenpho system to develop a five-stage Phoredox process. He found that adding an anaerobic zone to the head of the plant led to reliable phosphate removal. Subsequent simplification of the Phoredox process by increasing the size of the primary anoxic reactor and thus increasing denitrification efficiency and negating the need to have a secondary anoxic and re-aeration reactor gave rise to the three-stage Phoredox Process (Fig. 2.11). This increase in dinitrification led to less nitrates being returned to the head of the plant, and so increased BEPR (Toerien *et al.*, 1990; Bryan,1993).

Barnard in 1983 (as cited by Seviour *et al.*, 1999) suggested further modifications to the Phoredox system to improve plant performance when nitrate is returned in wastewater with otherwise unsuitable characteristics. The 'Johannesburg Process' was subsequently developed from the Phoredox system to overcome the detrimental effects of nitrate on BEPR, by introducing an endogenous sludge denitrification vessel in the return line (Barker and Dold, 1996). High biomass concentrations in the vessel were intended to ensure that denitrification would occur at a respectable rate, but the design increased the anoxic or unaerated biomass to a level which restricted the size of the primary anoxic or anaerobic zones (Barnard, 1994).

A further modification of the basic Phoredox process to avoid the problem of nitrate inhibition of P removal was also developed at the University of Cape Town and is called the ' UCT Process' (Wentzel *et al.*, 1991). In this system, the RAS recycle passes into the anoxic rather than the anaerobic reactor, and a mixed liquor recycle runs from the anoxic reactor to the anaerobic reactor (r-recycle). This configuration was attempting to ensure that the nitrates in the RAS is denitrified in the anoxic reactor before reaching the anoxic zone (Toerien *et al.*, 1990; Danesh and Oleszkiewicz, 1997). A later development, which was called the ' Modified UCT Process' (Fig. 2.12) divided the single anoxic zone into two compartments, allowing for improved individual control of the mixed liquor and RAS streams (Wentzel *et al.*, 1991; Bryan, 1993), and such plants are operating globally. This configuration generally uses long (> 20day) sludge ages, which sometimes results in problems with filamentous bulking (Wentzel *et al.*, 1991).

The above are the more common nutrient removal systems used globally. However, there are various other modifications and adaptations, many of which are patented and beyond the scope of this investigation.

2.7. MICROBIAL COMMUNITY ANALYSIS OF ACTIVATED SLUDGE PROCESSES

The activated sludge treatment of sewage is probably today's most important biotechnological process. In the past decades great progress has been achieved in process engineering, but our current knowledge on the structures and dynamics of the involved microbial communities, and consequently our understanding of the microbiology of the activated sludge process, is still very limited. Although it is generally believed that the microbial community of activated sludge systems is highly diverse and specialized (Amann *et al.*, 1996), it is difficult in the absence of persuasive data, to decide what is meant by a normal microbial community, or if in fact it exists. Microbiologist have been faced with methodological problems which have hindered attempts to understand the microbial ecology of these complex systems (Amann *et al.*, 1996; Mudaly *et al.*, 2000).

2.7.1 Conventional cultivation dependent techniques

Traditional isolation techniques employed by microbiologist to isolate, cultivate and identify microbial consortia was restricted to culture- dependent methods such as viable plate counts or most probable number techniques (Pike *et al.*, 1972; Banks and Walker, 1977). However, for their inevitable selectivity towards certain organisms these methods represent no adequate means of addressing this question. There is no single medium which will support the growth of all organisms present in any activated sludge plant (Wagner *et al.*, 1993). Standard plate counts reflect not so much the actual bacterial community structure of the activated sludge but rather the selectivity of the growth media for certain bacteria. For oligotrophic to mesotrophic aquatic habitats, direct microscopic counts can exceed viable cell counts by 2-4 orders of magnitude. In eutrophic water this effect is less pronounced, but recoveries from activated sludge even with optimized media are only between 0.85% and 14% (Wagner *et al.*, 1993; Kampfer *et al.*, 1995).

Currently there is no doubt that in most cases most of the microscopically visible cells are viable, but do not form visible colonies on plates. In activated sludge this is mainly caused by :

- clumping of the cells in the activated sludge flocs prevents a quantitative release of individual bacteria and consequently leads to an underestimation of the number of active cells determined by viable plate count techniques,
- the cultivation conditions are not suitable for all bacteria; this might apply both to common strains and to hitherto uncultured bacteria (Snaidr *et al.*, 1997).

Even if we can isolate and culture these populations, such information often tells little or nothing about their activity and function within the activated sludge plants. The increasing reliance on rapid commercial identification systems like API or Biolog for isolates can often provide an incorrect identification as these systems have been optimized mainly for clinical isolates for which they were originally designed (Knight *et al.*,1995).

2.7.2 Alternative techniques

Therefore, due to the biases associated with cultivation dependent methods, focus was targeted to developing suitable alternate technology for microbial community analysis of environmental samples. Hiraishi, (1988) utilized bacterial respiratory quinone profiles to monitor different bacterial populations in activated sludge. However studies applying a chemotaxonomic approach have had limited success due to intrinsic limitations since given the complexity of activated sludge communities, it is difficult to translate relative numbers of this biomarker into cell numbers. Another technique that was investigated included the immunofluorescence approach and facilitated identification and enumeration at the cellular level (Cloete and Steyn, 1987).However, some of the limitations of this technique include :

- Inhibition by extracellular polymeric substances in sludge which hinder antibody penetration,
- non-specific binding of antibodies to detritus particles and fungal spores results in high levels of background fluorescence,
- generation of antibodies requires a pure culture of the organism and not all bacteria in activated sludge have been cultured. Therefore the method is dependent on culture dependent technique

However it should be stressed that owing its high specificity, immunofluorescence will remain a valid and powerful method for conducting community analysis in activated sludge processes (Hiraishi *et* *al.*, 1998).

Jorgensen *et al.* (1992), attempted to estimate total biomass activity of activated sludge using its ATP content and respirometry with O_2 uptake rates, and measured biomass viability by its ability to hydrolyse fluorescein diacetate (FDA). Results suggested that the first two methods may be useful for sludges , but FDA hydrolysis correlate very poorly with other parameters they measured. However, application of enzyme systems as indicators of biomass activity have had some success using dehydrogenase activity as determined by tetrazolium salt reduction method. This method showed promising results when conducting viability determinations of filamentous bacteria in foams (Nybroe *et al.*, 1992).

In order to understand the functions of different microorganisms in activated sludge, a technique called Microautoradiography (MAR) was developed and could be applied to study the physiology of these organisms in situ. It is a technique that can be used to study the microscale distribution of radio-labeled compounds in a specimen. The compound appears in the cell as a result of adsorption of a tracer or by uptake of a labeled substrate. After appropriate sample handling, the radio-labeled sample is placed in contact with a layer of radio-sensitive emulsion. The emissions from the radioactive sample interact with silver bromide crystals suspended in the emulsion during an exposure time ranging from hours to weeks. When the emulsion is developed using standard photographic procedures the silver grains appear on the top of the radioactive structure , which can be viewed clearly microscopically. The main problem of using MAR has been the lack of proper identification of the microorganisms. However this problem has been overcome by the development of a technique that allows to perform a simultaneous detection of the activity of certain microorganisms by MAR and identification of the microorganisms by FISH on the same sample. The functional groups characterized by MAR can be identified to the extent that gene probes (typically 16S or 23S rRNA oligonucleotide -targeted probes) are available and include group, genus or species level. The combined method allows researchers to identify unknown bacteria that facilitate processes such as BEPR by conducting DNA extractions, PCR, clone libraries and developing new probes that can be targeted against unknown active microorganisms. However, this method necessitates extensive experience in using MAR and FISH, and if possible, access to a confocal scanning laser microscope (Nielsen *et al.*, 1998).

2.7.3. Molecular methods for systems analysis

2.7.3.1 DNA - based techniques

There are a variety of formats for r-DNA based analysis of community structure. A common approach is based on selective recovery of total community 16S rDNA sequences. This is accomplished using the Polymerase Chain Reaction (PCR) and primers complimentary to universally conserved regions of 16S rRNA gene to selectively amplify the rRNA gene sequences prior to cloning them in an appropriate plasmid vector (MacGregor *et al.*, 1997). This serves to generate a clonal library of system population diversity, each population represented by a unique 16S rRNA- containing plasmid clone. Sequencing and/or genetic "fingerprinting" analysis based on Restriction Fragment Length Polymorphism (RFLP) of clones can then be used to sort out the library and estimate the total number of unique 16S rDNA's. This also provides a rough estimate of abundance. Comparative sequencing of the library provides precise identification of individual populations and the foundation for developing complementary DNA-based tools (MacGregor *et al.*, 1997).

In addition, several fingerprinting methods have been developed for rapid cross- community comparisons of population complexity and similarity. One of these techniques is Denaturing Gradient Gel Electrophoresis (DGGE) that separates double-stranded DNA fragments of identical length according to the melting property of each unique DNA fragment. A population of homologous DNA fragments is amplified from the total community DNA using PCR primers that flank a region of the within a conserved gene. The primers are designed to hybridize to all members of the target population, which may be very general, (e.g., all life or domain level)or more specific (e.g., genus or species level). The amplified DNA is most commonly a region of the 16S or 23S rRNA (ca. 500 nucleotides in length). One of the primers is extended at the 5' terminus with a long stretch of GC nucleotides (the GC clamp) to prevent complete denaturation of the amplified DNA fragment during electrophoresis. The amplificates is separated by electrophoresis at a constant elevated temperature in a acrylamide gel containing a linear ascending gradient of denaturants (urea and formamide) (Wawer and Muyzer, 1995). Due to the differences in nucleotide sequence , individual DNA fragments exhibit different electrophoretic mobilities resulting from the transition between duplex structure and a partially denatured form in the gel. Usually a resolvable band is defined as a unique 'ribotype', which represents bacterial sequences with the same electrophoretic mobility. The number of bands resolved is considered a rough estimate of ribotype diversity within the community, and the intensity of each band reflects the abundance of the individual ribotype. Unfortunately, these interpretations may not be true for bacteria containing multiple copies of rRNA operon (Nubel *et al.*, 1996). However, the DGGE banding pattern can be used as a community signature of an environmental sample.

Another fingerprinting technique examines the Terminal RFLP (T-RFLP) of PCR- amplified 16Sr DNA's. In this application, one of the two primers is fluorescently -labeled at the 5' end. The amplified rDNA products are then digested with restriction enzymes having four- base recognition sites, and analyzed using an automated DNA sequencer. Since the rDNA amplification products are tagged with a fluorescent dye at only one terminus, following enzyme digestion only the terminal fragment derived from each sequence population will be detected. Comparative information provided by each T-RFLP pattern includes fragment length and restriction site sequence (i.e.ribotype), and peak area (i.e.abundance). Computer analysis of the T-RFLP for 686 amplifiable sequences from 1102 16S rRNA sequences of the Ribosomal Database Project (RDP) indicated that those sequences could be classified into233 different terminal restriction fragments (ribotypes) (Lui *et al.*, 1997). Thus T-RFLP is a sensitive and rapid way to assess the microbial diversity (the total number of terminal fragments) and obtain a distinctive fingerprint for each microbial community.

However, the aforementioned DNA based techniques should be considered an approximate assessment of community structure. This is because they introduce certain biases in population representation. For e.g., the recovery of an individual population (as represented by a unique 16S rRNA sequence) may vary with population -specific variations in lysis/DNA extraction efficiency, PCR amplification, cloning bias, and differences in16S rRNA cellular gene copy number (Hiraishi *et al.*, 1998; Lui *et al.*, 1998).

Another emerging technology included the use of the 'DNA Microchip' which has shown promising results for studies in microbial physiology, ecology and determinative microbiology. It provides several advantages over conventional hybridization techniques. The chip allows for simultaneous hybridization to a large set of hundreds or even thousands of probes complementary to the small or large subunit rRNA's. This is possible because it utilizes a high-density micro-array, consisting of a matrix of thousand's of individual gel elements bound to the surface of a glass slide. Each element (ca. 5-100 microns on a slide) contains a DNA probe complementary to a target sequence. The chip is a powerful tool that can be applied to monitor the microbial diversity and dynamics of natural or engineered environments at extremely high population and temporal resolution (Guschin *et al.*, 1997).

2.7.3.2 Ribosomal RNA based techniques

The advent of rRNA approach to microbial community analysis has created a new dimension in activated sludge population dynamics. Rapid nucleic acid sequencing is based on nucleotide sequence comparisons of ribosomal RNA's (rRNAs) or their genes extracted from naturally occurring biomass (Olsen *et al.*, 1986). This has made it possible to group microorganisms according to their evolutionary relationships, commonly referred to as a ' phylogenetic classification system'. Since, molecules and not organisms are isolated, the method is not limited to species that are amenable to laboratory cultivation. Bacterial ribosomes are particles with sedimentation coefficients of 70S. They are composed of two subunits , a 30S and a 50S particle, each of which is a complex of specific ribosomal RNA and proteins. The 50S subunit contains two rRNA molecules-a 23S rRNA and a 5S rRNA (Mathews and van Holde, 1990).

The r RNA- based methods significantly reduce or eliminate many of the biases associated with DNA based methods (Amann *et al.*, 1995; Stahl, 1995). This is because more disruptive techniques can be used to recover the RNA (unlike DNA, the rRNA is not subject to mechanical shear) and hybridization provides for a more direct measure of active microbiota abundance. The use of group and species specific DNA probes complementary to the rRNA is now well established in several different analytical formats (Stahl,1995). The most common formats employ membrane supports to immobilize total rRNA extracted from the sample or to retain the rRNA within individual cells using a fixation step prior to hybridization (Stahl and Amann, 1991). Population- specific rRNA abundance is then determined using hybridization of labeled probes (either radioactively or fluorescent- dye labeled) to the membrane- immobilized rRNA or to fixed cells. Individual populations are quantified by measuring the amount of radioactive probe bound to the membrane support or using microscopy and image analysis to count individual cells to which hybridization has conferred fluorescence (FISH) (Amann *et al.*, 1990b).

Another key advantage of the oligonucleotide probe- based method is the ability to construct probes of varying levels of specificity(Stahl and Amann, 1991; Amann *et al.*, 1995). When viewed as a linear target (ca. 1 500 nucleotides) for probe binding to different regions(ca. 15-25 nucleotides), the 16S rRNA presents a mosaic of both conserved and variable target regions. The most highly conserved regions are nearly invariant among all life forms, whereas the most variable regions often provide discrimination between species. A variety of such probe sets have been developed and applied to the study of natural and engineered systems. These include species, genus, family and order specific probes for nitrifiers, methanogens and sulphate- reducing bacteria (Raskin *et al.*, 1994; Mobarry *et al.*, 1996). A useful feature of this probe design strategy is the creation of a hierarchical set of probes. A hierarchical probe set provides for analysis validation. For example, if a more general probe fully represents the larger phylogenetic group, then the sum of the specific probe hybridization values should equal that obtained using the more general probe. Although the hierarchical probe approach has provided for the analysis of complex systems at varying levels of resolution, and for essential consistency checks, its use has yet to be fully realized(Lui *et al.*, 1998).

Another common technique that is used is the 'Quantitative dot-blot hybridization'. A quantification of a certain 16S rRNA compared with total 16S rRNA can be obtained by dot-blot hybridizations of a directly isolated nucleic acid mixture with universal and specific oligonucleotide probes. The relative abundance is calculated by dividing the amount of specific probe bound to a given sample by the amount of hybridized universal probe. However, it should be remembered as for chemotaxonomic markers, relative rRNA abundance cannot be directly translated into cell numbers, since cells of different species vary in their ribosomal content ranging from 10^3 to 10^5 ribosomes per cell. The relative rRNA abundance should be a good indication of relative physiological activity of the respective population (Stahl, 1995).

2.7.3.3 Fluorescent *in situ* hybridization

The use of hybridization *in situ* for counting and identifying organisms was proposed by Olsen *et al.*(1986). As demonstrated by the immunofluorescence approach, fluorescent probes yield superb spatial resolution and can be detected instantaneously by epifluorescence microscopy. Fluorescently labeled rRNA targeted oligonucleotide probes (usually 15-30 nucleotides in length) have been used successfully for rapid identification of bacteria (Amann *et al.*, 1995) and therefore a suitable tool for determinative, phylogenetic and environmental studies in microbiology. The probes are able to enter fixed bacterial cells and once inside they form stable associations (hybrids via hydrogen bonding between complementary nucleotides) with the 16S rRNA in the ribosomes. If the complementary sequence for the probe is not present in the ribosome, stable hybridization does not occur and the probe is washed from the bacterial cell. Thus the 'targets' for the oligonucleotide probe are the ribosomes of which there are up to 10^4 per actively growing bacterial cell. In order to observe when hybridization occurs, the probes also contain a 'reporter' molecule, which is referred to as a 'fluorochrome'. Cells in which the fluorescently labeled probe has hybridized with the rRNA in the ribosome can be directly visualized by epifluorescent microscopy. The technique is called FISH or 'whole cell probing' (Amann *et al.*, 1995; Lindrea *et al.*, 1999). The fundamental steps when applying FISH technology include :

- Designing and synthesizing the probe with the fluorochromes
- sample fixation to improve cell permeability
- teflon coat microscopic slides and attach sample
- hybridization of the probe with the target nucleic acid under optimized conditions
- view under epifluorescent microscope and could use image analysis to count cells (discussed in detail later).

FISH or whole cell probing is one most widely applied techniques when conducting microbial community analysis of environmental samples, especially activated sludge (Lindrea *et al.*, 1999).

2.7.4 The phylogenetic classification system

The fact that DNA is the expressive machinery through which natural selection, and therefore evolution operates implies that it is a valuable tool for mapping evolutionary relationships between microorganisms. Phylogeny thereby seeks to place bacterial taxonomy on a foundation of natural classification. Genotypic information i.e., sequence information, is superior to phenotypic information for classifying and relating organisms. The advantages of sequence information include

- more readily, reliably and precisely interpreted, and
- innately more informative of evolutionary relationships than phenotypic information.

The elements of a genetic sequence are also well defined and are restricted in number. The description of two organisms as being similar is based upon mathematically defined relationships making the determination more objective (Woese,1987). In addition, sequence data can be readily accumulated creating a " data base" which can be referred to for phylogenetic analysis as new sequence information becomes available (Olsen *et al.*, 1986).

2.7.4.1 Ribosomal RNA- the perfect molecular chronometer

A molecule whose sequence changes randomly in time can be termed a chronometer. All sequences are not of equal value in determining phylogenetic relationships and rRNA is presently the most useful molecular chronometer. A good molecular chronometer should have the following characteristics:

- they should have a random clockwise behavior,
- they should occur in all organisms and different positions in their sequences should change at different rates making their evolutionary range all encompassing, and
- they should have a large size made up of many functional domains which are evolutionary separate from one another (Woese,1987).

The most compelling reason for using rRNA as a chronometer is that they can be sequenced directly(and therefore, rapidly) by means of the enzyme reverse transcriptase. Since the genetic sequences dealt with exist in only their present state (past states cannot be known directly), the biologist cannot measure this change by comparison of some original to some final state. However, two different versions of the same molecule (from two different organisms) have at some past time shared a common ancestor. The difference between two extant sequences can be expressed as evolutionary time by measuring the sequence distance between them and assuming a relative rate of change for each lineage (Woese,1987, 1990).

2.7.4.2 The universal phylogenetic tree

Woese (1987) and Woese *et al.*, (1990) carried out a large comparative analysis of rRNA sequences, to create a phylogeny or natural division of all living organisms on earth. Three 'Domains' or kingdoms were determined representing the three main evolutionary lines and referred to as Bacteria, Archaea and Eukarya (Fig.2.13). A matrix of evolutionary distances was calculated from an alignment of representative 16S rRNA sequences from each of the three kingdoms. This was used to construct a distance tree, based upon those positions represented in all sequences in the alignment in homologous secondary structural elements. Line lengths on the three are proportional to calculated distances (Woese,1987).` Of special importance here is that the organisms with prokaryotic cells arise from two quite separate evolutionary pasts, an idea that never emerged in the earlier studies on bacteria (Woese, 1987; Pace, 1997)

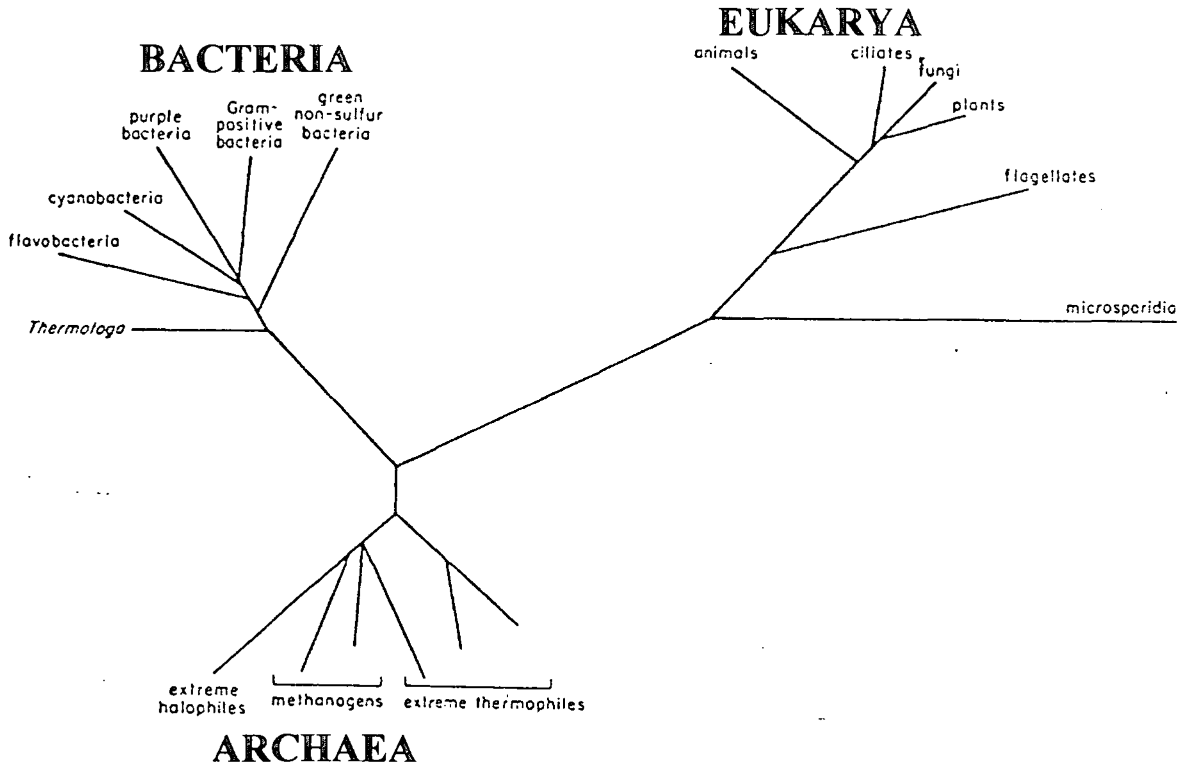


FIGURE 2.13 Universal phylogenetic tree showing the three domains (Woese, 1987)

The bacterial domain is subdivided based on their rRNA sequences into 'phyla'. These phyla are further divided into groups, and eventually into genera and species, and these groupings are in almost all cases quite different to the 'classical' classification of prokaryotes (Olsen *et al.*, 1986).

Microbiologist are currently trying to reconcile the phylogenetic and determinative information for these organisms.

In the past decade the number of identifiable bacterial divisions has more than tripled to about 40 due in significant part to culture-independent phylogenetic surveys of environmental microbial communities. Figure 2.14 represents the division-level diversity of the bacterial domain as inferred from representatives of the approximately 8000 bacterial 16S rRNA gene sequences currently available. Although 36 divisions are shown, several other division level lineages are indicated by single environmental sequences, suggesting that the number of bacterial divisions may well be over 40 (Hugenholtz *et al.*, 1998). The majority of the bacterial divisions however, are poorly represented by cultured organisms.

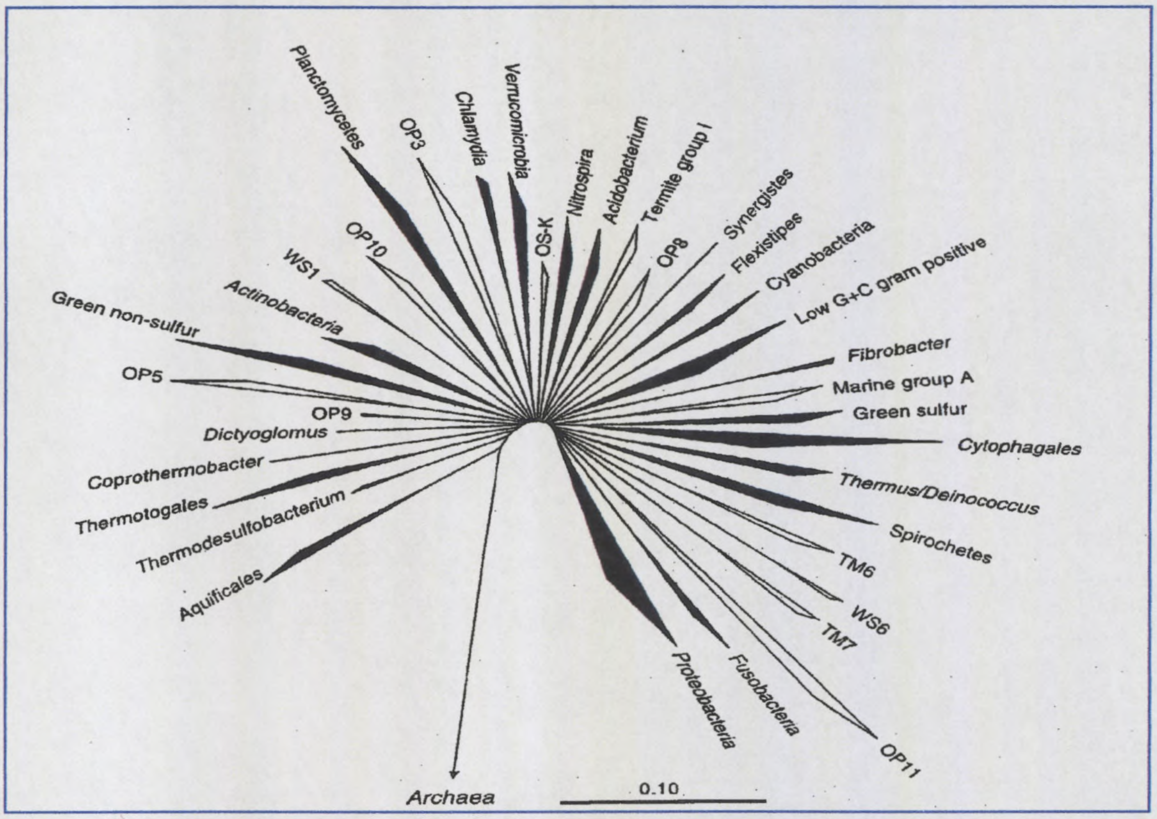


FIGURE 2.14 Evolutionary distance tree of bacterial domain showing currently recognized divisions. The scale bar indicates 0.1 change per nucleotide (Hugenholtz *et al*, 1998)

Division- level groupings of two or more sequences are depicted as wedges. The depth of the wedge reflects the branching depth of the representatives selected for a particular division. Divisions which have cultivated representatives are shown in black. Divisions represented only by environmental sequences are shown in the outline (Fig. 2.14).

2.7.4.3 Phylogenetic approach to identifying bacteria in activated sludge

With the advent of the novel molecular techniques, including the rRNA approach, microbial community analysis of activated sludge systems has been greatly enhanced. Amann *et al.* (1990a) by comparative analysis of 250 complete and partial 16S rRNA sequences were able to design an oligonucleotide specific for members of the Kingdom *Bacteria* (positions 338-355, according to *E. coli* 16S rRNA numbering) referred to as probe 'EUB 338'. Subsequent investigations in activated sludge showed the kingdom *Bacteria* to be predominant with the percentage EUB to be approximately 79% (Mino *et al.*, 1998; Mudaly *et al.*, 2000). Within the class *Proteobacteria,* the alpha, beta and gamma sub-classes predominated and within the class *Firmicute* (Gram-positive bacteria) the *Actinobacteria* sub-class (Gram positive bacteria with high G+C DNA content)(Bond *et al.*, 1999)

A) The *Proteobacteria*

The ancestral phenotype of the phylum is undoubtedly (purple) photosynthetic, which justifies the alternate name i.e. purple bacteria. Photosynthetic capacity has been lost many times in this phylum, resulting in various non-photosynthetic sublines. These 'purple bacteria' contain most but not all of the traditional gram- negative bacteria. However, the arrangement of classically defined families, genera and even species within this phylum is haphazard. Photosynthetic species are grouped with non-photosynthetic ones; heterotrophs are paired with chemolithotrophs; anaerobes are paired with aerobes, etc (Woese, 1987). This group as a whole, however, make up one of the few phyla that cannot be defined by a simple signature. *Proteobacteria* are subdivided into four subclasses i.e. alpha, beta, gamma and delta subclasses. Two helices in the 16S rRNA (positions 184 to 193 and 198 to 219 *E. coli* numbers , help to define and distinguish the four subdivisions (Woese,1987). These subclasses comprise many of the well known culturable wastewater bacteria :

- among others the alpha subclass encompasses *Caulobacter* spp., *Nitrobacter* spp and some

Zoogloea spp.

- members of the genera *Alcaligenes* and *Comamonas, Sphaerotilus* spp., some *Zoogloea* spp. and most of the autotrophic nitrifiers are members of the beta subclass
- among others, *Acinetobacter* spp., *Aeromonas* spp, Enterobacteriaceae, fluorescent pseudomonads and *Vibrio* spp. belong to the gamma subclass
- the delta subclass encompasses sulphate reducing bacteria and myxobacteria (Woese,1987; Snaidr *et al.*, 1997; Hugenholtz *et al.*, 1998).

Probing activated sludge with fluorescent derivatives of these oligonucleotides have revealed that the microbial consortia of various municipal treatment plants are dominated by Proteobacteria (Wagner *et al.*, 1993). According to Wagner *et al.* (1993) and Manz *et al.* (1994), members of the beta subclass play the major role of the microbial consortia in municipal activated sludge and bacteria of the alpha and gamma subclasses are less abundant.

B) Other important groups : ' Flavobacter-Bacteroides' phylum, Gram- positives with a high G+C DNA content.

Cytophagae and flavobacteria have been isolated regularly from biological sewage treatment facilities (Manz *et al.*, 1994). The common gram-negative filamentous bacterium *Haliscomenobacter hydrossis* frequently encountered in activated sludge is a member of this phylum. Cultivation of bacteria belonging to the 'Flavobacter-Bacteroides' phylum from activated sludge can require from 10 to 24 days. For monitoring this phylum *in situ*, Manz *et al.* (1994) developed a 16S rRNA-targeted oligonucleotide probe (CF) specific for many species of the genera *Cytophaga, Flavobacterium and Saprospira.* Probing *in situ* of activated sludge originating from two municipal sewage treatment plants revealed that 8-23 % of all bacterial cells could be detected simultaneously with probe CF (Wagner *et al.*, 1994b; Manz *et al.*, 1994). The microbial community of a dairy wastewater plant was shown to be even numerically dominated by spindle-shaped bacteria belonging to the above phylum (Manz *et al.*, 1994). In these studies bacterial cells hybridizing with probe CF have been most frequently detected in the cores of the flocs.

Molecular taxonomists found significant rRNA homologies between actinomycetes and a group of related bacteria, all characterized by Gram-positive staining and a high G+C DNA content, and consequently created a group named 'actinomycetes branch' or "Gram-positives with high G+C content of DNA.' (Woese,1987). Many species in this phylogenetic group have biotechnological or medical importance. Rapid and reliable monitoring of Gram positive bacteria with a high G+C content in activated sludge is necessary because representatives of the 'actinomycetes branch' have frequently been ascribed an important role in sewage purification processes. For instance, actinomycetes have been connected with malfunctions such as foaming (Seviour *et al.*, 1999). Roller *et al.* (1994) constructed a 23S r RNA-targeted group -specific oligonucleotide probe HGC for this important taxon. Approximately 25% of all bacteria in an activated sludge sample taken from a plant treating dairy waste could be specifically visualized using probe HGC and the FISH technique. In the same study only 9% of all bacteria in an activated sludge sample originating from a municipal sewage treatment plant with chemical phosphate precipitation were identified as Gram positive bacteria with a high G+C DNA content (Manz *et al.*, 1994). Full scale BEPR process showed a high abundance of members of the 'actinomycetes branch' using group-specific probe HGC. This was not possible using standard plating techniques (Wagner *et al.*, 1994b).

2.7.4.4 The EUB/DAPI ratio

The applicability of fluorescent oligonucleotide probing to activated sludge samples can be tested with the Bacteria -specific probe EUB. Dual staining of activated sludge samples with probe EUB and the DNA intercalating dye 4,6 diamidino-2-phenylindole (DAPI) (Hicks *et al.*, 1992) reveals that in all sewage treatment plants hitherto examined, most of the microbial cells revealed by DAPI also emitted probe-conferred fluorescence. When excited with light at a wavelength of 365 nm, the DNA-DAPI complex fluoresces bright blue, while unbound DAPI and DAPI bound to non-DNA material may fluoresce a weak yellow. In order to provide a dark background for fluorescing bacteria, nucleopore filters are stained with Sudan black for 12 hrs prior to filtration (Porter and Feig, 1980). They found that the minimum period required to expose the sample to DAPI for effective visualization and counting of cells was 5 min.

The fact that *ca.* 80% of total cells hybridized with the bacterial probe proved that the bulk of cells present in activated sludge samples were bacteria that had sufficient rRNA for detection and were permeabilized for oligonucleotide probes by the standard fixation procedures. The 'high EUB/DAPI ratio' indicated that most bacteria in activated sludge are metabolically active (Wagner *et al.*, 1993;Wagner *et al.*, 1994b; Manz *et al.*, 1994). Bacteria stained with DAPI that do not hybridize with the probe EUB are either metabolically inactive or not permeable for oligonucleotide probes by standard fixation procedures (Wagner *et al.*, 1993). Researchers conducted a comparison of the EUB/DAPI ratio with total viable counts on different media for an activated sludge sample from a sewage treatment plant in Munchen, Germany. Results showed that 76 % of all bacteria were detected by hybridization *in situ* with probe EUB, whereas only 3-19 % of total bacteria can be cultivated even on optimized media (Manz *et al.*, 1994).

Therefore, in contrast with cultivation-dependent techniques, *in situ* hybridization has shown the potential to obtain a more complete view of the diversity and dynamics of the microbial consortia involved in the activated sludge process.

2.8 CONCLUSIONS FROM LITERATURE REVIEW

South Africa is a water scarce country and a concerted effort will have to be made on the path of all the role players to protect this valuable resource. This responsibility does not lie solely with government, but also with industry to 'clean up their act' and discharge effluent that subscribe to municipal discharge standards. With regards to the latter, much effort is placed on environmental biotechnology as a science that uses naturally occurring microorganisms to remove contaminants from the environment. It involves the integration of molecular life sciences and engineering sciences that complement each other and focus on providing a cleaner environment.

Edible oil industries generate large volumes of effluent during the refinery process. Conventional effluent treatment technology has its limitation with regards to providing good quality final effluent. Biological treatment processes such as activated sludge have shown potential to treat such effluents. In order to fully understand degradation pathways of microorganisms involved in activated sludge and further optimize such processes it is essential to have an accurate representation of the microorganisms present. Conventional microbiological culturing techniques have their limitations and do not provide a true reflection of the microbes present. Novel molecular techniques such as FISH and DGGE have overcome this limitation, and now provide biotechnologist with tools required to obtain an accurate representation of the microorganisms present, and thereby further optimize these engineered processes.

CHAPTER THREE

DEVELOPMENT OF PRETREATMENT TECHNOLOGY FOR EDIBLE OIL EFFLUENT

3.1 INTRODUCTION

Among the variety of physical, chemical and biological processes developed for water treatment, each has inherent limitations in applicability, effectiveness and cost. Physical processes such as precipitation, adsorption or air stripping, for example, transfer pollutants from the aqueous to a second phase, but the pollutant is not destroyed. Chemical oxidation may be slow to moderate in rate and selective, or rapid and non- selective, thus generating appreciable reactor or oxidant cost. Aerobic biological treatment is limited when the feed is either recalcitrant to biodegradation or inhibitory or toxic to the bio-culture. Effective treatment of a particular wastewater, given these limitations, may require a combination of available processes in such a way as to exploit their individual strengths and thus attain the desired water characteristics (Scott and Ollis, 1995).

Combination of chemical and biological treatment is often the way to optimize the overall process (Andreozzi *et al.*, 1998). Four wastewater contaminant types are identified which can benefit from combined processes and these include :

- recalcitrant compounds
- biodegradable wastes with small amount of recalcitrant compounds
- inhibitory compounds, and
- formation of dead-end biological or chemical products

The design key for such two step systems lies in choosing processes that complement each other and lead to a synergistic effect (Scott and Ollis, 1995).

Primary pollutants from an edible oil refinery include fats, oils and greases(Steffen *et al.*, 1989).

These are large macromolecules or recalcitrant compounds that are not easily biodegradable. These recalcitrant compounds include substances such as polyphenols and lipids (Beccari *et al.*, 1998). Physico-chemical methods can be employed to break these compounds to smaller, biodegradable fractions (Scott and Ollis, 1995). Emulsified lipids from the major fraction of the fats, oils and greases wastewater parameter. Failure of activated sludge process under lipid overloading is caused by the adverse effect of lipids on sludge settleability (Hrudey, 1982). Therefore, these industrial effluents have to be pre-treated prior to being subjected to biological treatment.

Pretreatment may be defined as a process or processes that prepares a wastewater to a condition so that it can be further treated in a conventional secondary biological process (Kiely, 1997). Some of the common pretreatment technologies applied in the edible oil industry include physical and chemical processes. Chemical pretreatment as wet air oxidation and ozonation have also been considered for the abatement of toxic compounds. Wet air oxidation with the addition of H_2O_2 was used for the integrated treatment of OME and showed a marked decrease of COD (Chachouk *et al.*, 1994). Ozonation is usually adopted for water disinfection but it also has a high potential as a pretreatment method. The characteristic of ozone is that it is rather selective towards double bonds and targets the double bonds of unsaturated fatty acids, thereby increasing biodegradability of the effluent (Andreozzi *et al.*, 1998).

Coagulation and flocculation processes involve the elimination of suspended colloidal particles from a liquid. This is caused by the addition of chemicals that destroy electrical charges and induce aggregation of these particles. Some of the common coagulants used include Aluminum based coagulants such as aluminum sulphate, Ferric coagulants such as ferric chloride/sulphate or organic polymers. Flocculation is the particle transport phase, which promotes the interaction of particles to agglomerate into large units that can settle readily and be removed. Flocculation is aided by stirring the coagulant. (Letterman *et al.*, 1999). Current wastewater treatment processes in the edible oil industry include the application of coagulants followed by the physical process of Dissolved Air Flotation (DAF). However, the final effluent generated from these processes are still high in COD and FOG and do not subscribe to municipal discharge regulations and therefore have to be treated biologically (Eroglu *et al.*, 1990).

The aim of this aspect of the research was to investigate various pretreatment technologies that could be applied for the primary treatment of edible oil effluent, optimize conditions and select the ideal technology that could be used to prepare the effluent for biological treatment.

3.2 MATERIALS AND METHODS

The process of refining crude oil fluctuates amongst the various companies in the edible oil industry and this impacts on the effluent chemical characteristics. Therefore, for the purpose of this investigation, a sample industry was selected from the Pietermaritzburg region on Kwazulu-Natal that refines sunflower seed oil. The overall layout of the plant is explained in APPENDIX 1. Due to a confidentiality agreement, the company will be referred to as X edible oil industry.

3.2.1 Effluent collection

The site of effluent collection was located at a drain at the end of the refinery process and hence the effluent was untreated. Composite samples were collected in 25 L (22) containers and transported to the laboratory for further processing. On receipt, the COD was measured using a photometer (Nova 60) from Merck (APPENDIX 2) and FOG using standard methods (Standard Methods, 1989) and the effluent stored in the cold room (4^0 C)to prevent any biological activity, until required.

3.2.2 Coagulation and Dissolved Air Flotation

The common coagulants that were assessed to pre-treat the raw edible oil effluent included Aluminium sulphate (alum) and ferric chloride. The coagulants were prepared as per method outlined by Pryor and Freese (1998) (APPENDIX 3). Both alum and ferric chloride were tested individually using the following method :

- A conical flask containing 800 mL of pH adjusted effluent (pH 7.5), was placed on a magnetic stirrer and the sample stirred at 300 rpm.
- The coagulant was added at a concentration of 10 ppm (previously optimized) and stirred at 300 rpm for two minutes.
- The stirring speed was subsequently reduced to 40 rpm for 15 min.
- Subsequently, the sample was allowed to settle for 15 min and the COD of the supernatant measured (APPENDIX 2).

The sample was subsequently transferred to a portable laboratory scale DAF unit that was custom built at the Darvil Wastewater Works. The operation of the DAF unit was as follows :

- The pressure cylinder was filled with water up to the halfway mark and the lid was closed.
- The inlet valve on the pressure cylinder was opened to allow synthetic air to enter the water in the cylinder (dissolved air). The inlet valve was closed when the pressure reached 400 kPa (4 Bar).
- The pressure cylinder was then shaken vigorously to allow an even distribution of dissolved air.
- The outlet valve on the pressure cylinder was opened, allowing tiny air bubbles enter the DAF unit containing the flocculated effluent sample (800 mL). The outlet valve was closed when the level of the liquid in the DAF unit reached 1000 mL.
- The sample in the DAF unit was allowed to stand for approximately 5 min to allow the bubble-fat globule agglomerates to rise to the surface of the vessel. The scum layer is subsequently skimmed off the surface. A sample was obtained from the flocculated liquor for COD measurements.

3.2.3 Application of commercial flocculant

A silica based commercial flocculent with the trade name C40 was purchased from a company called Chemserve Trio. The chemical characterization of the flocculent was not within the scope of this investigation. Jar test were performed to optimize the concentration of the coagulant required. Experimentation was conducted at bench scale. Upscale to pilot scale will be explained in Chapter 4.

The raw edible oil effluent was placed in a 25 L vessel and the pH was adjusted to pH 7. A stirrer was set in place over the vessel. While stirring, approximately 110-150 g of the coagulant C40 was gradually added to the effluent until floc formation occurred and there was a simultaneous color change from a milky white to a clear effluent, resulting in a concentration of 4.4 - 6 g/L. The concentration of C40 used varies with each batch of effluent due to the differences in the organic strength and chemical composition of the effluent. The coagulated and flocculated effluent was then allowed to settle for 24 hrs at room temperature (20^0C). The COD of the clear supernatant was measured.

When comparing the performance of the above pretreatment technologies, the ideal process was selected based on the efficiency of COD removal.

3.2.4 Chemistry of oil effluent

The detailed chemistry of the raw and pre-treated effluent was determined using a Gas Chromatography- Mass Spectrometry (Finnagin MAT GCQ) (GCMS). Analysis was conducted on three sets of samples over the period of the investigation.

3.2.4.1 Preparation of sample

The raw and pre-treated oil were prepared separately using the following procedure :

- A volume of 150 mL sample was transferred into a 500 mL separating flask.
- Added 50 mL of dichloromethane (DCM) and the sample was shaken for 15 min on a Labcon shaker in order to extract the organic layer. This procedure was repeated with 50 and 40 mL of DCM to facilitate complete extraction of the organic layer.

- Rota-evaporated the organic solvent to +- 5 mL. The sample was dried with nitrogen and made up to 2mL with DCM.
- The sample was cleaned by passing hexane and DCM through a C 18 Sep-pak cartridge (Anatech).
- Once the sample (2 mL) was passed through the Sep-pak cartridge, it was collected in a vial and crimped and ready for GCMS analysis.

3.2.4.2 GCMS Procedure

The operating conditions of the GC is mentioned in Appendix 4

3.3 RESULTS

3.3.1 Pretreatment Technology

The results showing the efficiency of COD removal of the different pretreatment processes are tabulated.

TABLE 3.1 Pretreatment of edible oil effluent with ferric chloride showing COD removal efficiency. (Samples are listed as 1,2 & 3)

SAMPLE	COD (mg/L)			% COD REMOVAL		
	1	2	3	1	2	3
RAW EFFLUENT	5 945	6 710	6 920			
TREATED SAMPLE	2 920	3 750	3 720	50.8	44.1	46.2
TREATED SAMPLE AFTER DAF	1 852	1 920	1 899	68.8	71.4	72.6

TABLE 3.2 Pretreatment of edible oil effluent with Alum showing COD removal efficiency. (Samples are listed as 1,2 &3)

SAMPLE	COD (mg/L)			% COD REMOVAL		
	1	2	3	1	2	3
RAW EFFLUENT	5 945	6 710	6 920			
TREATED SAMPLE	3 259	3 820	3 964	45.2	43	42.7
TREATED SAMPLE AFTER DAF	2 095	2 220	2 470	64.8	66.9	64.3

TABLE 3.3 Pretreatment of edible oil effluent with C40 showing COD removal efficiency. (Samples are listed as 1,2 &3)

SAMPLE	COD (mg/L)			% COD REMOVAL		
	1	2	3	1	2	3
RAW EFFLUENT	5 945	6 710	6 920			
TREATED SAMPLE	1 561	1 720	1 695	73.7	74	75.5

TABLE 3.4 Comparison of the COD removal efficiencies of three pretreatment processes.

PRETREATMENT PROCESS	% COD REMOVAL (MEAN)
FeCl + DAF	70.9
ALUM + DAF	65.3
C_{40}	74.5

The effectiveness of such pretreatments was primarily evaluated on the basis of percentage of COD removal. Table 3.1 tabulates the results of FeCl as a coagulant and shows that approximately 47 % of the effluent COD was removed after treatment and 70 % after DAF. The COD of the raw effluent differed since they were obtained at different times and from different batches. The results when applying Alum showed approximately 43 % effluent COD removal.
Approximately 65 % of the COD was removed after treatment with DAF (Table 3.2). The assessment of the efficacy of the commercial flocculent C40 showed effluent COD removal of approximately 74 % (Table 3.3).

Comparison of the effectiveness of the pretreatment processes investigated showed C40 to be superior. In addition to providing comparatively better COD removal capacity, the use of the commercial coagulant as a pretreatment of choice when upscaling experiments was further justified since the process was not as laborious and there was no need for DAF. A further advantage was that C40 was slightly cheaper than Alum and FeCl and therefore would not add to cost if the pretreatment was to be applied to larger scale.

3.3.2 Chemistry of oil effluent

Due to the comparatively superior performance of the commercial coagulant, C40, it was used as a pretreatment method of choice. The detailed chemistry of the raw and flocculated effluent was determined using a GCMS. The detailed compound spectrum is reflected in Table 3.5.

TABLE 3.5 Compounds present in raw and flocculated effluent

RAW EFFLUENT		FLOCCULATED EFFLUENT	
COMPOUND	**AREA %**	**COMPOUND**	**AREA %**
Hexanal	10. 38	Toluene	4. 86
Alpha- Pinene	22. 05	Octenol	6. 19
Camphene	1. 67	Cineole	2. 14
Cyclohexane	0. 43	Filifolone	1. 36
Beta- Pinene	1. 57	Pinocarveol/ Pinocarvone	16. 03
Furan	1. 10	Furan	2. 49
Para- Cymene	9. 53	Para-Cymene	2. 98
L- Limonene	3. 62	Cis- Limonene	1. 24
Butylbenzene	5. 21	Butylbenzene	1. 74
Tricyclo- heptane	0. 87	Tricyclo- heptane	23. 51
Isopropenyl Benzene	1. 83	L-Camphor	1. 98
Nonanal	0. 70	Trans-P-Menthenediol	7. 47
Cyclopentene	1. 26	Cyclopentene	2. 91
Alpha- Campholene	0. 77	Bicycloheptanone	2. 41
Phellandrene Epoxide	0. 60	Dihydrocarvone	2. 24
Pulegone	0. 47	Trimethylnorpinan	1. 50
Nonenal	3. 29		
Terpineol	0. 24	Terpineol	9. 01
Dodecene	4. 65		
Dodecanol	1. 64	Borneol- L	5. 53
Endobornyl Acetate	1. 30	Endobornyl Acetate	3. 57
Trans- Decadienal	7. 69		
Beta-Elemene	0. 84		
Aristolene	0. 57		
Calarene	7. 10	Calarene	0. 84
Cyclooctane	3. 44		
Octadiene	6. 28		
Beta- Bisabolene	0. 94		

◊ The area % reflects the relative abundance of the compound in the sample and not the actual concentration, since this would require the use of standards.

The chromatogram (Appendix 4 & 5) provides detailed description of the various compounds present in the raw and flocculated effluent and summarized in Table 3.5. A total of 28 organic compounds were isolated from the raw effluent and 20 from the flocculated effluent, indicating that some of the compounds are removed during the flocculation phase. Compounds such as furan, para-cymene, limonene, butylbenzene and calarene were present in both the raw and flocculated effluents but in relatively lower concentrations in the latter. However, tricyclo-heptane, terpineol, cyclopentene and endobornyl acetate although occurring in both effluents, the relative concentrations were higher in the flocculated effluent. Hexanal, pinene, camphene and a host of other compounds (Table 3.5) were not present in the flocculated effluent and therefore removed with the flocculent. In addition compounds such as toluene, octenol, cineole etc. were present in the flocculated effluent only. These effluents contain various essential oils that emanate from the refinery process. It should be noted that, although the above compounds have been identified from GCMS library searches with a greater than 80 % probability of its presence in the sample, confirmation requires the analysis and comparison to the appropriate standards.

3.4 DISCUSSION

Industrial wastewaters are in several cases characterized by a remarkable content of organic substances. Complete abatement of wastewater pollutants can be hardly achieved by the adoption of single treatment methods. Combination of chemical and biological treatment is often the way to optimize the overall process and minimizing economic cost (Andreozzi *et al.*, 1998; Bertanza *et al.*, 2001). Emulsified lipids are commonly present in the wastewater's of edible oil manufacturing industries and form the major fraction of the FOG wastewater parameter. The COD of these untreated wastewaters range from 8 000 to 10 000 mg/L, depending on the source (Eroglu *et al.*, 1990). Failure of activated sludge processes under lipid overloading have been reported and evidenced by a dramatic increase in effluent turbidity (Hrudey, 1982). Therefore, these effluents have to be pre-treated to remove some of the FOG using physical/ chemical methods in order to render the effluent amenable to biological treatment. In addition, large macromolecules such as soluble polymers may not be easily biodegradable due to their size or lack of reactive sites. Chemical pretreatment can break these compounds into smaller biodegradable fragments.

Free oil and grease which are not emulsified, presents no serious problem with respects to it's removal from water because it tends to float and agglomerate and removed using fat traps. However the emulsified oils are far more difficult to remove and have to be treated chemically. Inorganic coagulants such as ferric chloride, alum, sodium hydroxide, lime etc. break down these emulsions by balancing or reversing the surface tension on each side of the interfacial film, neutralize stabilizing electrical charges or precipitating emulsifying agents (Sengul, 1989).

Ferric chloride showed comparatively good removal of organic matter and suspended solids when used as a coagulant (approx 47 %) and alum was marginally less at 43 % (Table 3.1 & 3.2). Compared to other coagulants such as sodium and calcium hydroxide, coagulation utilizing aluminum or iron salts has been effective for de-emulsifying oily wastes (Eckenfelder and Musterman, 1995). Previous research by Sengul (1989) using a ferrous salt showed a COD removal of 59 % and when using alum a COD removal efficiency of 70%. However, the coagulant dosages were much higher i.e. 500 ppm as opposed to the optimized concentration used during the present investigation. In addition the raw effluent concentrations were also much higher i.e. 43 000 mg/L. The difference in the organic removal capacity of alum and ferrous salt between our finding and Sengul (1989) could be attributed to various reasons. In addition to difference in coagulant dosage and raw effluent concentration, another reason could be difference in the chemistry of the oil effluent constituents which is dependent on the refinery process. Eroglu, *et al.* (1990) showed that when comparing the efficacy of coagulants such as ferric chloride, alum and lime to reduce the organic content of edible oil wastewater's, ferric chloride was found to be most effective, thus supporting our findings. During the physico-chemical treatability studies, ferric chloride COD removal capacity was approximately 36 %. However, our findings showed a slightly higher COD removal capacity of 47 %.

In addition to the use of coagulants, the current technology of choice for the primary treatment of edible oil wastewaters include DAF. (Eroglu *et al.*, 1990; Ozturk *et al.*, 1990; Khan and Akhtar, 1998). The DAF units have been used to remove free fatty materials from acidic wastewaters and

satisfactory removal efficiencies have been obtained when compared to conventional fat traps (Ozturk *et al.*, 1990). The principle of DAF is based on the fact that when pressure on the water is reduced, dissolve air is released in extremely fine bubbles. These bubbles attach themselves onto suspended material present in the medium and cause them to float to the surface where they concentrate and can be removed by skimming. Analysis of the findings show that using ferric chloride as a coagulant removed 47 % (mean) (Table 3.1) and the combined treatment with DAF removed a further 23 % of the organic matter, giving a total COD removal of 70.9 % (Table 3.4). The performance of alum was slightly weaker with 43 %(mean) (Table 3.2) initially and when combined with DAF, the total COD removal capacity was 65 % (Table 3.4). Comparison of the efficacy of the two treatment processes showed that ferric chloride and DAF performed marginally better than alum and DAF (Table 3.4). These findings substantiates previous research by Eroglu *et al.* (1990) showing better removal capacity of ferric chloride and DAF combined treatment. The addition of these coagulants prior to DAF are important since they serve to destabilize these oily emulsions.
Laboratory scale dissolved air flotation studies to treat edible oil effluents by Ozturk *et al.* (1990) showed very high fatty material removal efficiencies and COD removal efficiency of approximately 80 %. Present findings i.e. 70 and 65 % COD removal respectively, using ferric chloride and alum combined DAF systems compares favorably with other researchers.

The application of C40 was done to compare the efficacy of commercial coagulants to the conventional inorganic coagulants. With regards to COD removal C40 performed better than ferric chloride - DAF and alum -DAF combined systems i.e. 74 % as opposed to 70 and 65 % respectively (Tables 3.3 & 3.4). Unlike the two step coagulant -DAF process, the C40 method involved a one step process therefore making it more practical for large scale application, without adding to the cost.

The company from which the coagulant C40 was purchased refuse to provide information on the chemical structure. However close inspection of the physical structure and texture of the grains revealed good resemblance to some sort of organoclay. Bentonite which is a organoclay is known to be used to remove oil from water (Alther, 2000). It is made up of two or more mineral oxide layers. These layers are stacked parallel units of silica and alumina sheets. These layers are expandible and known for the absorption capacity. For these clay particles to adsorb or bind an organic molecule, there must be opposite electrical charges that attract. Clays with a high cation exchange capacity (CEC) such as bentonite (60-100+ MEQ) have a large number of negative charges on their surfaces. In addition, the pore size of the clay will also impact on the binding of organic molecules as well as the surface bonding (Alther, 2000). Previous research by Beccari *et al.* (1999) involved the use of bentonite for the pretreatment of olive oil mill effluent (OME)prior to anaerobic digestion. Their findings showed that when applying bentonite to pre-treat OME, at a concentration of 4g/L, they achieved a COD removal efficiency of 42 %. Subsequent trials to enhance the anaerobic treatability of OME using higher concentrations of bentonite i.e. 10 & 15 g/L produced marginal increases in COD removal i.e.47 and 45 % respectively. The latter also shows that increase in the coagulant concentration from 10 to 15g/L bentonite did not increase the COD removal, rather resulting in a slight decrease (Beccari *et al.*, 2001). When comparing the latter to our findings, the COD removal efficiencies of C40(bentonite) was relatively good at 74%, even at much lower coagulant dosage concentrations of 4.4 to 6 g/L.

Comparison of the efficiencies of the three pretreatment processes (Table 3.4) clearly indicates that C40 not only to be superior in COD removal capacity i.e. 74 %, but also more practical for larger scale processes and more cost effective. In addition, it involved a single step and not a two stage process such as coagulation/flocculation and DAF. Therefore, C40 was selected as a pretreatment of choice for further investigations and bio-treatability studies involving the application of activated sludge to treat edible oil refinery effluent.

For the purpose of this research, it should be noted that the chemical composition of the effluent produced is ultimately dependent on the source crude oil and the refinery process employed which is variable amongst the different factories. Most of the previous research conducted in this field focused primarily on characterizing edible oil effluents on the basis of the COD/BOD, pH, total suspended solids and fats/oils and greases and not investigating the detailed chemistry. The effluent used during the course of this investigation was obtained from the refinery process only. In terms of volume and load ,the largest proportion of the effluent discharged by an edible oil plant arises from refining operations. Typically about 80 % of the effluent volume is attributable to the refinery process (Steffen *et al.*, 1989). In the refinery process, effluents are generally discharged from the degumming stage (gums), the neutralization stage (soapstock and soapy water) and the bleaching stage (spent earth), as well as the various steam and cooling operations and general washing.

The chemical composition of the primary pollutants in edible oil effluents are mainly fats and oils (Boyer, 1996). Following neutralization of the crude oil and subsequent soap splitting, water is also used for washing giving rise to effluents known as soapy water and acid water. These can contain considerable quantities of fats and oils and high levels of sodium and sulphates. Detergents used in wash-down operations often emulsify oil leading to high levels of oil in plant effluent (Steffen *et al.*, 1989). Table 3.5 presents a description of organic compounds identified from composite samples of raw and flocculated effluents. Although the actual concentrations of the compounds were not determined using standards, the area percentage on the GCMS chromatogram provides the relative abundance of the compound in the sample.

Twenty eight organic compounds were identified from the raw effluent and twenty remained in the effluent after flocculation. The coagulant C40 removed compounds such as hexanal, alpha-pinene, camphene, cyclohexane etc. Para-cymene, limonene, butylbenzene and calarene were present in both effluents but in lower concentrations in the flocculated effluent, indicating removal during the flocculation process. Although the area % of tricyclo-heptane, terpineol, cyclo-pentene and endobornyl acatate was greater in the flocculated effluent, no conclusions could be drawn on the relative concentrations since standards were not used. The occurrence of toluene, octenol, cineole etc. in the flocculated effluent only and not the raw effluent could be due to various reasons. The most plausible could be chemical interaction between substances in the raw effluent and the coagulant C40 resulting in transformation of the original compounds into new products.

The source of the hexanal and cyclohexane in the raw effluent can be attributed to loss of hexane during the hexane recovery process and washing during the refinery process (Steffen *et al.*, 1989). Refining of edible oil involves processes designed to neutralize free fatty acids present in the oil by introduction of a alkali, usually caustic soda, and removal of phospholipids, color bodies and other soluble and insoluble impurities. The latter usually end up in the effluent (Anderson, 1996).

Detailed chemical analysis of the raw and flocculated effluents showed a large proportion of essential oils (Table 3.5) which are known to have high commercial value. Essential oils are volatile chemical components usually of plant origin and are known to occur in sunflower seeds. These essential oils are usually produced commercially from plants using different extraction techniques and distillation processes. However, during the current investigation these essential oils were released into the effluent during the refinery process which involves degumming and neutralization using high temperatures (Guzinski, 1996). With reference to Table 3.5, a large number of essential oils were identified. In the raw effluent, the common essential oils included alpha pinene, camphene, para-cymene, limonene, cyclo-pentene, terpineol, dodecene, beta -elemene, calarene and beta-bisabolene. In the flocculated effluent, cymene and limonene were also found but at lower concentrations. In addition, toluene, octenol, cineole, pinocarveol, camphor, terpineol, borneol and calarene were also detected.

With regards to relative abundance, it can be seen that alpha-pinene was predominant in the raw effluent and pinocarveol in the flocculated effluent. It is known that bio-transformation of essential oils do occur, but it generally mediated by microorganisms and enzyme systems (Duetz *et al.*, 2001). Alpha pinene is a monoterpene that can be biologically transformed by the bacteria *Bacillus pallidus* into pinocarveol using the enzyme alpha-pinene monooxygenase. Limonene can also be transformed into carveol by *Pseudomonas* sp. and *Rhodococcus opacus*. However, during the current research, transformation of these essential oils could not be attributed to biological systems, but rather to chemical reactions facilitated by the interaction of the coagulant C40 with the components of the oil effluent. Transformation of alpha- pinene and limonene from the raw effluent could account for the high pinocarveol concentration in the flocculated effluent. This hypothesis could also be used to justify the increase in certain essential oils e.g. terpineol in the flocculated effluent and the existence of new compounds in the flocculated effluent e.g. toluene, octenol and cineole that were non-existent in the raw effluent. Limonene can be transformed biologically into alpha-terpineol (Duetz *et al.*, 2001; Tian, 2001; Wong and Li, 2001).

Essential oils are known to have a substantial commercial value due to their extensive uses in the food, pharmaceutical and fragrance industries. The conventional extraction and purification techniques for the commercial production of these oils are expensive and add to the cost of these products. Current investigations show that large amounts of these oils are present in the sunflower oil effluent. Tea- tree oil is known for it's wide application in the medicinal and aromatherapy industries. Of the 10 major constituents that make up tea-tree oil (Coldstream Plantation, 2001), during the current investigation, 9 have been found to occur in the raw effluent e.g. pinene, cymene and limonene. Therefore, a waste product such as edible oil effluent could be used to derive valuable by-products i.e. essential oils at a fraction of the cost. However, the success of the process would ultimately depend on the cost of extraction and purification of the essential oils. A thorough literature search revealed no information leading to using waste edible oil effluents to produce valuable essential oils.

Conventional methods of producing essential oils from plants include distillation procedures, of which steam distillation is most common(Guzinski, 1996). A more recent development in the production of these oils includes a technique called "supercritical extraction". It involves the extraction of essential oils with supercritical carbon dioxide which is applied under pressure (10Mpa) and therefore turns liquid. This method is usually sufficient to obtain the well-soluble volatile essential oils, while the co-extraction of undesired higher molecular weight substances is limited (Stateva and Savova, 2001). However, optimization of this process for the extraction and purification of essential oils from vegetable oil effluents would require further adaptations. The latter was not within the scope of this investigation.

3.5 CONCLUSION

- Detailed chemical investigations of the oil effluent revealed the presence of valuable essential oils amongst other organic products.
- Valuable essential oils could be extracted and commercialized before treatment of the effluent stream.
- Pretreatment of the oil effluent is crucial in preparing the effluent for biological treatment, since it served to reduce the high FOG load.
- The application of the commercial coagulant C40 showed comparatively better performance than ferric chloride, alum and DAF and therefore was selected as a method of choice for pretreatment of the raw effluent obtained from industry.

CHAPTER FOUR

LABORATORY SCALE BIOLOGICAL TREATMENT PROCESS

4.1 INTRODUCTION

Increased concern over a clean environment by the public and regulatory authorities, is forcing the implementation of stringent regulations in improving the quality of environment not only in industrialized but also in developing countries (Ozturk *et al.*, 1990). In South Africa there are 16 edible-oil processing plants, which produce approximately 300 000 tons of vegetable oil annually (Mkhize and Bux, 2001). The industry consumes nearly 2 million m^3 of water per annum and approximately 40 % of incoming water is discharged as effluent into sewers (Steffen *et al.*, 1989). The effluents discharged to sewers contain high quantities of fats, oils and greases, sulphates, phosphates and other pollutants and has a high organic as well as inorganic loading. Although the general concepts are the same, the physical and chemical characteristics of the effluent that emanates at the end of the refinery process varies amongst the industry. The latter is dependent on various aspects, including the source of the crude oil that is refined (Beccari *et al.*, 2001). Experimental surveys indicate however that striking differences in the quantity and quality of effluents may be observed from one plant to the other within the same subcategory, mainly because of different processing experiences and habits. Therefore, in-situ experimental information needs to be derived from each plant, on a case by case basis (Orhon *et al.*, 1999).
Since it is crucial to work with effluents that do not vary markedly with regards to the chemical properties amongst the batches collected, the current research was limited to obtaining the effluent from a single company.

Current method of effluent treatment in the in the edible oil industry is restricted to either physical separation of oil and grease via a gravity fat trap or dissolved air floatation and subsequently pH correction before discharge. Even after application of these physico-chemical techniques, the remaining emulsified grease tends to clog sewer pipes and pumps and often don't subscribe to municipal discharge standards (Eroglu *et al.*, 1990; Mkhize *et al.*, 2000; Mkhize and Bux, 2001). Globally, trends are currently focusing on applying biological processes for treatment and final polishing of the effluent. Majority of the agro-industries are compatible with biological treatment, with or without a pretreatment step and the activated sludge process is the preferred treatment scheme (Orhon *et al.*, 1999). Eroglu *et al.* (1990) conducted research on the comparative evaluation of treatment alternatives for wastewater's from an edible oil refining industry. Results showed that activated sludge proved successful in removing most of the fats, oils and greases and the anaerobic filter removed approximately 60 % of the sulphates. Difficulties in the anaerobic treatment of olive mill effluents are mainly connected with the presence of biorecalcitrant and /or inhibiting substances, essentially polyphenols and lipids (Hamdi *et al.*, 1991). However, a significant increase in methane production rate from OME previously fermented aerobically with *A. niger* was experienced. In addition, preliminary aerobic treatment with specific microorganisms facilitated shorter residence time for the anaerobic process (Beccari et al., 1999).

Therefore, the emphasis of this aspect of the research focused on developing and optimizing a biological treatment process i.e. activated sludge in order to successfully treat sunflower oil effluent. The aims were :

- wastewater characterization and large scale pretreatment of the oil effluent
- development of laboratory scale activated sludge process
- operation of the activated sludge process
- optimization of the process for maximum COD removal
- microscopic analysis of activated sludge flocs, and
- critical analysis of data.

The investigation subscribed to using effluent from a single factory (Company X), since previous research (Orhon *et al.*, 1999) has shown that developing remediation technology in the edible oil waste sector has to be subjected to a case by case approach. In addition, the effluent used was from the refinery process, since it comprised a major portion of the final effluent.

4.2 MATERIALS AND METHODS

4.2.1 Summary of the oil refinery process at Company X

The process of refining edible oil varies amongst the different companies in the industry. It involves various stages (Fig. 4.1) and is explained as follows :

4.2.1.1 Degumming

During the course of this investigation crude oil was imported and stored in tanks, hence there was no milling on site at Company X. Refining can be said to begin in these tanks, since oil -insoluble matter separates by gravity. The oil is then pumped via a pump centre to the degumming process. Here gums are separated into hydratable and non-hydratable types and it is important that they be removed to produce good quality final oil. Hydratable gums are removed simply by adding hot water(70^0 C). Non-hydratable gums require prior treatment with 0.1% citric acid in order to render them hydratable. When the hot water (3 % of total volume) is added to the oil, it is then retained in a precipitate holding tank for 1 hr facilitating separation of the gums. In addition, 70-80 L of 3% caustic soda is added which helps to flocculate the gums. Caustic also increases the pH from 3 to pH 5. The mixture including insoluble gums are then subjected to centrifugation (7000 rpm) which separates the gums from the oil. The company X applies physical refining only.

4.2.1.2 Bleaching

In addition to color removal, bleaching acts as a further purification step in the refining process. Assessing the color of the oil actually determines the amount of bleach to be added. This step is conducted under vacuum and at raised temperatures (100^0 C). Bleaching clay (2 %w/v) is mixed with the oil and agitated for 45 min. The mixture subsequently passes through a series of filters and eventually passes out clean oil. This process removes trace metals, pigments such as chlorophyll and caratenoids and various oxidation products. The oil is cooled to 35^0 C and then passes to the winteriser unit.

4.2.1.3 Winterising

Unrefined sunflower oil contains wax from the husk which causes the undesirable cloudy appearance at lower temperatures (10^0C) when they begin to crystalize. Typical wax content varies between 200 to 2000 mg/kg and this must be reduced to 10 mg/kg if adequate cold stability is to be obtained (Steffen *et al.*, 1989). The oil enters the winteriser at 35^0 C. The reduction of wax in the oil is affected by carefully cooling the oil to 10^0 C, followed by filtration to remove the crystalized wax.

4.2.1.4 Physical refining and deodorising

The de-waxed oil after winterising goes to a vacuum de-aerator to remove the air and moisture. The deodorization process involves steam distillation under vacuum. It's purpose is to remove residual free fatty acids, aldehydes and ketones which are responsible for bad odors and flavors in oil and decolourising oil by thermal decomposition of the pigments. The decomposition products from the pigments are subsequently distilled off. The oil then enters the top heating section of the stripper (heat exchanger) and is heated to 264^0C using a thermal fluid (such as Dowtherm) from a vapouriser. The oil is then refined by flowing over the top series of trays, counter current to the flow of stripping steam (pressure of 3 bar) which is injected below the bottom tray. The process is continuous. High temperatures results in the vapourisation of free fatty acids. De-acidification and deodorization occurs under vacuum and 3 bar steam. The refined oil flows down 7 compartments (with subsequent decrease in the temperature in each compartment) down to the holding section, which provides retention necessary for the heat bleaching of some of the oils, before a final series of trays enables steam stripping of all the remaining odoriferous material. Energy utilization is maximized since exiting oil transfers some of its heat to the incoming feed before being pumped to polishing filters and on to storage. The fatty acid chamber (160^{0}C) is where the fatty acid is in vapor form and returns to liquid at lower temperatures.

The Company X refines approximately 240 tons/day of crude oil.

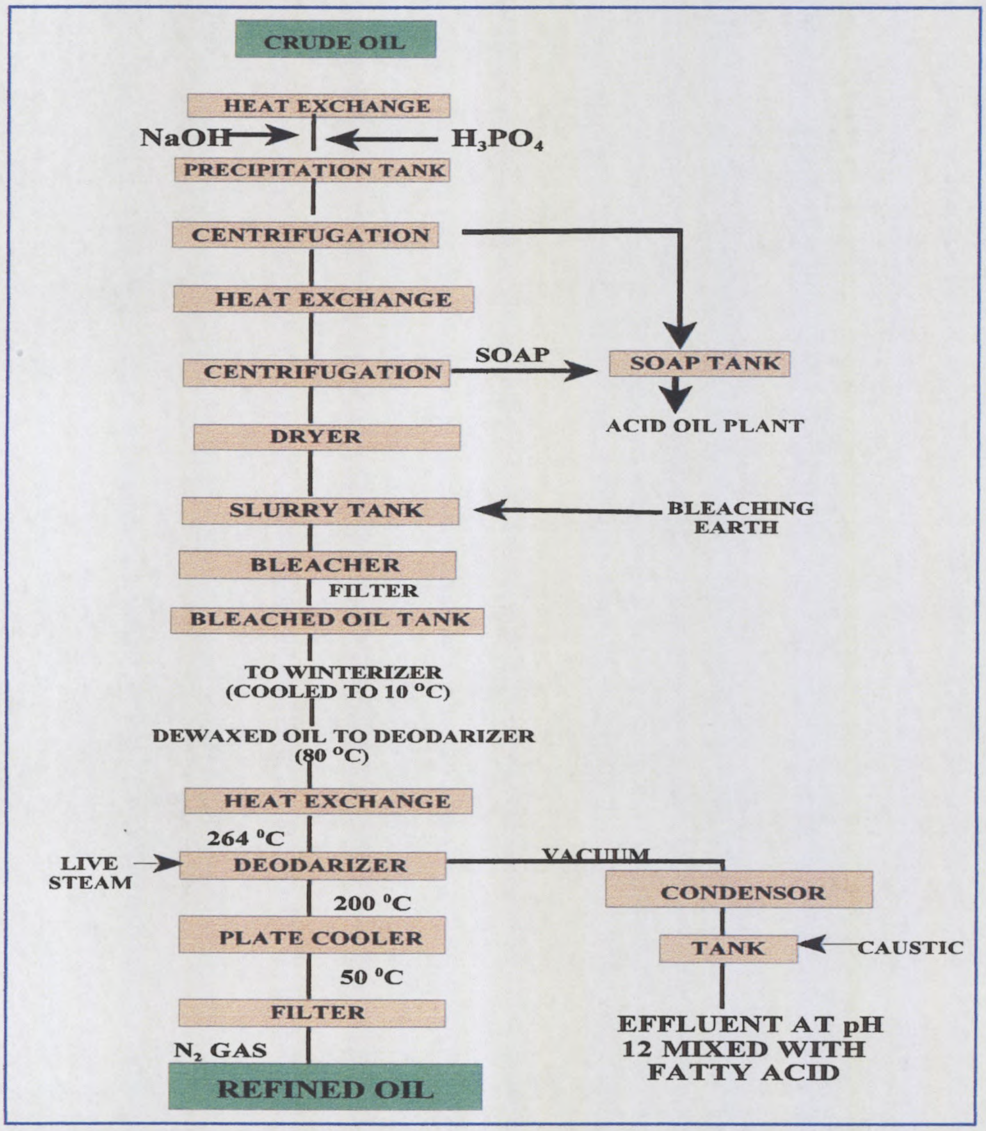


FIGURE 4.1 Diagram of Refinery process at Company X

4.2.2 Laboratory scale pretreatment process

4.2.2.1 Collection of effluent

Composite effluent samples were collected from the drain at the end of the refinery process. The volume collected comprised of 22 x (25L)containers, giving a total volume of 550 L. The company was situated approximately 90 km north west of Durban. The samples were transported immediately to the laboratory for processing which included a battery of chemical test to characterize the influent. The balance of the samples was stored in the cold room at 4^0C to prevent further biological activity. It should be noted that collection of sample was ultimately dependent on the regular operation of the refinery process. However, during the course of this investigation, the problems that hampered regular supply of effluent were as follows :

- Regular interruptions in the refinery process due to problems in obtaining crude oil
- alternating between refining soya, cotton seed and sunflower oil without prior notification which was problematic since the investigation was limited to effluent of the sunflower oil refinery process
- an explosion at the company which disrupted the refinery process for 3 months.

The above hurdles are quite common when investigating industrial problems as the industries rarely place priority on research needs of the project, and therefore progress is subjected to conditions at the industry. The effluent collection period spanned during the entire period of the laboratory trials i.e. May 2000 until October 2001.

4.2.2.2 Effluent parameters measured

On receipt of the effluent from industry, samples were immediately obtained and the following chemical analysis were conducted :

- COD. This analysis was conducted in order determine the strength of the effluent and calculate the dilution factor required for laboratory scale treatment studies. A colourimetric method was used using the Merck SQ 118 Photometer (Method No. 14690) (Appendix 2).

The COD varied amongst the different batches of effluent collected.

- FOG , The determination of the fats, oils and greases is crucial for the pretreatment and preparing the effluent for biological treatment. The procedure involved applying Standard Methods (1989) (Appendix 6).
- Total phosphate, The TP is measured in order to correct the nutrient ratio i.e. C:N:P of the effluent prior to biological treatment. The method involved using the Merck SQ 118 photometer (Method No. 14842) (Appendix 7)
- Total Kjeldahl nitrogen, The TKN is indicative of the total nitrogen content of the wastewater. Similar to TP, it is measured in order to balance the nutrient conditions of the effluent prior to activated sludge treatment. The Spectroquant analysis method 14537 was employed using the Merck photometer (SQ 118) (Appendix 8).

4.2.2.3 Large scale pretreatment procedure

The effluent was processed immediately on receipt. The batch was separated into 2x 275 L batches. One batch was stored at 4^{0}C for future use. A volume of 275 L was dispensed into a 300 L vessel in order to facilitate the flocculation and coagulation process at room temperature (20^{0}C). The initial pH of the effluent was acidic but on addition of the flocculent, the pH became basic (approximately pH 9). The pH was further adjusted to pH 7 by the addition of sulphuric acid. The flocculent (C40) was added to the reactor with slow stirring. As the powder was added, flocs started to form and settled to the bottom of the container, thus changing the texture of the effluent from milky to clear. The approximate weight of C40 that was added was 2210 g for 275 L of effluent, giving a final C40 concentration of 8 g/L. However, the amount of C40 required for complete flocculation did vary amongst the batches. The latter was due to fluctuation in the FOG component of the oil effluent which was dependent on the refinery process. The time taken until breakthrough(i.e. clearing from milky to clear) was reached was approximately 10 min. The supernatant (flocculated effluent) was allowed to remain for 24-48 hrs in the vessel to facilitate efficient removal of the emulsified fats, oils and greases. On completion of the flocculation process, the supernatant is transferred to a 300 L feed vessel which is situated in the cold room.

4.2.3 Laboratory scale unit description

4.2.3.1 Unit set-up

The pilot scale activated sludge process was manufactured by the Department of Civil Engineering, University of Cape Town. The unit was set up next to the cold room, thus facilitating easy supply of influent which was situated in the cold room at 4^0C (Feed Tank). The temperature of the room in which the pilot plant was located was maintained at 20^0C (± 1^0C) using air conditioners during the summer month's. The temperature of the room during winter was within the above limits. The unit and the accessories was purchased as separate pieces and had to be assembled in accordance with the suppliers specifications. The latter took approximately 3 months. The laboratory scale process was operated for approximately 18 months (May 2000-October 2001).

4.2.3.2 Unit design and configuration

During the initial period of the investigation, the pilot plant was operated based on the 3- stage Phorodox process used for biological phosphate removal. However, due to a change in the oil refinery process i.e. phosphoric acid was replaced with citric acid, the total phosphate concentration of the effluent was within the discharge standards. The pilot plant configuration was subsequently changed to the modified Ludzak- Ettinger configuration focusing primarily on carbon and nitrogen removal. A schematic design of the pilot plant is given in Fig. 4.2. The reactor configuration comprised of the following : an anoxic (AX) zone (V=8 L); first aerobic (AE1) zone (V=10 L); and second aerobic (AE2) zone (V=10 L). The settler (2.5 L), was positioned downstream of the reactors at an angle of 60^0 to the perpendicular. Although the latter configuration follows that of an MLE process, an additional aerobic zone was present. Therefore the process can be regarded as an adapted MLE process (Fig. 4.3).

After the flocculation and coagulation process, the influent was transferred to the perspex

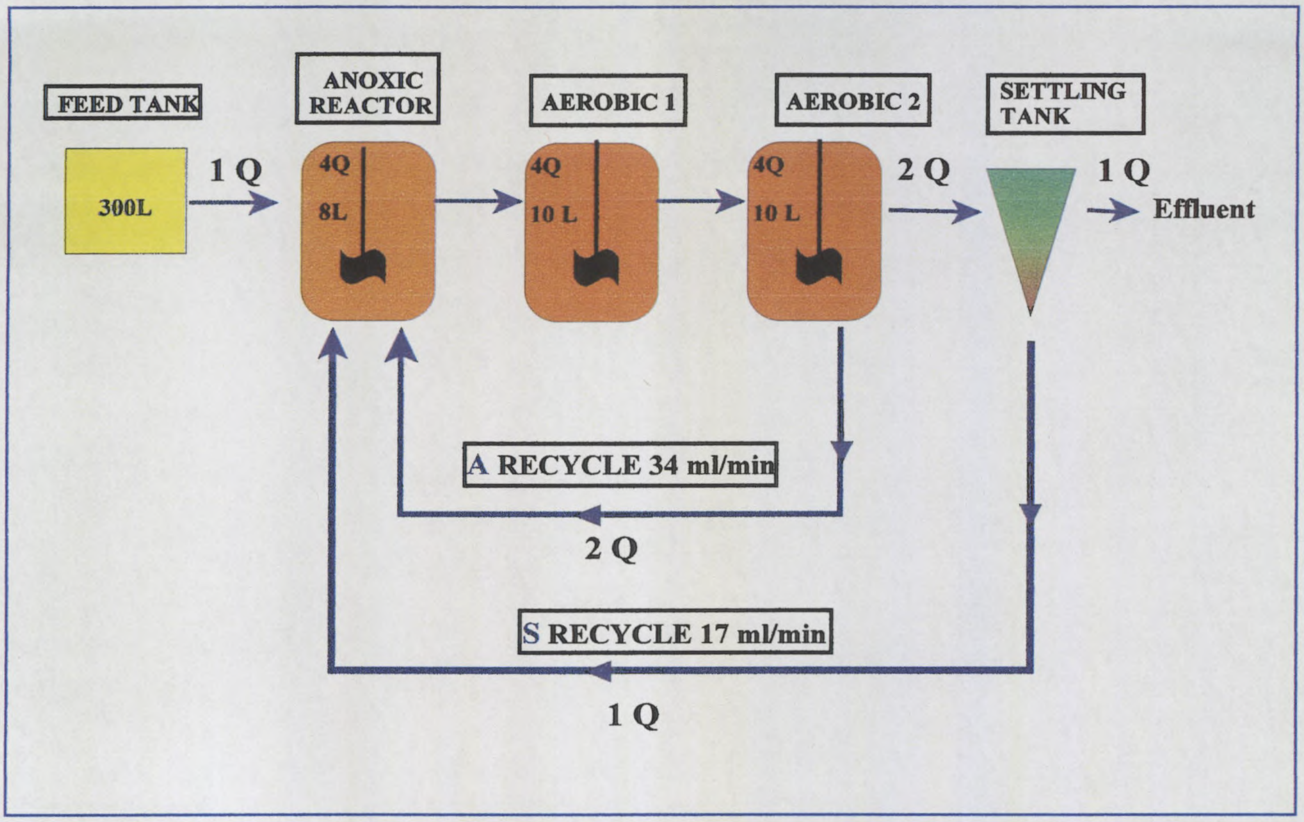


FIGURE 4.2 Schematic representation of the laboratory scale unit modeled upon the modified Ludzak- Ettinger process.

feed tank (V=300 l) which was located in the cold room(4^0C) in order to limit any biological activity prior to treatment. Target influent flow rate (Q_i) was set at 17 ml/min (24.48 L/d). An aperture in the cold room was used to house the silicon tubing which was connected to a multi-chanal peristaltic pump (Gilson) which was used to set the flow rate. The tubing was immersed in water set at room temperature before feeding the influent directly to the AX reactor. The latter facilitated increasing the temperature of the influent before it entered the biological reactor. The S-recycle is the flow that is pumped from the clarifier (settling tank) to the AX reactor, with a set ratio of 1:1 with respect to Q_i . The A-recycle, was pumped from AE2 to the AX reactor at a set ratio of 2 :1, with respect to Q_i. The recycle flows were controlled using a multi-chanal peristaltic pump manufactured by University of Cape Town (Civil Engineering).

Sludge was wasted on a daily basis and a volume of 1. 75 L was withdrawn from AE2. The system sludge age (R_s) was therefore maintained at 16 d i.e., the unit volume capacity =28 L; sludge wasted = 1. 75 L. However, the R_s did vary during the course of the investigation. The calculation of sludge age is as follows : $R_s = {}^{V}/_{Q}$ where V= total volume in the reactor and Q= flow rate. The oxygen uptake rate (OUR) was measured in AE2 using a dissolved oxygen (DO) probe (WTW) and meter (HiTech Microsystems). Air supply to the aerobic reactors (AE1&2) was facilitated using the OUR meter that controlled the aquarium pump, which was switched on at a DO concentration of 2.0 mg/L and switched off when the DO concentration of the reactors reached 5.0 mg/L. The meter which was operated in an OUR mode recorded the OUR over a 24 h period. Temperature and OUR results were then downloaded onto a computer using a RS232 adapter and data was subsequently manipulated using Lotus 1-2-3 software.

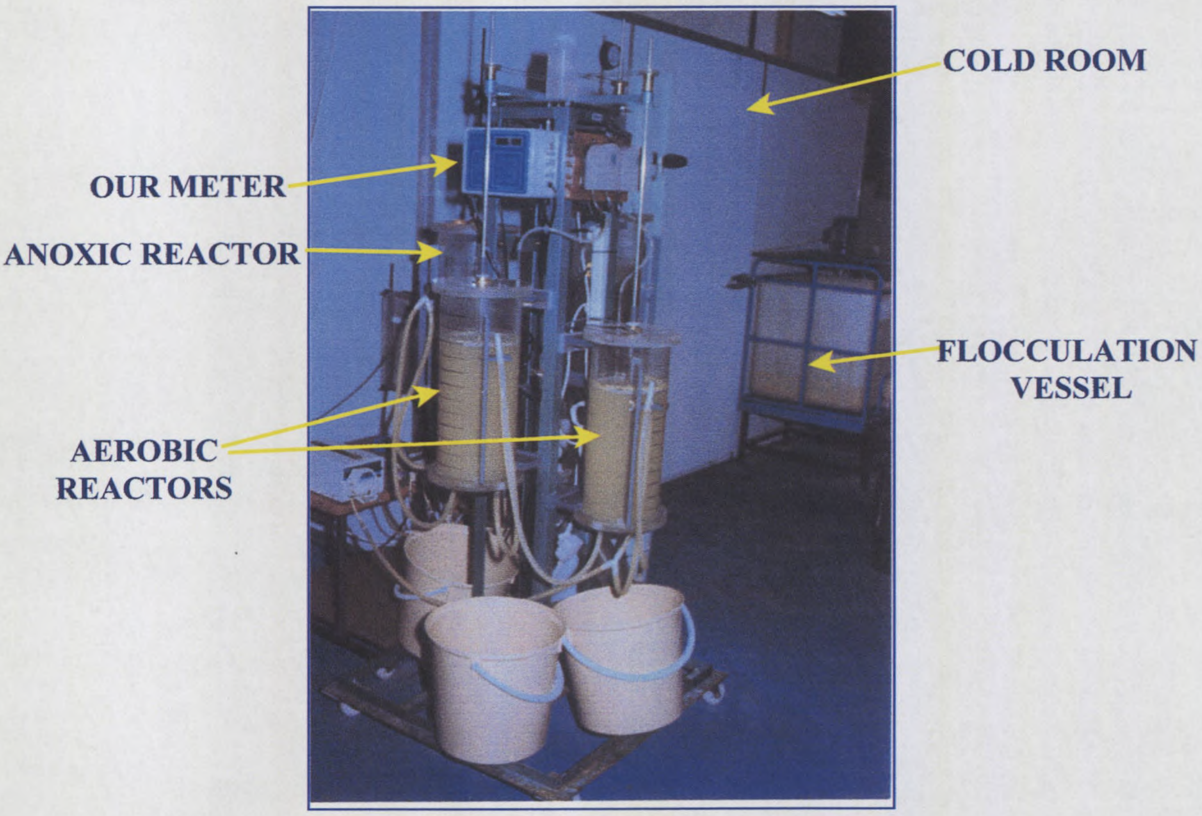


FIGURE 4.3 Photograph of the adapted MLE process showing the cold room and flocculation vessel in the background.

4.2.4 Pilot plant operation

4.2.4.1 Acquisition of seed inoculum

Mixed liquor was obtained from the aeration tank of Darvil wastewater works which is situated in the Pietermaritzburg region of Kwazulu-Natal. The reason for selecting the latter wastewater works was that most of edible oil industries were localized in this region, and their final effluent were discharged into Darvil. Therefore the sludge would have already been exposed and acclimatized to edible oil effluents. The MLSS of the Darvil seed sludge was approximately 3 500 mg/L. The desired MLSS concentration of the system was 2 000 mg/L. Therefore, 16 L of Darvil mixed liquor was decanted into the pilot plant and 12 L of water was added giving a total volume of 28 L. This rendered a final suspended solids concentration of 2 000 mg/L. The inoculum was fed diluted edible oil effluent (COD ± 300 mg/L) for a period of 2 weeks in order to further acclimatize the microorganisms in the sludge to the edible oil effluent.

4.2.4.2 Wastewater characterization and feed supplementation

The major limitation when applying biological methods for the treatment of industrial effluents was the varying strength and chemistry of the effluent and dependency of effluent supply from the industry. Therefore, to overcome the latter problem, the effluents had to be manipulated and corrected before being subjected to activated sludge treatment. The parameters of the effluent that were characterized prior to treatment were :

COD : The COD of the effluent after pretreatment was still high and therefore was diluted with distilled water in order to satisfy the objectives of the research i.e. subjecting the treatment process to incremental increase in COD from 500 mg/L to 1800 mg/L (at full strength after pretreatment i.e. undiluted). The COD was not fractionated due to inconsistencies in the chemical composition of the effluent batches collected. During the course of this investigation, the organic strength of the effluent was represented by the COD.

FOG : Since the effluent was comprised primarily of fats and oils, it was essential to determine the FOG and manipulate it i.e. pre-treat and reduce in order to prevent organic shock load of the system.

Nutrients : Due to changes in the refinery process, nitrogen and phosphorus became limiting in the effluent during the early stages of the treatment process. Therefore, the TKN and TP of the effluent was determined. In order to maintain the integrity of the biological system, nitrogen and phosphorus was added to the effluent at a C : N : P ratio of 100 : 10 : 1. These salts, ammonium chloride (NH_4Cl) for N and Potassium dihydrogen phosphate (KH_2PO_4) for P was added directly to the feed tank after the flocculation procedure.

pH : The pH of the effluent was corrected to pH ± 7 prior to biological treatment by the addition of acid in the feed tank. The volume of acid added to neutrality was ultimately dependent on the pH of the batch of effluent collected.

4.2.4.3 Phasic approach to treatment process

The design, operation and optimization of a laboratory scale process to treat the edible oil effluent was dependent on nature and regular supply of effluent and manipulating the process to achieve maximum organic removal. This approach necessitated the compartmentalization of the laboratory scale process into phases that had common operating conditions during the course of the investigation. Other factors that supported this approach included inherent problems with the laboratory scale process resulting in system failure, varying nature and strength amongst effluent batches and disruption in effluent supply. The laboratory scale treatment process was initiated on the 18 May 2000 and continued until October 2001. The period under investigation was divided into five phases as explained in Table 4.1.

4.2.4.4 Pilot plant maintenance

The feed tank was cleaned with water periodically prior to addition of new batch of effluent. The flocculation vessel was also rinsed with water and the settled slurry was collected by Wastetech. Agitation of the reactors was terminated briefly on a daily basis in order to brush and clean the interior of the vessels. The silicone tubing from the pumps were cleaned daily by gentle squeezing and brushing.

TABLE 4.1. Activity during the phases of the laboratory scale treatment process.

PHASE	PERIOD	ACTIVITY
1	18/5/00 - 24/5/00	Pilot plant operated as a Phoredox Process for biological N & P removal. Effluent was not pre-treated and system failed after five days due to very high FOG loading (approximately 8 200 mg/L)
2	25/5/00 - 05/6/00	Due to high FOG, an efficient pre-treatment protocol was developed and optimized. The pilot plant was operated as Phoredox process. Although, the FOG was low, the phosphorus removal capacity was poor and COD removal efficiency decreased. Process was terminated.
3	06/6/00 - 21/7/00	Refinery process was changed, i.e. citric acid was used instead phosphoric acid. Therefore the effluent contained minimal amount of P and there was no need for nutrient removal. The process configuration was changed to facilitate carbon removal only. In order to maintain the biological integrity of the system the effluent was supplemented with nitrogen and phosphorus. However an erroneous setting of the dissolved oxygen meter i.e. limit 6 mg0_2/L instead of 5 mg 0_2/L. This resulted in a bulking problem and subsequent loss of MLSS and poor overall COD removal efficiency. Influent COD concentration was maintained at 500 mg/L.
	21/7/00 - 27/ 7/00	The process was shut down completely and completely and the system including tubing was cleaned thoroughly
4 A	28/7/00 - 16/9/00	The process was restarted with fresh Darvill seed inoculum. The MLSS was 2546 mg/L. The sludge age was maintained at 16 d. The COD of the influent feed was increased to 800mg/L. The free and saline ammonia (FSA) concentration in the effluent was high. In order to solve the latter problem, the feed supplementation ratio was changed from 100:10:1 to 100:5:1 (C:N:P). In addition, the system configuration was also changed to introduce an anoxic zone and the process was operated as a modified MLE process. Sustaining constant influent feed concentration was problematic due to precipitation caused by resident flocculent in the feed tank and variation amongst the different batches of effluent collected. Therefore in order to determine the efficiency of the process, COD removal rates were reflected as percentages (result section). The pH was maintained at pH ± 7.
4 B – 4F	17/9/00 - 02/2/01	Process remained as an MLE configuration. The influent COD increased to approximately 1200 mg/L. The MLSS was set at 3500 mg/L. On the 21/10/00, there was a shut down of the refinery process at the industry and subsequently there was no supply of feed influent. The final effluent had to be recycled and served as influent in order to sustain the system. The effluent supply normalized on 3/11/00 On 01/12/00 the effluent feed concentration was increased to approximately 1500mg/L. On 12/12/00 the influent concentration was increased to approximately 1800mg/L. However, it was difficult to maintain exact influent feed concentration (reasons mentioned previously) There was a disruption in influent supply for 1 month. However, the final effluent was recycled to sustain the system during this period. No analyses were conducted.
5	06/3/01 - 07/10/01	The process remained as an MLE configuration. However there was a decrease in the influent COD which was uncontrollable since the quality and strength of the influent was dependent on the supply from industry. The process parameters remained the same.

Marprene tubing (internal diameter of 4.8 mm and wall thickness of 1.6 mm, Aeromix) was used to connect the peristaltic pumps to the silicone tubing due to its resilient properties and durability. All T-pieces and weirs were cleaned daily to prevent common blockages and spillages. The DO probe was cleaned and calibrated daily (using a saturated sodium sulphite solution) and the membrane was changed weekly due to the oily nature of the effluent.

4.2.5 Determinand analysis

4.2.5.1 Sample collection and preservation

All glassware including test tubes and beakers were cleaned thoroughly by scrubbing with a brush and soaked overnight in a 20 %(v/v) sulphuric acid solution, followed by triple rinse with deionized water. The glassware was then oven dried and used when required. The samples were obtained routinely from the pilot plant and decanted into pre-washed and dried plastic honey jars. The samples were preserved according to Appendix 9.

4.2.5.2 Parameters measured

The parameters that were measured, frequency of measurement and methods of analysis of the effluent from the reactor included :

- COD (influent and effluent). The analysis was conducted photometrically using the Merck SQ118 photometer, Method No's. 14541 & 14555(Appendix 2). The measurements were conducted daily.
- FOG (raw effluent, influent and final effluent). The analysis was conducted using Standard methods, 1989 (Appendix 6). The analysis was conducted routinely.
- Total P (raw effluent, influent and final effluent). The analysis was conducted using the Merck SQ 118 photometer, Method No. 14842. The analysis was conducted routinely and when required (Appendix 7).
- Nitrogen (TKN) (raw effluent, influent and final effluent) Analysis was conducted using the Merck SQ118 photometer, Method No. 14537. Analysis was conducted routinely and when required (Appendix 8).
- Ammonia (influent and effluent). Conducted photometrically using the Merck SQ 118 Method No. 14739 (Appendix 10). Analysis was conducted when required..
- Nitrate (effluent). Conducted photometrically using the Merck SQ 118, Method No.

14542. Analysis was conducted routinely (Appendix 11).

- MLSS and MLVSS . Samples were processed according to Standard method 2540 D and 2540 E (Standard Methods, 1989). Samples were obtained from AE1. Analysis were conducted daily (Appendix 12).
- Sludge volume index (SVI) and diluted SVI (DSVI). Samples were processed according to Standard method 2710 D (Standard Methods, 1989).Samples were obtained from aerobic reactors. Analysis were conducted occasionally and especially during bulking phenomenon (Appendix 13).
- pH and Temperature. Measured online using a Beckman pH50 meter.
- OUR. Measured online with the probe immersed in AE2 over a period of 24 hrs. Information downloaded onto a computer (mentioned previously).

Due to large number of variables and changing operating conditions during the operation of the laboratory scale process, the statistical interpretation of the data was limited to application of mean and standard deviations. The statistician consulted indicated that it would be difficult to apply the multifactorail analysis of variance method..

4.2.6 Electron Microscopy

4.2.6.1 Preparation of sample

The period of investigation spanned from June 2001 to August 2001 when the process was operating at steady state. A 100 ml mixed liquor sample was obtained from the AE 1 reactor and decanted into a Schott bottle and transported to the Electron Microscopy Unit at the University of Natal, Pirtermaritzburg. In addition, a 100 mL mixed liquor sample was also obtained from the seed inoculum (Darvil sludge) which was used at the beginning of the treatment process. This served as a control. A volume of 1 mL of both samples was dispensed into separate 1.5 mL eppindorf tubes and centrifuged at 3000 rpm for 10 min using a biofuge (Heraeus). The pellets were re-suspended in deionised water and the centrifugation cycle was repeated. However, a third sample of mixed liquor from AE 1 was also centrifuged but re-suspended in 1 mL Diethyl ether (Saarchem, AR grade). The centrifugation cycle was repeated. This was done to remove the resident fats, oils and greases that was attached to the sludge flocs. The pellet was washed to get rid of excess diethyl ether by centrifuging with deionised water. The supernatant was discarded and the 3 sample pellets were ready for electron microscopy.

4.2.6.2 Use of electron microscope

At least 3 drops (approximately 0.1 mL) of mixed liquor from the pellet were placed directly onto stainless steel stubs, which were then fitted into a Peltier stage, cooled to 5 ^{0}C and viewed using a Philips model XL 30 (Holland) Environmental Scanning Electron Microscope (ESEM). The microscope was operated at 10 and 20 kV when viewing different samples. The ESEM chamber starting pressure was set at 6 Torr and carefully monitored and adjusted to slowly lower the relative humidity within the chamber. This procedure removes enough surface water from the sample to allow observation of topographical views of hydrated flocs (Liss *et al.*, 2002). Images of native hydrated flocs of the 3 sets of samples and their surface pore structure were stored on disks as TIFF files.

In addition the ESEM was also used to conduct an energy dispersive spectra of the extracellular matrix of the microbial flocs in the sample from reactor AE1. The results were reported as percentage weight of the various elements.

4.2.7 Chemistry of the effluent after treatment

The organic chemistry of the final effluent after biological treatment was determined using a GCMS. The procedure employed has been explained previously (Chapter 3, 3.2.4.1/3.2.4.2).

4.3 RESULTS

4.3.1 Pilot plant process

4.3.1.1 Wastewater characterization

The Company X produced approximately 195 m^3 of effluent per day from the refinery process, during a 24 h operational period. However, the flow rate was not critical for the current laboratory scale investigation. The characteristics of the raw wastewater, flocculated and corrected influent is shown in Table 4.2. Due to the large number of analysis conducted per parameter, the results are presented indicating the range i.e. highest and lowest values and the mean of all the analysis.

TABLE 4.2. Wastewater characteristics of the raw and prepared effluent

PARAMETERS	RAW EFFLUENT		POST-PRETREATMENT		PREPARED INFLUENT	
	RANGE	MEAN	RANGE	MEAN	RANGE	MEAN
PH	3 4.9	3.75	9.40 8.50	9.2	6.8 7.3	7.1
COD mg /L	6190 8470	7250	1800 1950	1890	310 1928	N/A
FOG mg /L	4990 6840	5950	250 1350	440	156 395	N/A
TP mg /L	2.80 492	4.72	1.95 202	3.95	1.51 197	N/A
TKN mg/L	1.00 37.00	7.42	1.00 22.00	6.72	1.00 13.00	N/A

* Range - Parameters indicating the minimum and maximum values measured during the course of the entire investigation.

* Raw effluent → effluent obtained from industry without any correction.

* Post - pretreatment → effluent that is pre-treated with flocculent

* Prepared Influent → effluent that is corrected after flocculation (pH adjusted /diluted/ supplemented)

Some of the notable features of the characteristics measured with reference to Table 4.2 are as follows :

- The pH of the raw effluent is acidic on collection and upon flocculation it becomes basic. The pH is corrected to neutral prior to being used as feed influent.
- The raw wastewater has a high organic pollution load as shown by the COD. However, approximately 74 % is removed upon flocculation. The prepared influent was diluted in accordance with the concentrations required for incremental COD increase during the course of the investigation.
- Large portion of the organic pollution load (COD) comprised of fats, oils and greases. Large amount of the FOG were removed during flocculation (± 92 %).
- The wide range of the total phosphate results were due to high phosphate concentration of the raw wastewater during Phase 1 &2 and limiting P conditions for the rest of the investigation. The above explanation also applies to the TKN. Both N &P were limiting nutrients in the raw wastewater and had to supplemented in the prepared influent during Phase 3-5.

For the sake of creating a clearer understanding of the results, the duration of operation the laboratory scale process and the results was divided into phases which encompassed common operating conditions.

4.3.1.2 Start -up process (Phase 1)

The process was initiated on the 18-5-2000 and was based on a 3 stage Phoredox configuration. The effluent from industry was not pre-treated and the high FOG concentration (8 200 mg/L) resulted in the decrease in the COD removal capacity i.e. from 82 % to 27 % within 3 days of operation with an influent COD concentration of 500 mg/L. Complete system failure was evident after 5 days of operation. In addition, the presence of a high FOG loading was evident by the formation of a oily scum layer on the surface of all reactors. The OUR recorded during the latter period of operation were low and varied between 8-10 mgO/L.h. No graphical data was presented due to short period of operation.

4.3.1.3 Phosphorus and carbon removal (Phase 2)

The process commenced on the 25-5-2000 and continued until 5-06-2000. The process configuration was based on a Phoredox design incorporating anaerobic and aerobic reactors which was equipped to remove phosphate biologically. During this phase the raw effluent was subjected to a pretreatment protocol to remove the high FOG. With reference to Figs 4.4 & 4.5 the following were observed. During the initial period comparatively good COD removal capacity was obtained with mean percentage COD removal of 70% and approximately 400 mg/L of COD was removed. However, within Day 4 of the process, the COD removal capacity was reduced (23%) with very low OUR being recorded i.e. 7 mgO/L.h (Fig 4.7). The FOG of the influent on the 26-5-00 was 150 mg/L and the effluent was 2.10 mg/L. The reduction in the efficiency of the process could be attributed to the fats coating the activated sludge flocs. There was also a steady decline in the MLSS over this period (Fig. 4.6). Throughout the phase there was more phosphate released in the effluent than introduced in the influent (Table 4.3). This could be attributed to various factors including the flocs in the aerobic reactor being starved of oxygen i.e. anaerobiosis due to the oil and therefore releasing the phosphates into the effluent

TABLE 4.3 Total Phosphate analysis during Phase 2

Results expressed a s mean values with sample standard deviation (SD) in parenthesis

PERIOD (DAYS)	INFLUENT TP (mg/L)	EFFLUENT TP (mg/L)
3	191 (3.75)	207 (5.54)
6	184 (9.51)	192 (7.76)
9	176 (4.02)	178 (3.30)
12	181 (6.75)	202 (2.19)

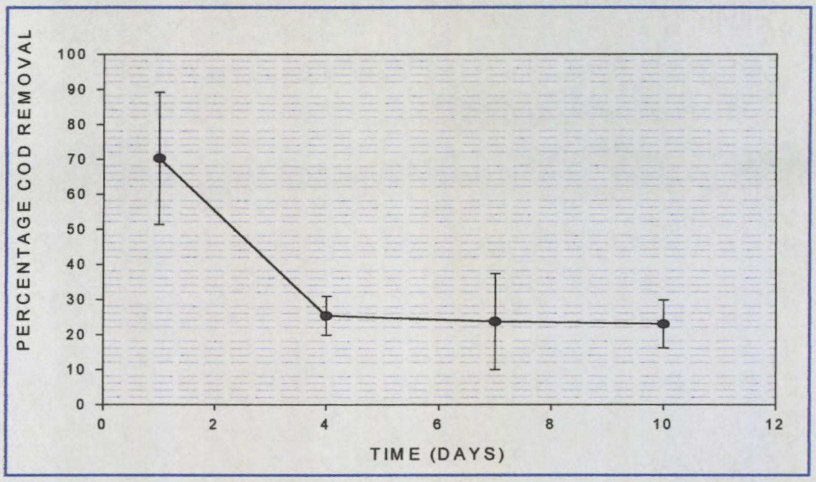


FIGURE 4.4 Percentage COD removal during Phase 2

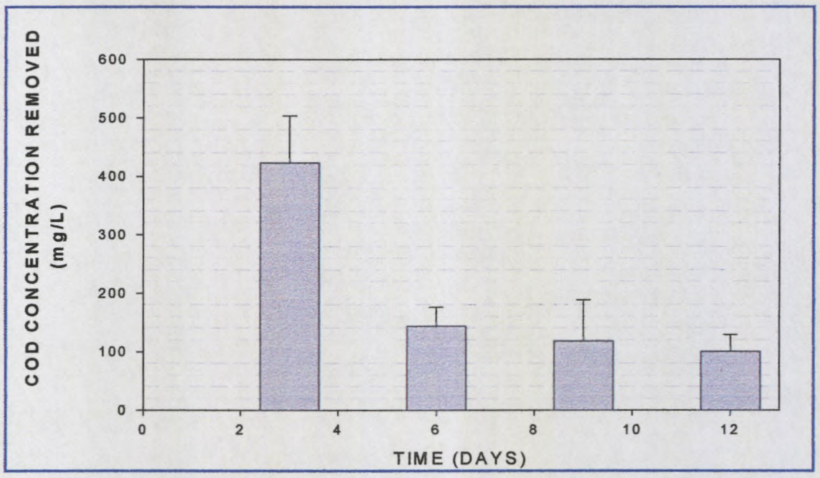


FIGURE 4.5 Concentration of COD removed during Phase 2. Histograms are means of multiple determinations(± standard error).

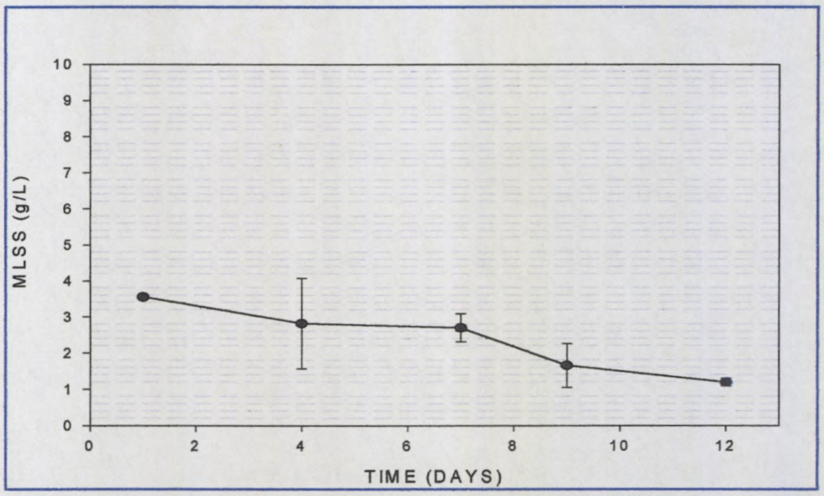


FIGURE 4.6 Mixed liquor suspended solids concentration during Phase 2

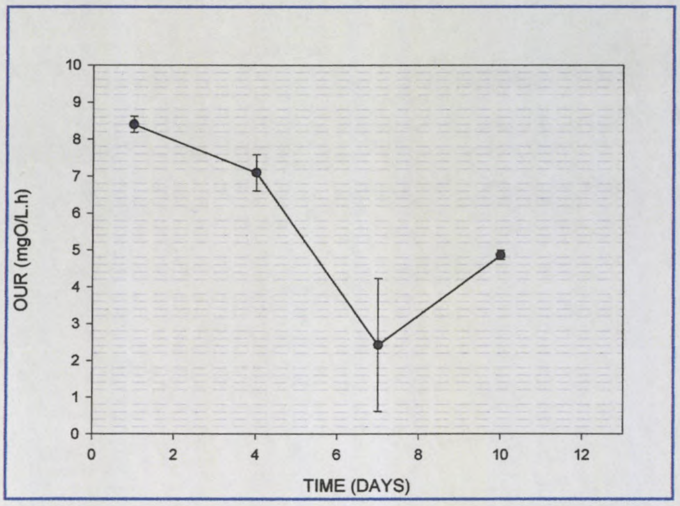


FIGURE 4.7 Mean oxygen utilization rate during Phase 2

4.3.1.4 Carbonaceous material removal (Phase 3)

The process configuration was changed from a Phoredox to a MLE process, focusing on carbon removal only (reason's were explained previously). The process was initiated on 6-6-2000 and due to nitrogen and phosphorus limitation in the effluent, N &P had to be supplemented in the effluent at a ratio of 100 :10 :1. The influent COD was maintained at 500 mg/L. Compared to phase 2, the amount of COD removed was higher (Fig's 4.8 and 4.9). In addition, there was marked improvement in the OUR indicating microbial activity and cell growth and COD consumption (Fig 4.10). However, during the latter period of phase 2 (Fig 4.6) and through phase 3 the MLSS steadily deceased, indicating a gradual loss of biomass (Fig 4.11). The histograms and line graphs presented are means of multiple determinations (± standard error).

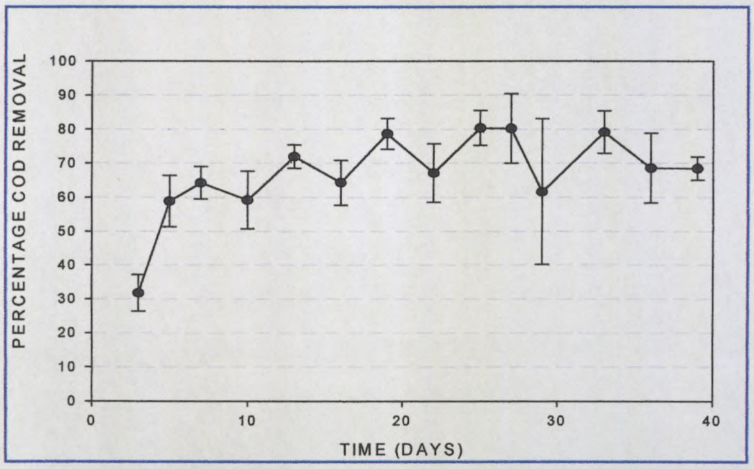


FIGURE 4.8 Percentage COD removal during Phase 3

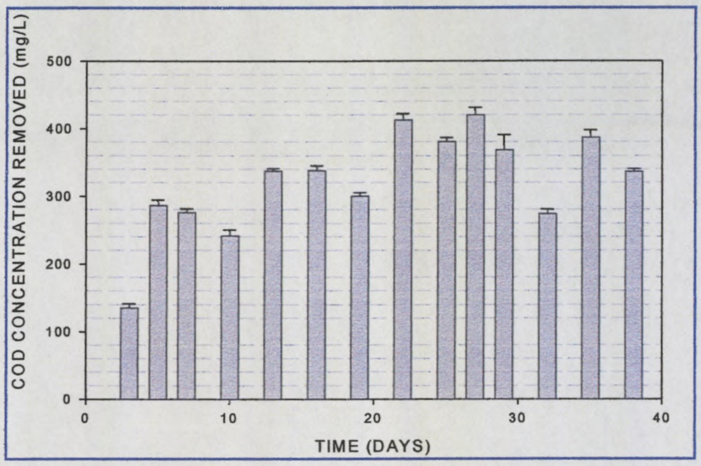


FIGURE 4.9 Concentration of COD removed during Phase 3

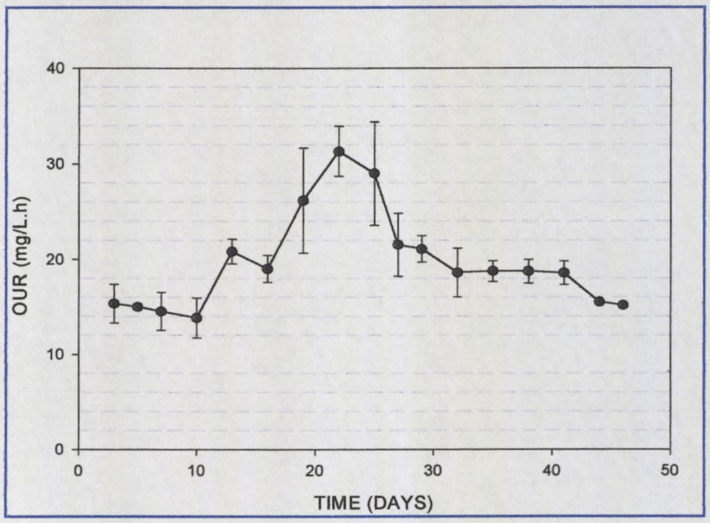


FIGURE 4.10 Mean oxygen utilization rate during Phase 3

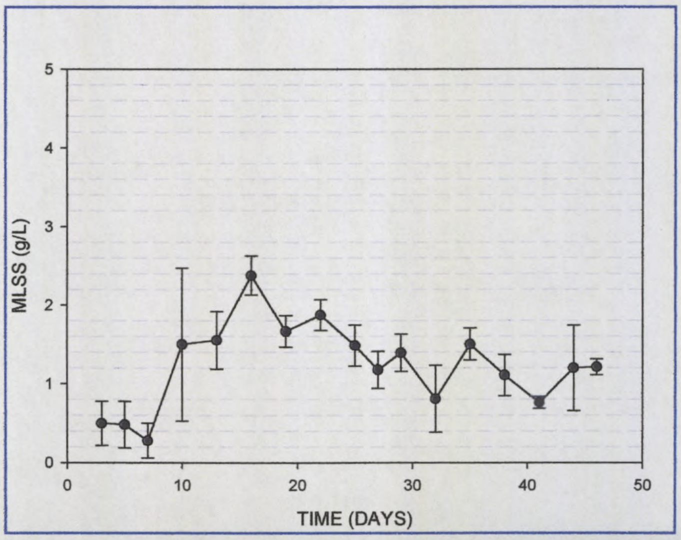


FIGURE 4. 11 Mixed liquor suspended solids concentration during Phase 3

Analysis of the effluent FOG results showed that the sludge was still retaining some of the oils. However, on the 5-7-2000 an operational problem occurred when the dissolved oxygen meter was erroneously set at 6 instead of 5 mgO/L.h. and the problem was only rectified after 24 h. This resulted in a bulking problem due to over aeration with high diluted SVI's being recorded i.e. 172 mL/g. The latter problem caused a loss of MLSS and a gradual decrease in the OUR (Fig's 4.10 and 4.11). Due to decrease in the efficiency, the process was shut down and the entire system including the tubing was cleaned.

4.3.1.5 Optimization of the process (Phase 4)

A) <u>Phase 4 A (28-7-00 – 15-9-00)</u>

The laboratory scale process was restarted with fresh seed inoculum from Darvil . The MLSS was 2546 mg/L. The COD of the influent feed was increased to 800 mg/L. The mean COD removal efficiency continued to improve and 13 days after the resumption of the process, the percentage removal was approximately 90% (Fig 4.12) and the concentration COD removed was approximately 722 mg/L (Fig 4.13). The high COD removal trend continued during most of this phase. In addition there was a marked improvement in the OUR during much of this phase (Fig 4.14). However, 35 days later, there was a slight decrease in the process performance i.e. reduction to approximately 75 % (Fig 4.12) but the process efficiency improved within a few days. During the latter period a dip in the OUR was also evident (Fig 4.14).

The FSA profile during this period was as follows :

- High effluent FSA concentration i.e. range 15 -27 mgN/L
- The introduction of an anoxic zone (MLE process) and reduction of the influent nitrogen concentration i.e. 100 :10:1 (C:N:P) to 100 : 5 : 1. eliminated the problem and the effluent FSA was reduced to 2-6 mgN/L, which was at acceptable limits
- The effluent TKN was also low i.e. range 1-9 mgN/L.

On correction of the process configuration and influent nitrogen supplementation, the above trend of low effluent nitrogen concentration continued for most of the investigation.

The COD of the influent feed which was measured on a daily basis varied slightly and could have been caused by some resident flocculent in the feed tank. This phenomenon continued throughout the investigation. In addition different batches of effluent were used within a phase. Although the influent COD concentration was controlled, the consistency in the chemistry of the different batches of effluent was uncontrollable. Therefore the results indicating trends were expressed as percentage and concentration of COD removed using mean and standard deviations. The time period 15-9-00, the process efficiency had reached approximately 90 % and it appeared that it had reached steady state.

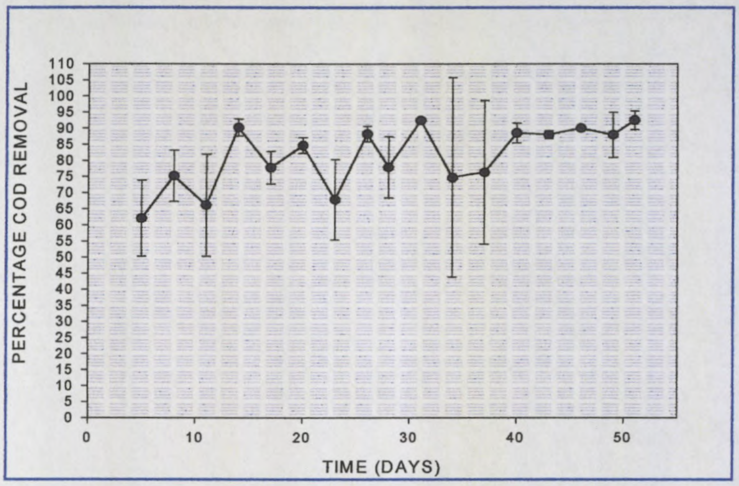


FIGURE 4.12 Percentage COD removal during Phase 4 A

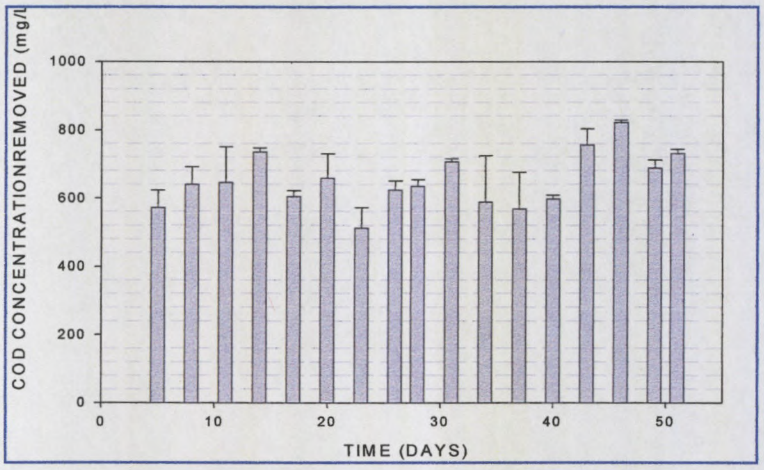


FIGURE 4.13 Concentration of COD removed during Phase 4 A

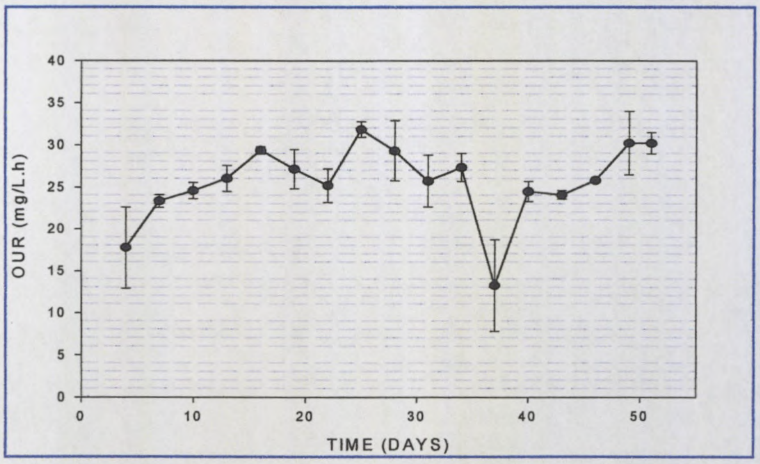


FIGURE 4.14 Mean oxygen utilization rate during Phase 4 A

B) Phase 4 B (16-9-00– 19-10-00)

This phase was a continuation of the previous but separated due to an increase of the influent feed concentration from 800 mg/L to 1 200 mg/L. The MLSS was consistent at approximately 3500 mg/L. During this period of investigation, the system appeared to have reached steady state with sustained good COD removal efficiencies. The percentage COD removed appeared to have peaked at 92 % and the range was between 80 - 90 % during the phase (Fig 4.15). The concentration of COD removed peaked at 1142 mg/L with a range of between 800 and 1142 mg/L (Fig 4.16). Throughout the phase the OUR remained high peaking at 38 mgO/L.h. (Fig 4.17) and the trend of the graph was similar to those reflecting COD removal . The mean MLVSS/MLSS ratios during this phase were consistent, ranging from 0.8 to 0.9 mgVSS/mgTSS. (Fig 4.18).

Once again there was a substantial variation in the influent feed COD concentration which resulted in an erratic F/M ratio (data presented later).

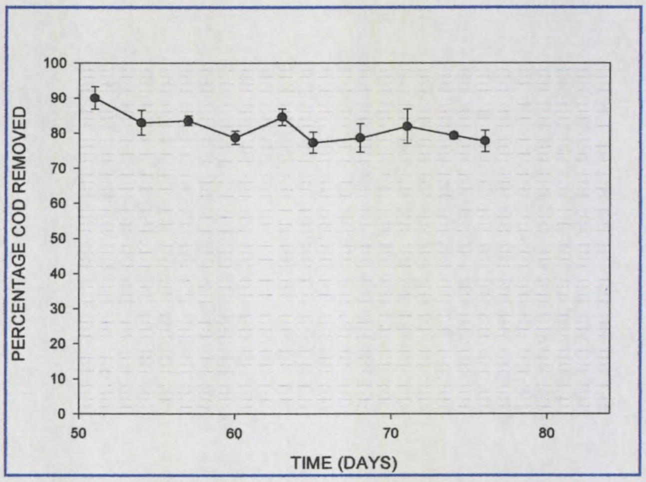


FIGURE 4.15 Percentage COD removal during Phase 4 B

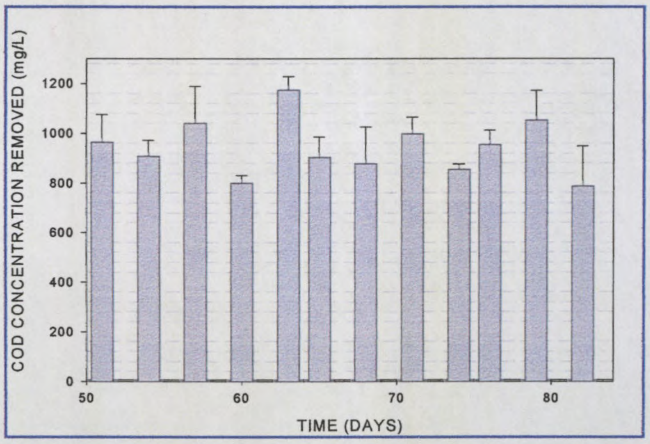


FIGURE 4.16 Concentration of COD removed during Phase 4 B

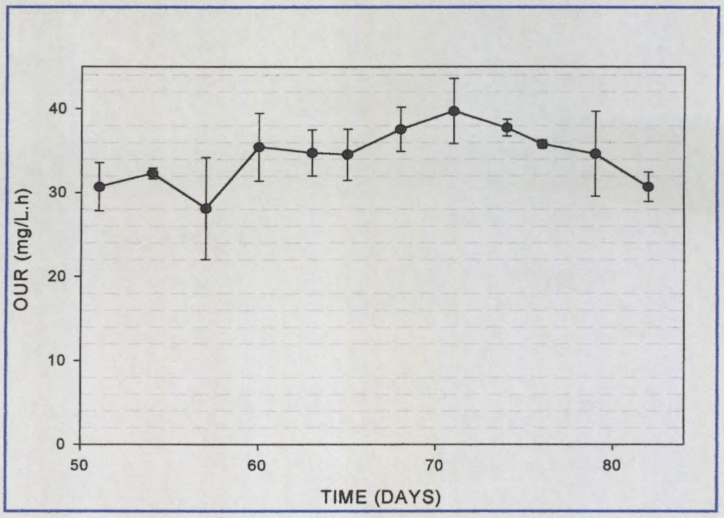


FIGURE 4.17 Mean oxygen utilization rate during Phase 4 B

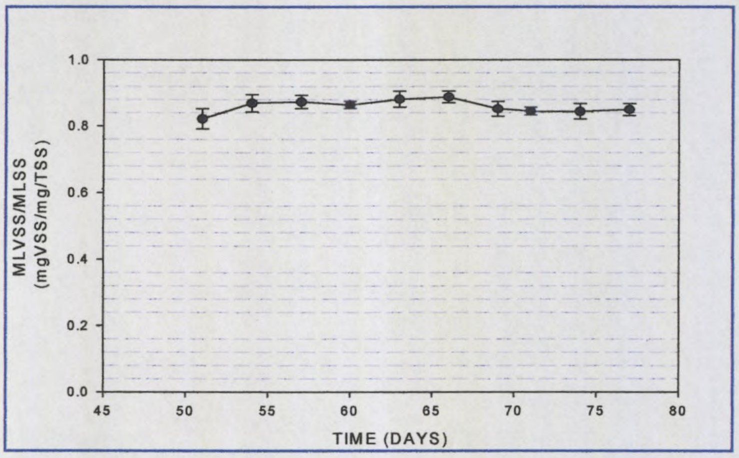


FIGURE 4.18 Mean MLVSS/MLSS ratios during Phase 4 B

C) Phase 4 C (20-10-00 – 5-11-00)

There was a disruption in the feed supply from industry. In order to sustain the system, the final effluent had to be recycled. The problem persisted from Day 82 until Day 91, when the influent supply from industry was resumed. During this period, there was a decrease in the COD removal efficiency and improvement when fresh influent was supplied (Fig 4.19). The decrease in the metabolic activity of the activated sludge microflora was also evident with the decrease in the OUR during the period of influent disruption. However, the OUR gradually increased on resumption of fresh effluent feed (Fig 4.20)

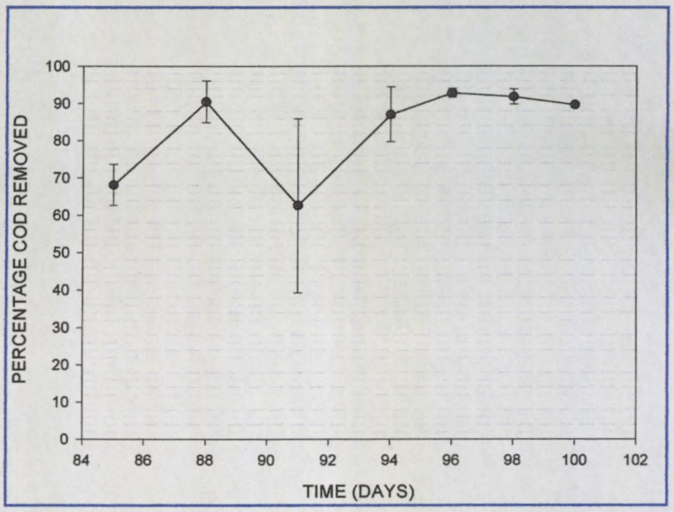


FIGURE 4.19 Percentage COD removal during Phase 4 C

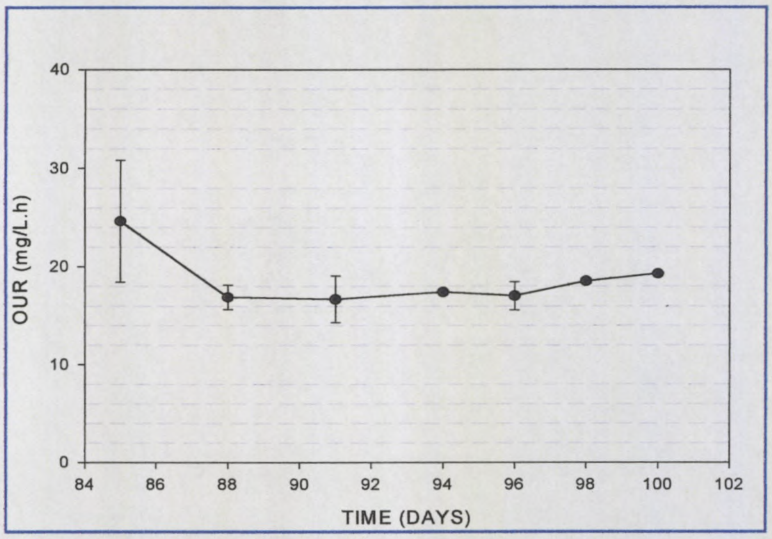


FIGURE 4.20 Mean oxygen utilization rate during Phase 4 C

D) Phase 4 D (9-11-00 – 30-11-00)

The high COD removal efficiency continued during phase 4D with a slight decrease in COD concentration removed towards the end of the phase (Figs 4.21 &4.22). The mean OUR also remained high, with a maximum of 38 mgO/L.h and decreased towards the end of the phase, reflecting a similar pattern as the COD removal graphs (Fig 4.23). The VSS/MLSS ratio remained relatively constant (Fig 4.24). There was periodic episodes of foaming during this period but decreased substantially towards the end of the phase

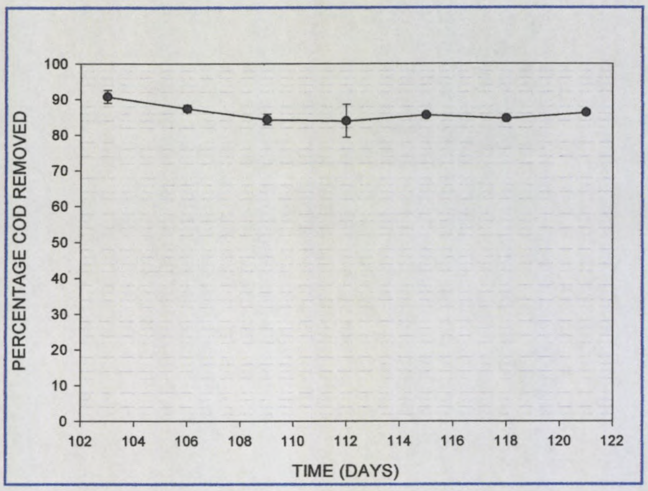


FIGURE 4.21 Percentage COD removal during Phase 4 D

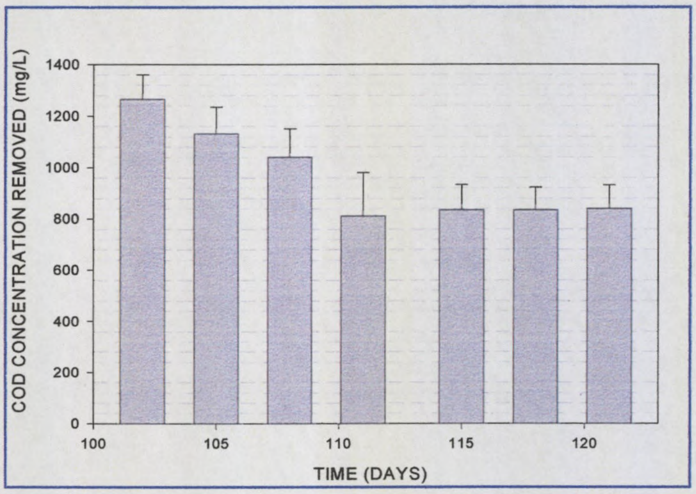


FIGURE 4.22 Concentration of COD removed during Phase 4 D

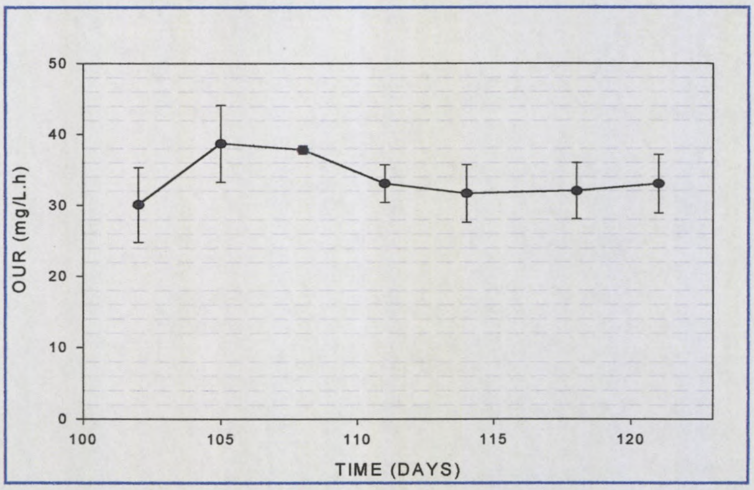


FIGURE 4.23 Mean oxygen utilization rate during Phase 4 D

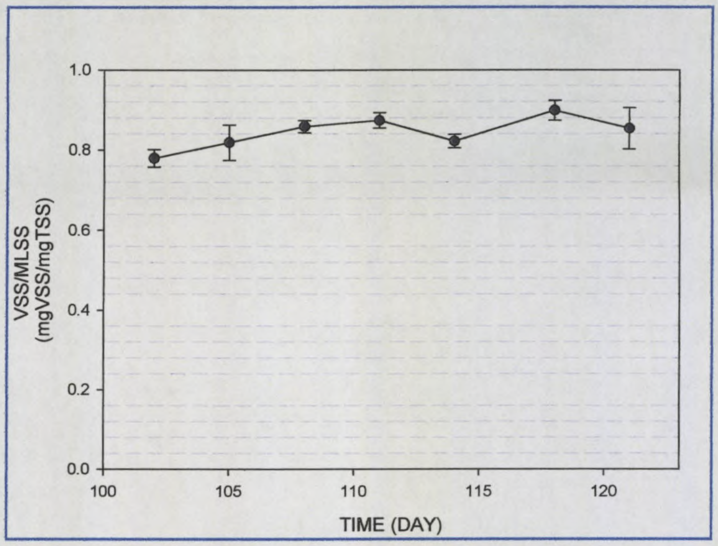


FIGURE 4.24 Mean MLVSS/MLSS ratios during Phase 4 D

E) Phase 4 E (1-12-00 – 1-2-01)

The influent feed concentration was increased to 1 500 mg/L. However, during the first period of the phase the COD removal efficiencies appeared erratic with a decrease and subsequent increase between days 125 and 130 (Fig 4.25). This could be attributed to either experimental error or perturbations within the activated sludge system. However, the latter is more probable since there was also a decrease in the OUR during the same period (Fig 4.27). On the 12-12-00 the influent feed COD was increased to 1 800 mg/L. The COD removal efficiencies improved with the highest being 1 550 mg/L COD removed (Fig 4.26). There was a substantial variation in the COD removal efficiency during the mid and latter portions of this phase. The decrease in COD removal between days 165 and 170 could be attributed to experimental error since high standard deviations were recorded (Fig 4.25).

There was a shut-down at the industry on the 27-1-01 and therefore termination in the supply of influent feed for a period of 5 weeks. The process was shut down and no analysis were conducted during this period. However, in order to sustain the biomass, final effluent from another edible oil company was obtained and served as feed influent. The viability of the microorganisms in the activated sludge was monitored by the OUR and remained within the range of 20 - 25 mgO/L.h.

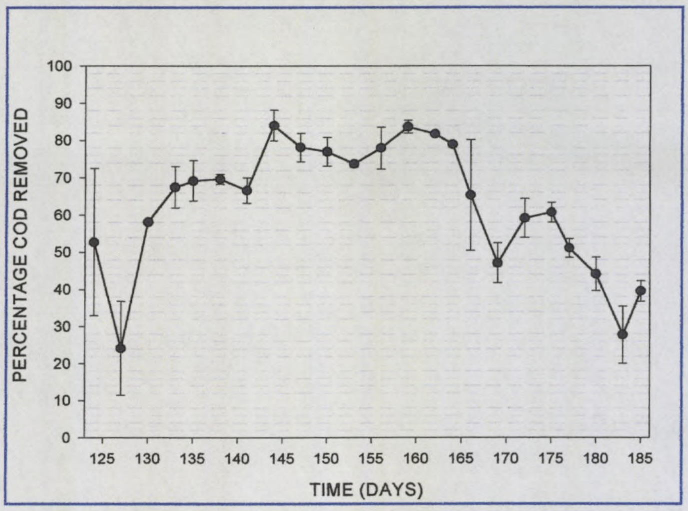


FIGURE 4.25 Percentage COD removal during Phase 4 E

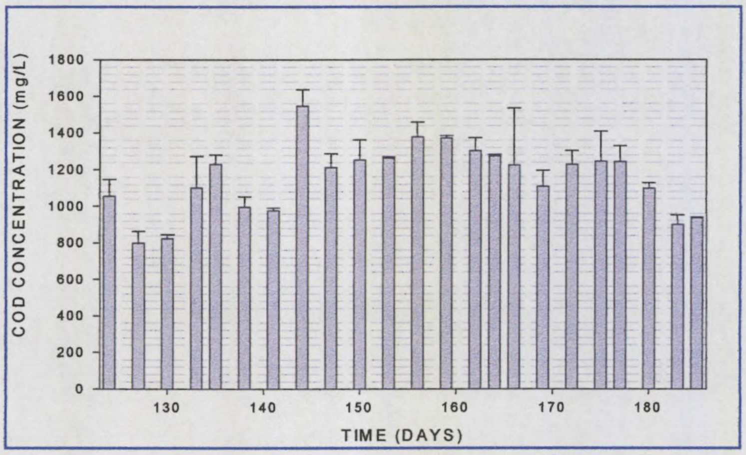


FIGURE 4.26 Concentration of COD removed during Phase 4 E

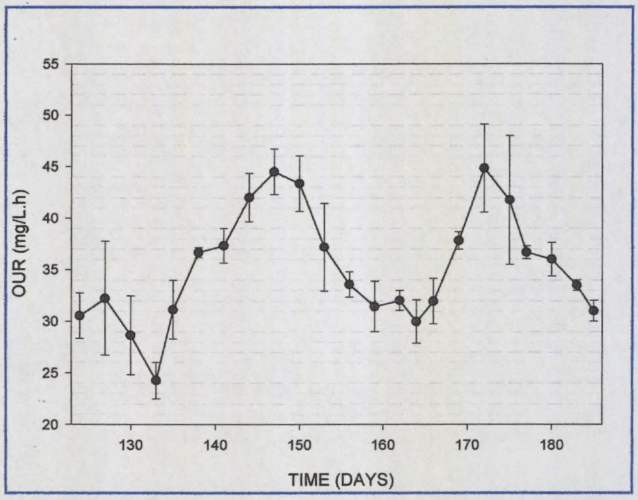


FIGURE 4.27 Mean oxygen utilization rate during Phase 4 E

4.3.1.6 Optimization of process - continued (Phase 5)

A) Phase 5 A (6-3-01 – 26-3-01)

The process was initiated and remained as an MLE configuration. The MLSS on start up was 2542 mg/L. However, the strength of the influent COD after pretreatment decreased slightly(compared to Phase 4 E) and the mean COD for the first 3 days was 1542 mg/L. The decrease in COD of the influent was dependent on effluent obtained from industry, a parameter which could not be controlled. The process performance on start up was good with 85 % COD removal at the beginning with a slight decrease as the phase progressed (Fig 4. 28). The maximum mean COD concentration removed during the first stage of the phase was approximately 1345 mg/L and from day 5 onwards there was a slight decrease in the concentration removed (Fig 4.29). The mean OUR was slightly erratic (Fig 4.30) but reflected a similar trend as the COD removal efficiency. The VSS/MLSS ratio was consistent and the range was between 0.8 and 0.9.

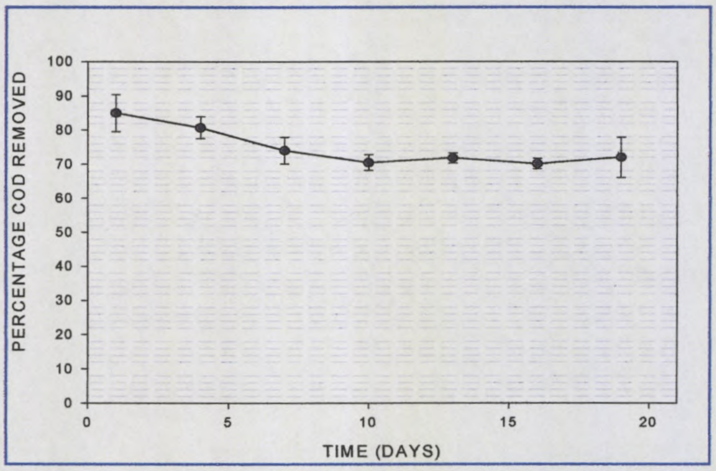


FIGURE 4. 28 Percentage COD removal during Phase 5 A

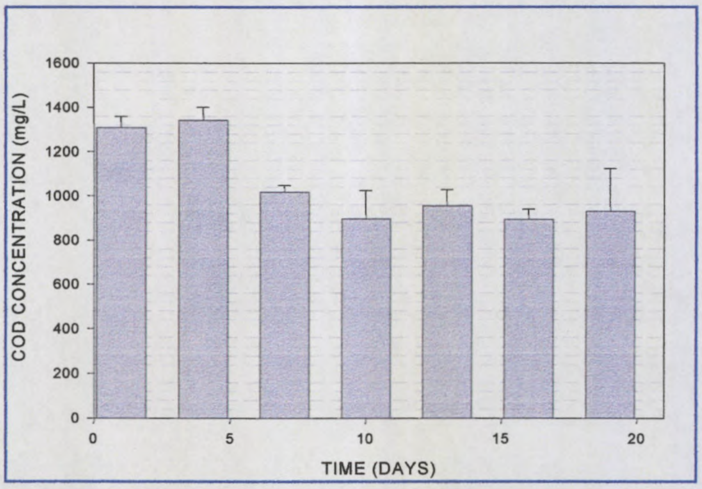


FIGURE 4.29 Concentration of COD removed during Phase 5 A

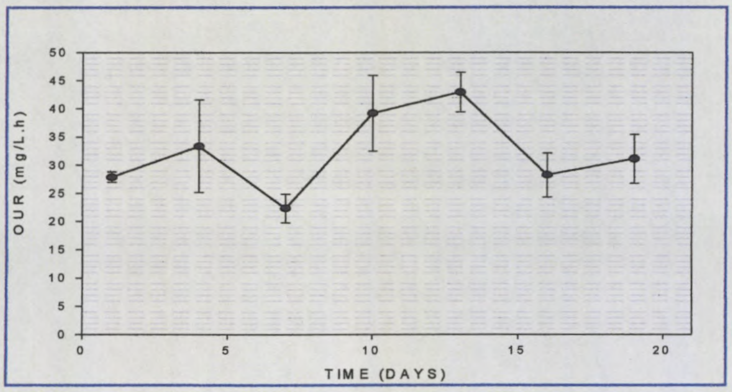


FIGURE 4.30 Mean oxygen utilization rate during phase 5 A

B) Phase 5 B (31-3-01 – 17-4-01)

Once again the influent feed obtained from industry was of low strength and the average mean influent COD was 701 mg/L. The percentage COD removal fluctuated between 78 and 88 % during this period (Fig 4.31). The COD concentration removed ranged from 461 mg/L to 680 mg/L (Fig 4.32). As expected, the relatively low removal efficiencies were due primarily to low influent feed concentrations.

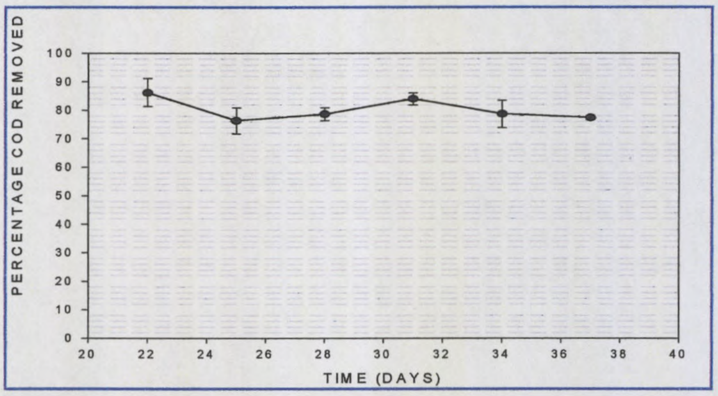


FIGURE 4.31 Percentage COD removal during Phase 5 B

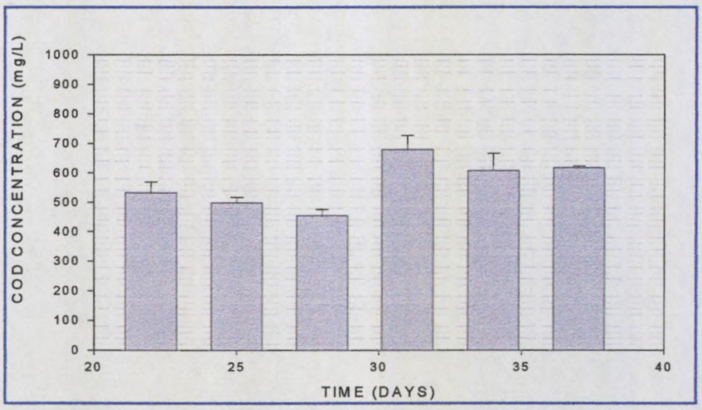


FIGURE 4.32 Concentration of COD removed during Phase 5 B

C) Phase 5 C (18-4-01 – 21-5-01)

The influent feed concentration improved with mean COD concentration at 1465 mg/L. The percentage COD removal during the phase peaked at 90 % removal and was consistent throughout most of the phase until day 72 when there was a decrease (Fig 4.33)and the maximum COD concentration removed was 1324 mg/L (Fig 4.34). However, as observed in previous phases, the trend of the OUR curve did not follow a similar pattern as the COD removal graphs (Fig 4.35). These erratic results could be attributed to experimental error as seen by the high standard deviations. Except for the first 7 days, the MLVSS/MLSS ratio was consistent averaging at 0.8 mgVSS/mgTSS (Fig 4.36).

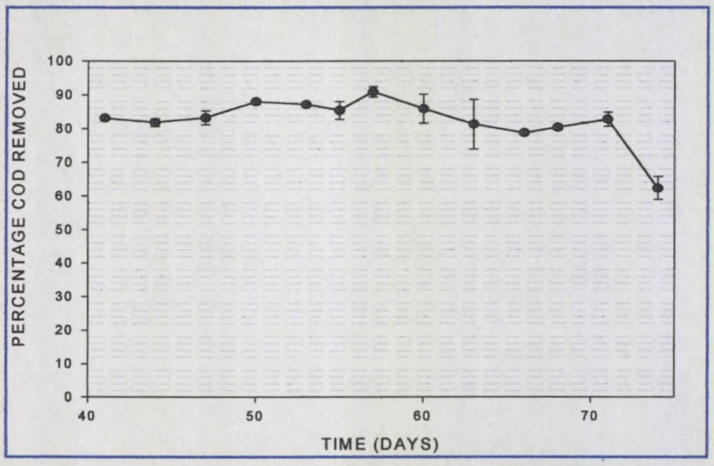


FIGURE 4.33 Percentage COD removal during Phase 5 C

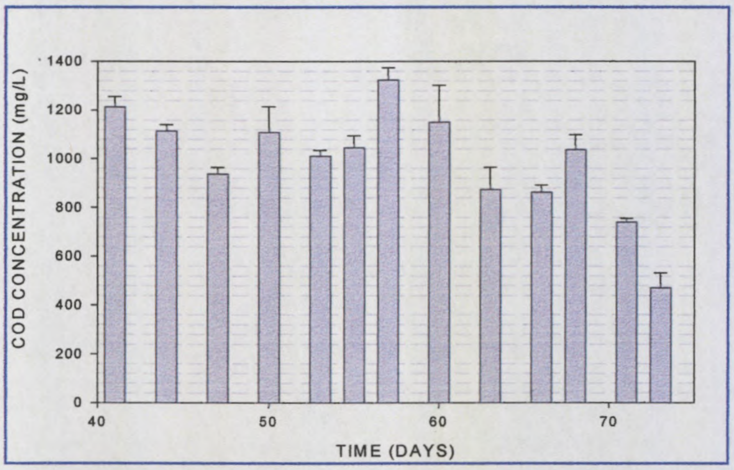


FIGURE 4.34 Concentration of COD removed during Phase 5 C

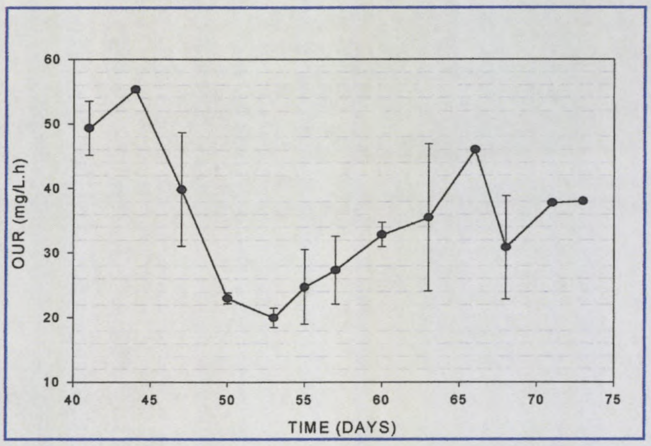


FIGURE 4.35 Mean oxygen utilization rate during Phase 5 C

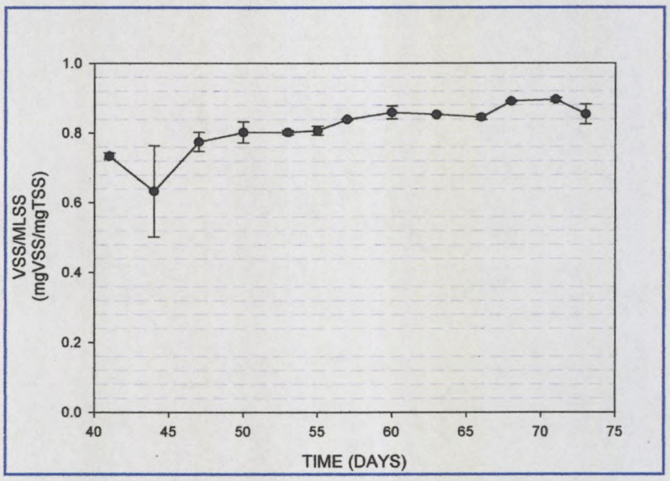


FIGURE 4.36 Mean MLVSS/MLSS ratio during Phase 5 C

D) Phase 5 D (22-5-01 – 13-6-01)

A change in the effluent batch from industry once again resulted in a lower influent feed COD concentration of 915 mg/L which fluctuated substantially during this phase. The decrease in COD removal towards the end of the previous phase was reversed during the first 5 days of this phase. From day 79 onwards, the percentage COD removal was consistent (Fig 4.37). The maximum COD concentration removed during this phase was 937 mg/L (Fig 4.38). The mean OUR was 32mgO/L.h. The MLVSS/MLSS ratio ranged between 0.82 and 0.90. During this period there was persistent foaming in the aerobic reactors but did not appear to impact on the system performance.

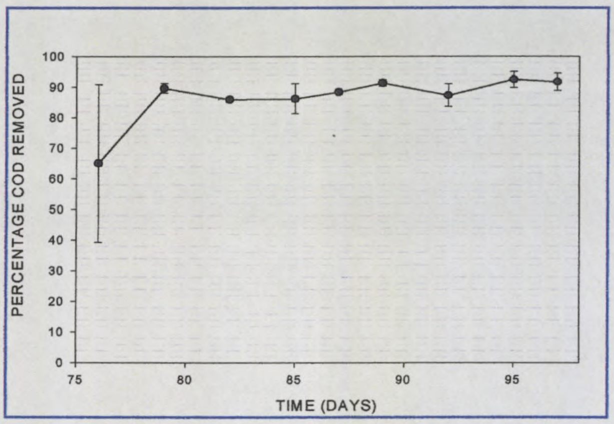


FIGURE 4.37 Percentage COD removal during Phase 5 D

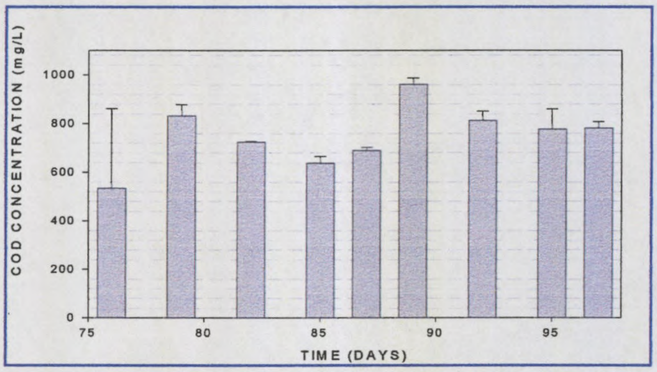


FIGURE 4.38 Concentration of COD removed during Phase 5 D

E) Phase 5 E (14-6-01 – 26-7-01)

During this phase the mean influent COD decreased to 712 mg/L. As the phase progressed, there was a gradual decrease in the percentage and concentration of COD removed (Figs 4.39 & 4.40). Although the mean OUR was erratic, the overall trend of the graph was similar to COD removal (Fig 4.41). The mean MLVSS/MLSS ratio was consistent and fluctuated between 0.8 and 0.9 (Fig 4.42).

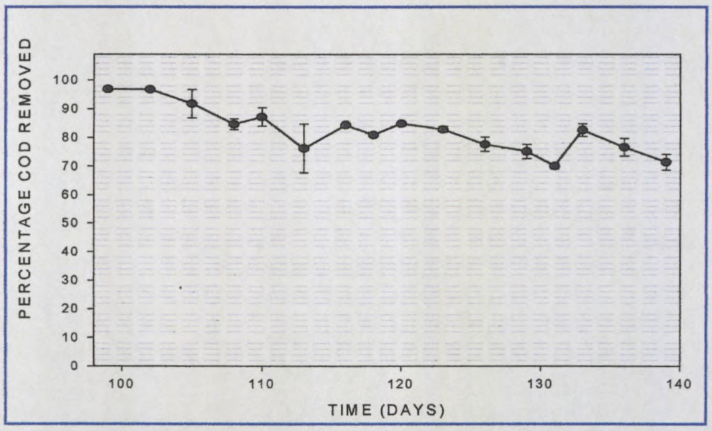


FIGURE 4.39 Percentage COD removal during Phase 5 E

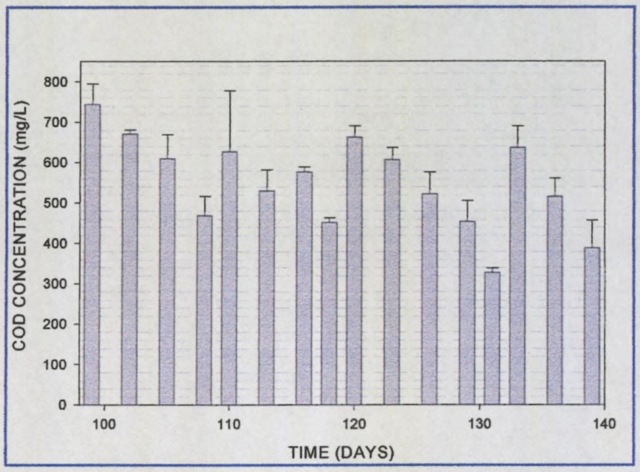


FIGURE 4.40 Concentration of COD removed during Phase 5 E

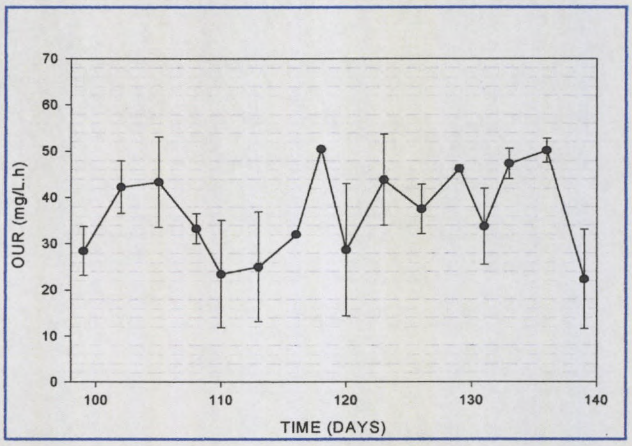


FIGURE 4.41 Mean oxygen utilization rate during Phase 5 E

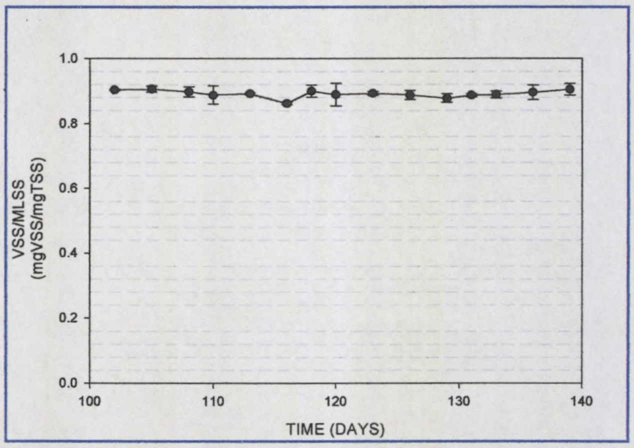


FIGURE 4.42 Mean MLVSS/MLSS ratio during Phase 5 E

F) Phase 5 F (28-7-01 – 7-08-01)

Introduction of a new batch of effluent resulted in an increase in the feed influent COD concentration when compared to the previous phase. The system responded immediately with improved COD removal capacity ranging between 80 and 93 % (Fig 4.43). The concentration removed was consistent ranging between 1000 and 1200 mg/L COD (Fig 4.44). The mean OUR ranged between 23 and 28 mg/L (Fig 4.45)and the MLVSS/MLSS ratio was consistent and ranged between 0.84 and 0.87 (Fig 4.46).

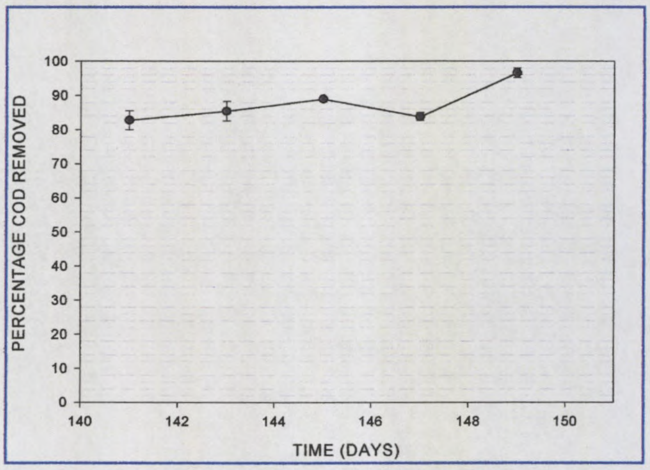


FIGURE 4.43 Percentage COD removal during Phase 5 F

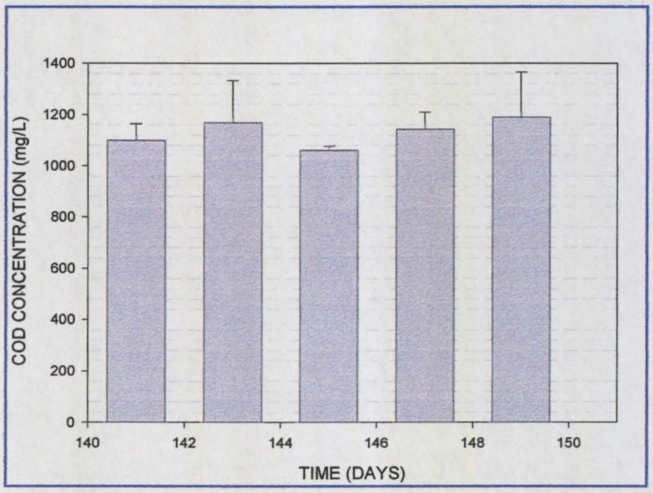


FIGURE 4.44 Concentration of COD removed during Phase 5 F

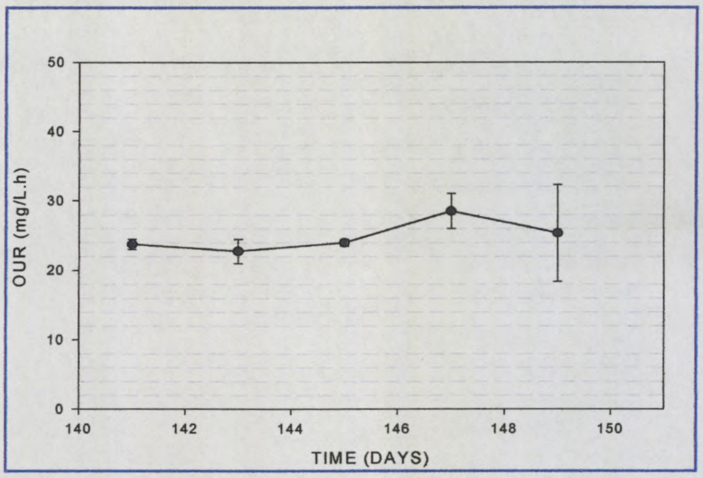


FIGURE 4.45 Mean oxygen utilization rate during Phase 5 F

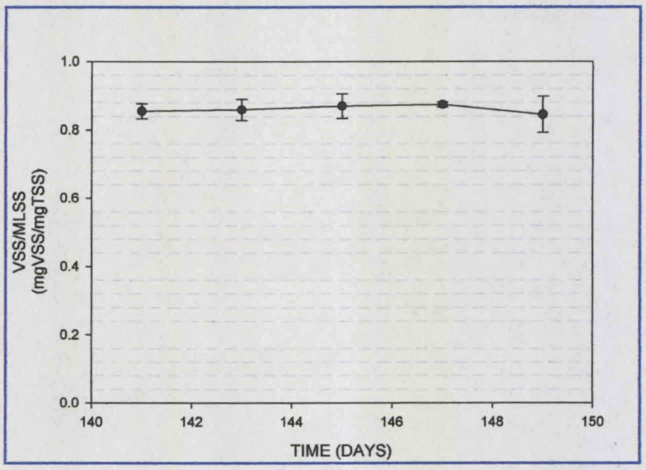


FIGURE 4.46 MLVSS/MLSS ratio during Phase 5 F

G) Phase 5 G (8-8-01 – 30-9-01)

The batch of effluent collected from industry was lower in strength than the previous batch. There was a gradual decrease in percentage COD removed but on day 172 there was a rapid decline. However the process corrected itself and the efficiency was normalized. Towards the end of the phase the process efficiency once again began to decrease (Fig 4.47). The concentration of COD removed fluctuated between 290 and 680 mg/L (Fig 4.48). However, the OUR also began to decrease and the mean OUR at the beginning of the phase was 31 mgO/L.h which subsequently decreased to 19 mgO/L.h towards the end of the phase. The MLVSS/MLSS ratio remained constant and ranged between 0.8 and 0.9 mgVSS/mgTSS..

The desired objectives of the of this aspect of the research was achieved and the process was terminated on the 2-10-01.

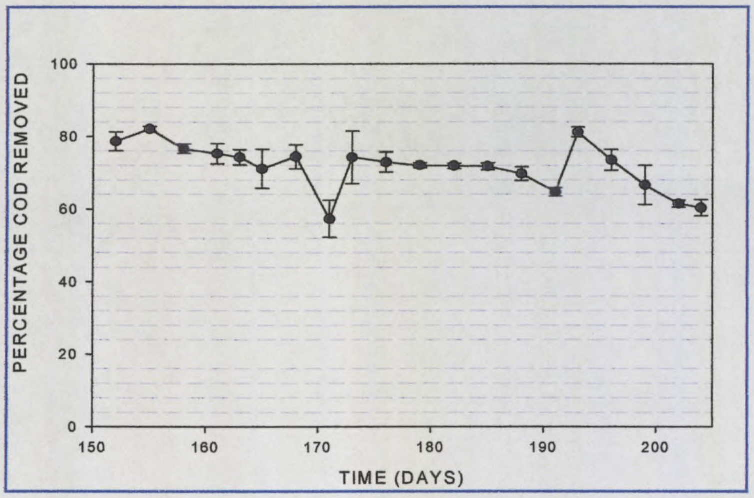


FIGURE 4.47 Percentage COD removal during Phase 5 G

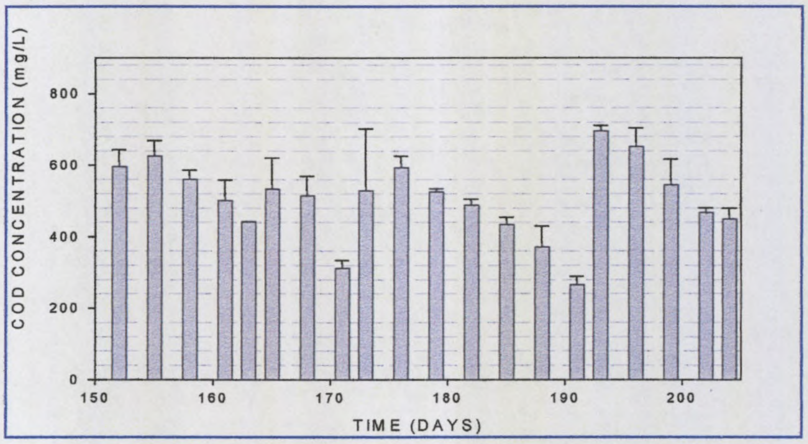
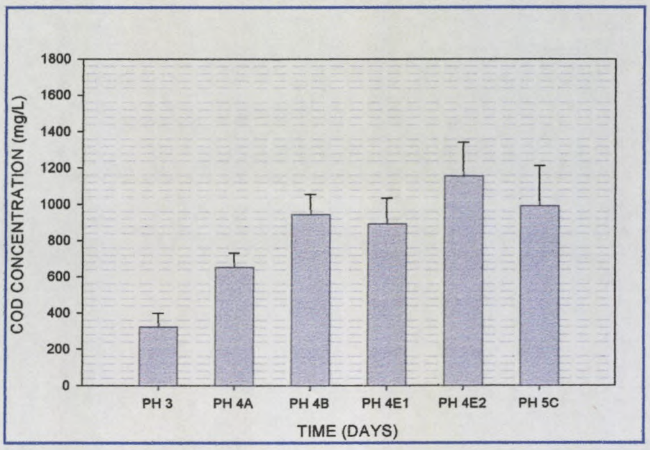


FIGURE 4.48 Concentration of COD removed during Phase 5 G

4.3.2 Process Performance

4.3.2.1 Performance with increase in COD concentration

One of the aims of the research was to assess process performance with increase in influent COD concentration. This was subjective to the COD of the raw effluent obtained from industry and operational limitations experienced during the treatment process. The phases presented in Fig 4.49 were selected subject to the process achieving steady state performance and to show increase in COD removed with increase in COD influent concentration. The influent concentration ranged from 500 to 1800 mg/L COD(undiluted after pretreatment). It also should be noted that the values reflected in Fig 4.49 are an average within the phase and not the actual values, which in some cases are higher. However, it reflects a trend of COD removed with increase in influent COD concentration for the entire treatment process.



- each bar reflects the mean value of COD removed during the respective phases

FIGURE 4.49 Increase in COD removed with increase in influent COD concentration (The increase in concentrations across phases and periods are reflected in Table 4.4)

TABLE 4.4 Increase in COD concentration across phases and F/M ratios

PHASE	PERIOD	INFLUENT COD CONCENTRATION (mg/L)	MEAN F/M RATIOS
3 (PH 3)	06/06/00 - 21/07/00	500	0.5476 (0.19)
4 A (PH 4A)	28/07/00 - 16/09/00	800	0.2740 (0.13)
4 B (PH 4B)	17/09/00 - 19/10/00	1200	0.4838 (0.14)
4 E1 (PH 4E1)	01/12/00 - 11/12/00	1500	1.3363 (0.31)
4 E2 (PH 4E2)	12/12/00 - 01/02/01	1800	0.9302 (0.42)
5 C (PH 5C)	18/04/01 - 21/05/01	1800	0.6133 (0.14)

(The F/M ratios are expressed as mean values with samples standard deviations in parenthesis)

With reference to Fig 4.49, the results show that as the influent COD concentration ($S_{ti)}$ is increased, the amount of COD removed by the process also increases. However, there was a slight decrease in concentration of COD removed during phase 4 E1 when compared to phase 4 B, although the concentrations were higher i.e. 1 500 mg/L. A similar trend was noticed during phase 5 C when compared to phase 4 E2. Maximum COD removal was achieved during phase 4 E2 and although the mean influent concentrations during phase 5 C were the same, the concentrations removed were slightly lower. It should be noted that 1800 mg/L COD was the maximum strength of the influent i.e. effluent from industry which is undiluted after pretreatment. The steady state performance of the process is presented in the graphs of the individual phases where the operating conditions were similar. The change in the color of the mixed liquor during the laboratory scale treatment process is presented in Fig 4.50.

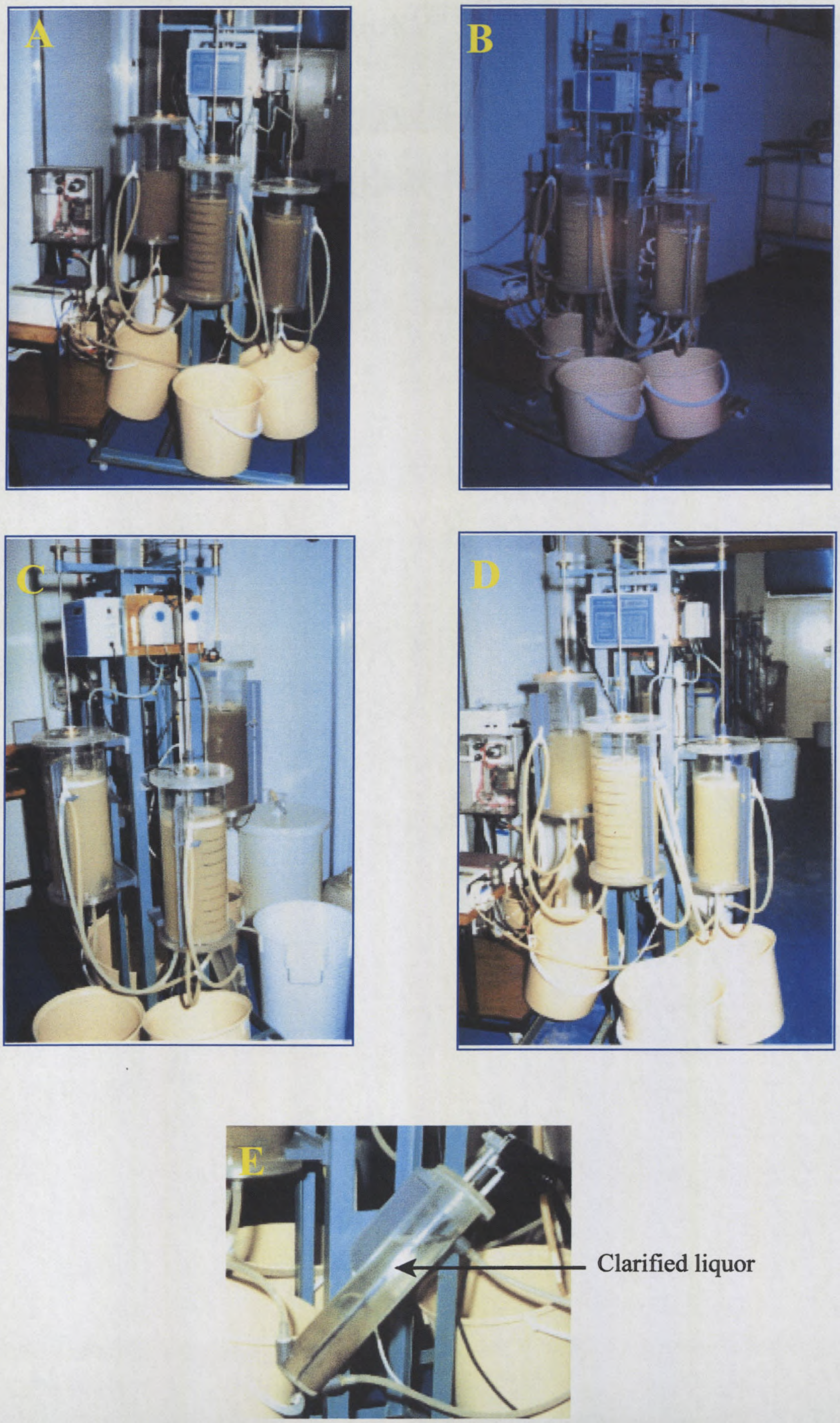


FIGURE 4.50 (A -D) Transition in the color of the mixed liquor during the laboratory scale activated sludge treatment process. (E) Photograph of clarifier.

4.3.2.2 Associated problems

During the period of operation of the laboratory scale treatment process, some of the major problems encountered were as follows :

- Disruption in the supply of effluent from industry which resulted in having to recycle the process final effluent to sustain the system
- blockages in the tubes due to high FOG (Phase 3) resulting in loss of MLSS and subsequent diminished process efficiency
- associated bulking problem also resulting in poor settling during phase 3 which recorded a DSVI of 244.5 mL/g.
- inconsistency in the industrial effluent strength (COD) amongst batches thus prevented achieving the desired process influent concentrations i.e. after pretreatment the S_{ti} was sometimes lower than desired for the phase
- occasional mechanical problems associated with peristaltic pumps thus effecting daily flow rates and infrequent disruption of electricity thus temporarily shutting down the process
- fractionation of the S_{ti} was not possible due to the variable nature and chemistry amongst the different batches.

4.3.3 Electron microscopy

4.3.3.1 Activated sludge floc structure

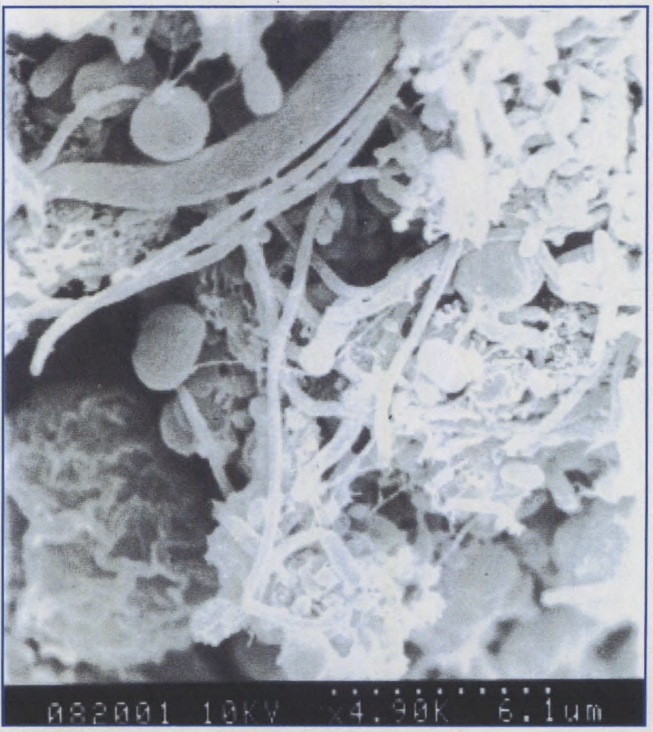
Three sets of activated sludge samples were subjected to Environmental scanning electron microscopic (ESEM) investigations. The Darvil(wastewater treatment plant) sludge sample served as a control since it was used as seed inoculum at the beginning of the process. The samples obtained from the laboratory scale reactor were prepared separately, one set of sample that was pre-treated with a non-polar solvent i.e. diethyl ether and the other a standard mixed liquor sample. The ESEM images of the Darvil sludge sample reveals the topography of the floc surface and only a portion of the entire floc in a partially hydrated state displaying an abundance of microorganisms (Fig 4.51 A). However, higher magnification reveals greater floc ultrastructural details. The typical floc surface was dominated by bacterial cells, both cocci and rod shaped that were partially embedded in a loose extracellular polysaccharide (EPS) matrix (Figs 4.51 B-D). The ESEM micrographs of the flocs that were subjected to the oil effluent revealed a different topographical structure. At low magnification, the flocs appear to coalesce and attach together, possibly due to the surface of the flocs imbibing some of the oil from the effluent (Fig 4.52 A and B)). In addition, a protozoan is clearly evident amongst the flocs (Fig 4.52 C).

Higher magnification reveals much more information on the ultrastructural details of the flocs. Although a dense matrix of microbial cells are visible, the surface structure of the flocs appear to be more flat and smooth and covered by a oil film (Fig 4.52 D). There is a noticeable difference between the flocs from the Darvil sludge and those from the oil effluent sample. At higher magnification the diversity of the micro-flora and especially bacteria is evident with cocci, short and long rods, and filaments clearly visible. The surface is less diffuse and although cells can be seen protruding there are more flat and smooth areas of highly condensed and less hydrated material (Figs 4.52 E and F)when compared to the control (Darvil). The sludge sample from the treatment process that was pre-treated with a non-polar solvent (diethyl ether), which removed most of the resident oil presented bacterial cells with a more defined structure clearly showing the shape of the bacteria. The floc appears more hydrated. Some of the EPS matrix is visible with the bacterial cells partially embedded in the loose EPS matrix (Fig 4.53).

A large number of samples were subjected to ESEM investigations during the course of the research and many images were captured. However for the purpose of this report, not all the images could be presented and a few were selected to best represent and depict the results

A

B



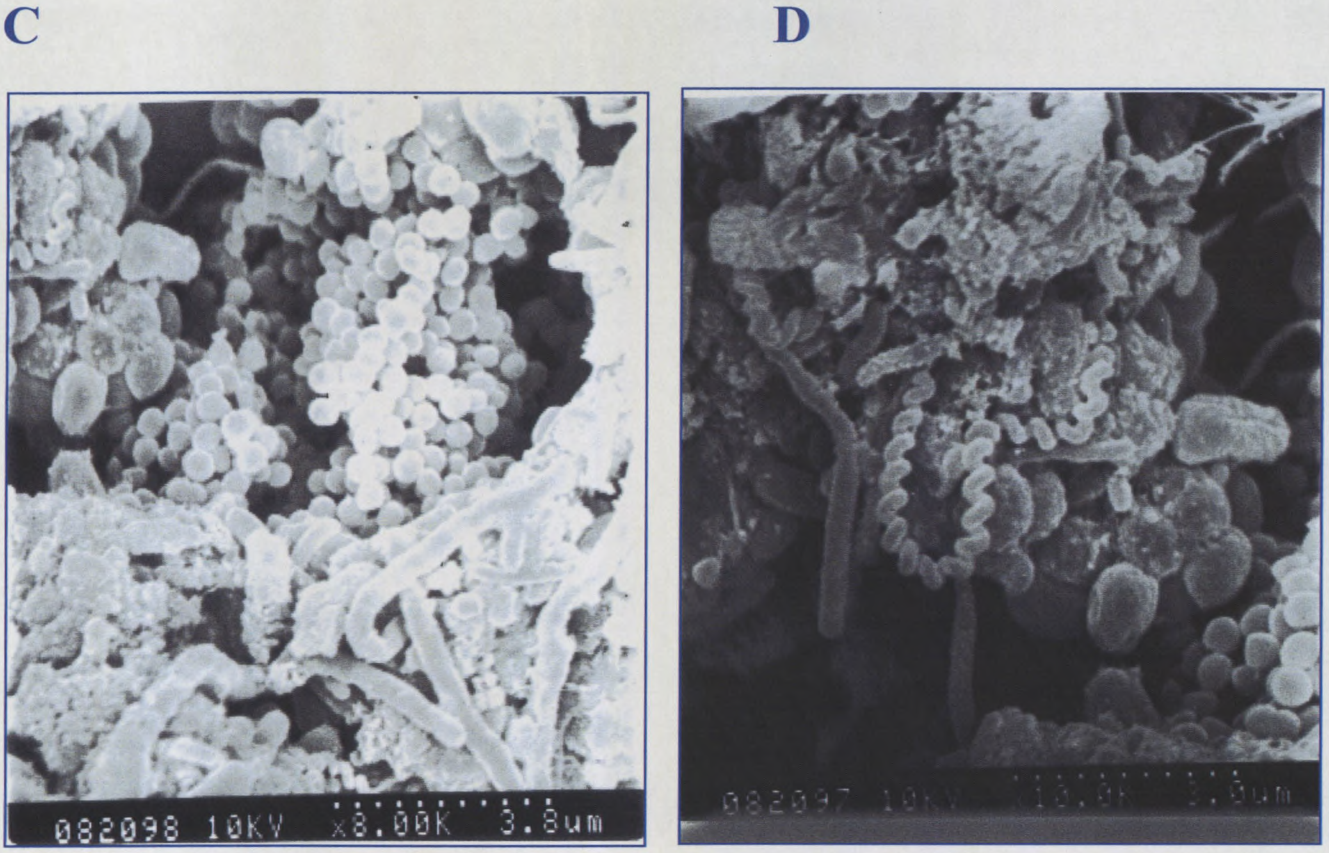
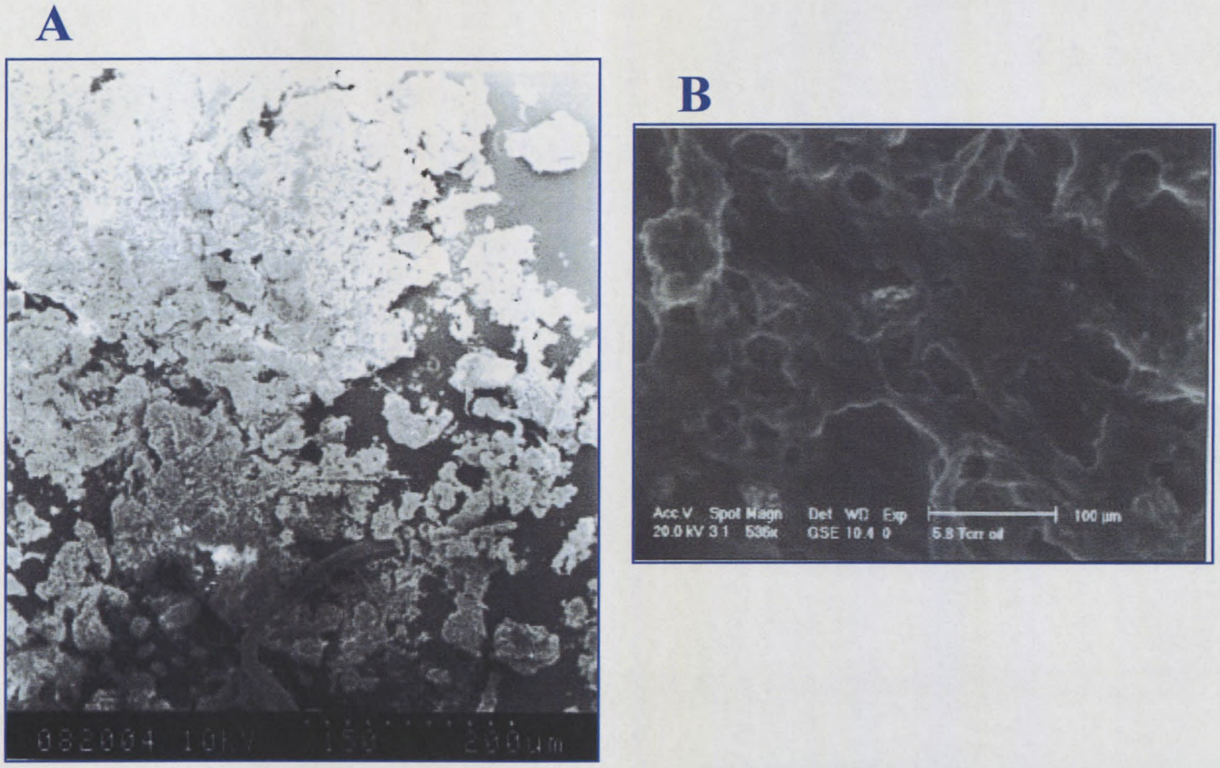


FIGURE 4.51 Environmental scanning electron micrographs of microbial flocs from Darvil at (A) magnification of(X 4000), and (B-D) at higher magnifications. Scale bars and magnification are reflected at the bottom of the micrographs



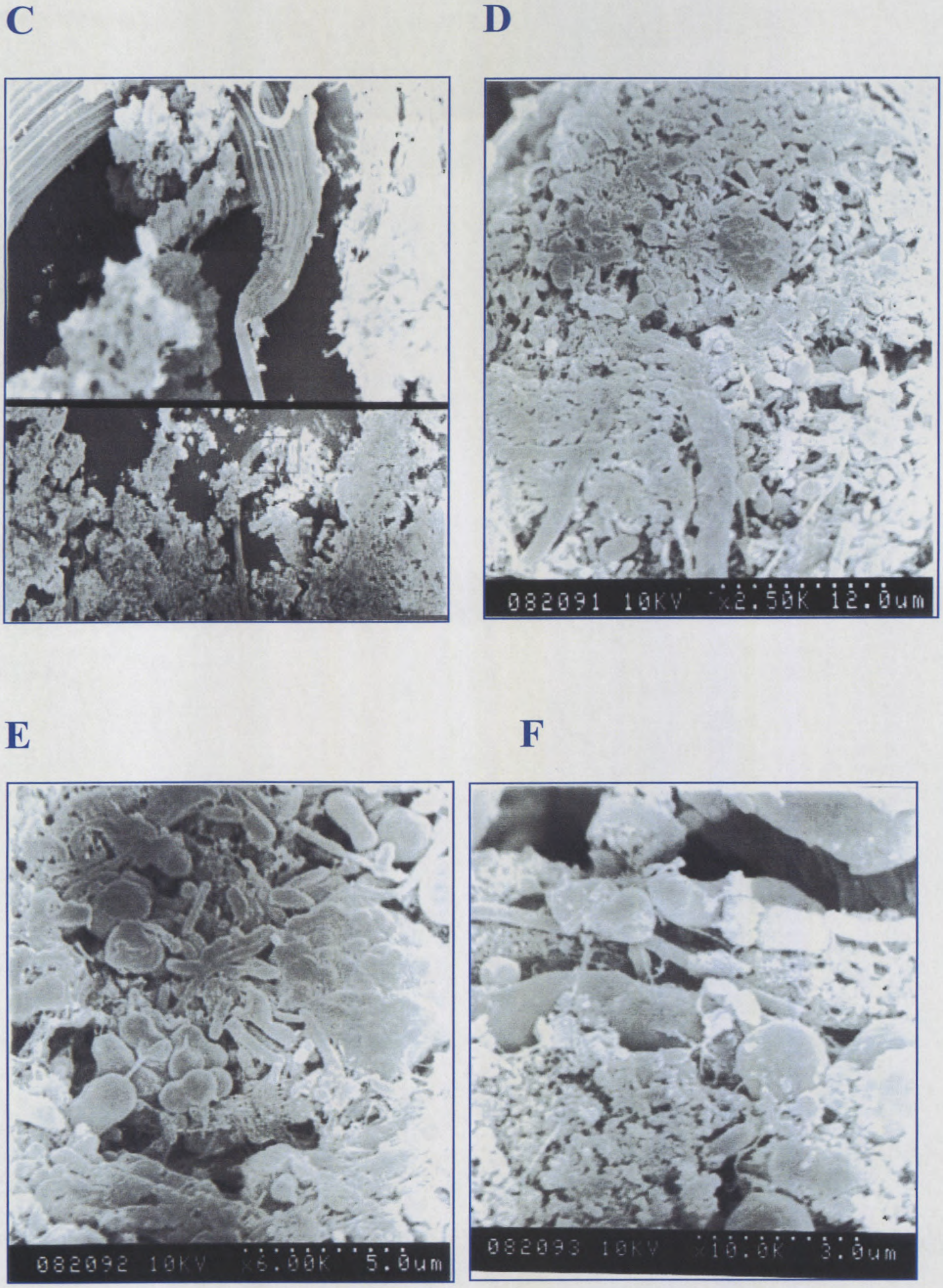


FIGURE 4.52 Environmental scanning electron micrographs of microbial flocs from the MLSS of the oil effluent treatment process at (A-B) low magnification, and (C) showing the presence of protozoa, (D-F) at higher magnification displaying floc structure. Scale bars and magnifications are reflected at the bottom of the micrographs.

FIGURE 4.53 Environmental scanning electron micrographs of a microbial floc from the oil effluent treatment process pre-treated with a non-polar solvent. Scale bars and magnification is reflected at the bottom of the micrograph.

4.3.3.2 **Energy dispersive X-ray analysis (EDAX)**

Two of the oil effluent samples which were obtained from the process approximately 7 weeks apart were subjected to energy dispersive spectroscopy to provide more detail on the physico-chemical nature of the microbial flocs. The spectra shows carbon and oxygen to be the predominant elements with both samples (Fig 4.54 A and B). The investigations conducted showed the distribution of the various elements in the extracellular matrix of the microbial floc, as a percentage weight of the total field examined are reflected in Table 4.5. The distribution of the major constituents i.e C and O between both samples are not very different with 47.66 % and 33.31 % (sample 3-7-01) and 56.78% and 23.19 % (sample 21-08-01) respectively. The other elements are present in both samples but at lower concentrations. Phosphorus is present in both samples at low concentrations (Table 4.5).

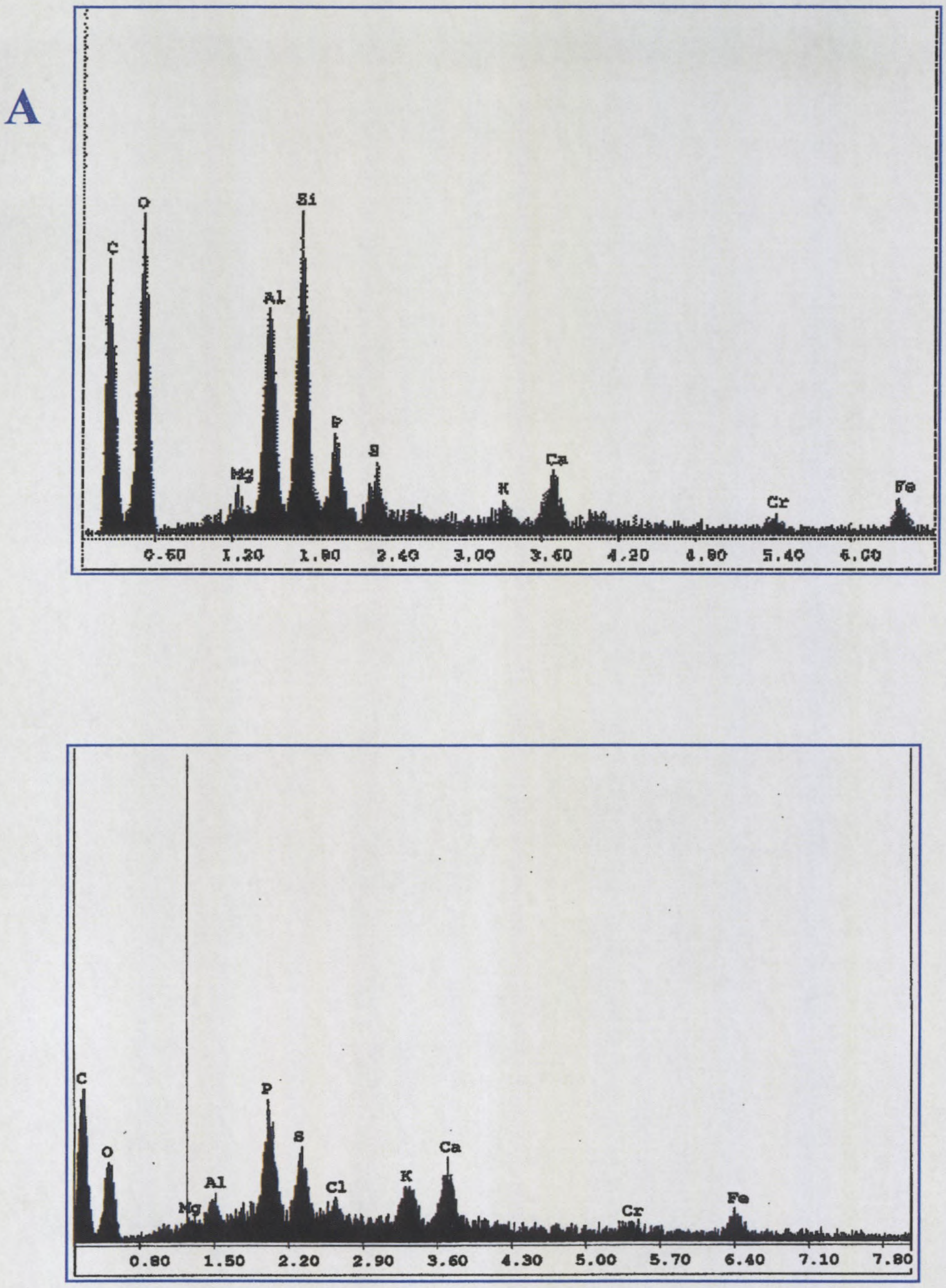


FIGURE 4.54 Energy dispersive spectra of a microbial floc for two samples (A) obtained on 3/07/01 and (B) obtained on 21/08/01

TABLE 4.5 Energy dispersive X-ray analysis showing distribution of elements

ELEMENT	03/07/01	21/08/01
	% WEIGHT	% WEIGHT
C K	47.66	56.78
O K	33.31	23.19
Mg K	0.73	0.60
Al K	4.57	1.34
Si K	5.97	Not Present
P K	1.84	4.34
S K	1.00	2.83
Cl K	Not Present	1.50
K K	0.55	2.42
Ca K	1.58	3.23
Cr K	0.75	0.96
Fe K	2.05	2.81
TOTAL	**100.00**	**100.00**

4.3.4 Chemistry of effluent after biological treatment

The final effluent after biological treatment was subjected to GCMS analysis to determine the compounds present and compared with the raw and flocculated effluent (Table 3.5). Investigations of the raw and flocculated effluent identified 28 and 20 compounds respectively. However, after biological treatment, 2 compounds were detected i.e. cyclohexene at a area %(relative abundance) of 67 % and methyl benzene at a area percentage of 33 % (Table 4.6 and Appendix 14). However, the compounds were identified from the search library and the percent probability was 59 and 72 % respectively, which is comparatively lower than the identification of compounds from the raw and flocculated effluents. The detailed compound spectrum of the final effluent does establish that most of the organic compounds and essential oils that were present in the feed (flocculated) influent were biodegradable and metabolized by the activated sludge microflora. The area % reflects the relative abundance of the compound in the sample and not the actual concentrations, since this would require the use of standards.

TABLE 4.6 Compounds present in the raw, flocculated and final effluent after biological treatment

RAW EFFLUENT		**FLOCCULATED EFFLUENT**		**FINAL EFFLUENT**	
COMPOUND	**AREA %**	**COMPOUND**	**AREA %**	**COMPOUND**	**AREA %**
Hexanal	10. 38	Toluene	4. 86	Cyclohexene	66.66
Alpha- Pinene	22. 05	Octenol	6. 19	Methyl \benzene	33.34
Camphene	1. 67	Cineole	2. 14		
Cyclohexane	0. 43	Filifolone	1. 36		
Beta- Pinene	1. 57	Pinocarveol/ Pinocarvone	16. 03		
Furan	1. 10	Furan	2. 49		
Para- Cymene	9. 53	Para-Cymene	2. 98		
L- Limonene	3. 62	Cis- Limonene	1. 24		
Butylbenzene	5. 21	Butylbenzene	1. 74		
Tricyclo- heptane	0. 87	Tricyclo- heptane	23. 51		
Isopropenyl Benzene	1. 83	L-Camphor	1. 98		
Nonanal	0. 70	Trans-P-Menthenediol	7. 47		
Cyclopentene	1. 26	Cyclopentene	2. 91		
Alpha- Campholene	0. 77	Bicycloheptanone	2. 41		
Phellandrene Epoxide	0. 60	Dihydrocarvone	2. 24		
Pulegone	0. 47	Trimethylnorpinan	1. 50		
Nonenal	3. 29				
Terpineol	0. 24	Terpineol	9. 01		
Dodecene	4. 65				
Dodecanol	1. 64	Borneol- L	5. 53		
Endobornyl Acetate	1. 30	Endobornyl Acetate	3. 57		
Trans- Decadienal	7. 69				
Beta-Elemene	0. 84				
Aristolene	0. 57				
Calarene	7. 10	Calarene	0. 84		
Cyclooctane	3. 44				
Octadiene	6. 28				
Beta- Bisabolene	0. 94				

4.4 DISCUSSION

The refining of edible oil results in the production of highly concentrated and large volumes of wastewater as opposed to other manufacturing processes in the industry (Rohbrechtbuck and Sekoulov, 1990). Being one of the key sectors within the agro-industry, the edible oil manufacturers are known to contribute to environmental pollution. The conventional technology used for the treatment of these wastewater's generally apply physico-chemical methods, which include dissolved air flotation (DAF) with the addition of poly-electrolytes. However, these treatment methods have produced satisfactory results with regards to COD removal efficiency and concentrations were still too high to satisfy municipal discharge standards (Ozturk et al., 1990; Mkhize and Bux, 2001). Therefore there was a need to develop alternate technology to satisfy the need for appropriate treatment of edible oil effluents.

Complete abatement of wastewater pollutants can hardly be achieved by the adoption of a single treatment method (Andreozzi *et al.*, 1998). . The current research focused on developing and optimizing suitable technology by integrating chemical and biological treatment methods. The chemical method involved selecting and applying the appropriate coagulants for the pretreatment of the effluent from industry (explained in Chapter 3) in order to remove the inhibitory compounds i.e. FOG and prepare the effluent for biological treatment. A laboratory scale activated sludge unit was operated and biological treatability studies carried out in this system. A phasic approach was adopted to the investigation for the following reasons :

- compare results within common process operational parameters, and
- determine the response of the system to incremental increases to influent COD concentration.

Subsequent to large scale pretreatment of the wastewater to reduce the high FOG content and remove COD, the effluent was characterized. The pH of the refinery wastewater on collection was acidic i.e. pH 3.75 and basic i.e. pH 9.2 after pretreatment and adjusted to neutral prior to serving as influent feed. The current findings substantiated previous research by Saw *et al.*(1987)that showed raw effluent pH measurements of pH 2 and COD range of 1010-8200 mg/L, which compared favorably with the COD of the effluent collected that ranged between 6190 and 8470 mg/L. It should be noted that the wide range was ultimately due to varying strengths of the effluent collected from industry which was dependent on the refinery process. The pretreatment process using flocculent removed approximately 74 % and 92 % of the COD and FOG respectively (Table 4.2). Previous research by Sengul, 1990 using coagulants such as poly-electrolytes when treating sunflower oil effluent showed COD and FOG removal rates of 76 and 77 %, respectively. Therefore, pretreatment has proven to be necessary and highly effective when adopting a two stage treatment approach. Biological treatment serves to remove the resident COD and satisfy municipal discharge standards.

The phosphates and nitrogen were limiting in the effluent collected from industry and therefore had to be supplemented in the influent feed at a C:N:P ratio of 100:5:1 in order to achieve the desired TP/COD and TKN/COD ratio and sustain biological activity. The ratio of nutrient supplementation is ultimately dependent on the characteristics of the raw wastewater and Saw *et al.* (1987) using anaerobic packed bed processes to treat edible oil wastewater, maintained a C:N:P ratio of 100:1.6:0.9. However, their research showed excess P in the treated effluent at all times indicating that phosphorus supplementation was not necessary. Ozturk *et al.*(1990) when conducting treatability studies on edible oil effluent using pilot scale activated systems initially identified a C:N:P ratio of 100:4.4:23. These findings were consistent with the previous results and indicated that N was the critical value and can be a growth limiting nutrient , while P was in excess in the medium. However, unlike the above findings, during the current research from phase 3 onwards, citric acid replaced phosphoric acid during the refinery process and therefore P was limiting in the effluent (influent feed) and had to be supplemented.

The laboratory scale treatment process was partitioned according to phases signifying common operational parameters and conditions. During phase1 the influent feed was not pre-treated resulting in a high FOG loading which resulted in the process failing within 3 days. This was evident with diminished COD removal efficiency and the low OUR being recorded. Previous studies have demonstrated the capability of activated sludge to treat emulsified lipids and provide removal efficiencies of as high as 80% (Mulligan and Sheridan, 1975). However, Hrudey, (1982) identified emulsified lipid overloading conditions that resulted in unacceptable effluent biochemical oxygen demand (BOD) and suspended solids level. These occurrences were known to occur in activated sludge plants that treated industrial waste with a high lipid loading.

When investigating activated sludge treatment of industrial wastewater, emphasis should be placed upon the choice of parameters for organic carbon removal. Assessment of substrate is the main concern for biological processes. Unlike inorganic substrates such as the nitrogen and phosphorus content of wastewater which can be quantified directly, assessment of organic's is not so simple since wastewater's contain a great variety of organic's which cannot be identified individually on a routine basis. Consequently, collective substrate parameters are traditionally used as indirect index values to characterize the overall organic content. The most common collective parameter in wastewater characterization and activated sludge modeling are the BOD and COD. However, the COD parameter is more commonly used since it reflects the appropriate electron balance between substrate, biomass and the electron acceptor ((Orhon *et al.*, 1999).

The emphasis of the treatment process during phase 2 was on carbon and phosphorus removal. The influent feed was pre-treated to remove the high resident fats ,oils and greases. During the initial period, good COD removal efficiency was observed with approximately 70 % and 400 mg/L COD being removed (Fig's 4.4 & 4.5). However, subsequent to Day 4 the removal capacity was decreased to 23 %. In addition, the OUR which is indicative of the metabolic activity of the microorganisms was also low (Fig 4.7). The activated sludge retained approximately 98 % of the FOG when comparing the influent and effluent FOG values. There could be various reasons for the system reduction in performance. However, the most likely would be failure of the process under lipid overloading. Banerji, (1974) suggested that the high FOG loadings may cause the activated sludge floc to become coated with hydrophobic material, thereby limiting oxygen transfer efficiency. The latter theory could explain the parallel decrease in the OUR over the period (Fig 4.7). It was also noted that since inception of phase 2, there was also a gradual decrease in the MLSS concentration (Fig 4.6). This effect was attributed to an adverse effect of lipids on sludge settleability resulting in loss of mixed liquor solids from the secondary clarifier. These observations were explained on the basis that lipids posses a specific gravity of less than 1.0 and, therefore, higher concentrations of lipids in mixed liquor were expected to reduce the specific gravity of the floc to the extent that settleability would be impaired (Hrudey, 1982).

The system performance with regards to P removal showed that more phosphate was released than present in the effluent (Table 4.3). The non-existent P removal of the system could be attributed to various factors. Some of the more probable causes for the phenomenon could be that the covering of the surface of the floc by a oily layer would have resulted in anaerobic conditions in the aerobic reactor, thereby releasing P into the medium. This is also account for the depleted OUR by the microorganisms in the system (Fig 4.7). A similar phenomenon was observed by Mkhize *et al.* (2000) when using a laboratory scale bioreactor to assess P removal from edible oil effluents whereby more P was released into the effluent. They deduced that this could be attributed to the insufficient anaerobic sludge mass fraction of 0.08 or 8 % of the reactor volume. The size of the settling tank also contributed to poor P removal due to prevailing anaerobic conditions as a result of extended sludge retention times. To support the latter explanation, Ekama *et al.*(1984) suggested that the synthesis of short chain fatty acids (SCFA's)or volatile fatty acids (VFA's) from the fermentation of influent readily biodegradable COD fraction was incomplete and the full acetate complement was not produced. Increasing the anaerobic reactor volume to a ratio of 0.2 (increased anaerobic mass fraction) improved the P removal capacity by 8 % (Mkhize *et al.*, 2000). This increase of the anaerobic mass fraction to 20 % of the total reactor volume has been reported to increase the fermentation of RBCOD to VFA's in the anaerobic zone of domestic wastewater treatment plants (Wentzel *et al.*, 1990; Rustrian *et al.*, 1999). However, during the current research increasing the anaerobic mass fraction to improve P removal could not be achieved, since during the proceeding phase 3, phosphorus was rather a limiting nutrient than in excess in the influent(change in oil refinery process) and therefore no need to remove biologically.

Since phosphorus was no longer a problem in the effluent and no need to remove biologically, the configuration was changed to MLE process, focusing on carbon removal only. Since inception of this phase, there was a improved COD removal efficiency when compared to phase 2. Although there were slight fluctuations on the daily COD removal rates (Fig 4.8), the overall trend showed a gradual increase in the concentration of COD removed. During the first few days of phase 3, the efficiency was low but improved subsequently (Fig 4.9). This initial lag could be attributed to following. The major part of lipids in wastewater are present as triacylglycerides (TAG) and a minor part as free long-chain fatty acids (LCFA) (Quemeneur and Marty, 1994). Little is known about the transformation of this fraction , mainly because organic matter is usually only regarded as COD and not as separate fractions with various transformation routes and kinetics. This high fraction of TAG in edible oil wastewater's meant that hydrolysis must take place before a conversion can take place in the biomass, thus accounting for the initial lag in response (Hwu *et al.*, 1998).

It should be emphasized that during the current investigation , OUR was routinely monitored to observe the system response to various parameters i.e. increased COD loading, varying nature of influent feed and operational problems. The OUR reflects the rate at which microorganisms use oxygen (Lilley *et al.*, 1997). Carbon (COD) and nutrient (N&P) mass balances were not conducted due to operational problems encountered (mentioned previously) . The temperature of the room in which the reactor was located was maintained at 20^0 C using 3 air-conditioner units since this minimized impact on temperature dependent metabolic processes. Varying temperatures especially in summer is pronounce due to marked changes in the OUR caused by the increased activity of the microorganisms (Lilley *et al.*, 1997).

The OUR increased during the first half of phase 3 (Fig 4.10) reflecting similar trends as shown with COD removal (Fig 4.9) i.e. an increase in the OUR and subsequent tailing of. Although it is not straightforward to use OUR to characterize the transformation kinetics of lipids in activated sludge(Dueholm *et al.*, 2001), it serves as a good measure of the metabolic activity and health of the activated sludge process. The MLSS followed the same pattern as the OUR with a decrease in the MLSS during the second half of the phase(Fig 4.11). The decrease in the MLSS and OUR during the latter part of phase 3 were symptoms of an operational problem that resulted in a bulking sludge and recording of SVI's as high as 172 mL/g resulting in loss of MLSS from the clarifier and a turbid final effluent. Concurrent gross microscopic examinations of a activated sludge floc from the process revealed an abundance of *S. natans* and lower numbers of *Microthrix parvicella* which is a common organism associated with bulking conditions. *M. parvicella* is able to take up LCFA under all electron acceptor conditions applied in nutrient removal plants (aerobic, anoxic and anaerobic) and this mode is hypothesized to give the organism a competitive advantage against floc-forming bacteria (Andreasen and Nielsen, 2000). In order to establish an improved control strategy against bulking filaments, better knowledge of lipid transformation under different electron acceptor conditions is needed(Dueholm *et al.*, 2001) The latter explains the abundance of the organism during the bulking conditions experienced. The bulking episode negatively impacted on process efficiency and with diminished MLSS concentrations and subsequent comparatively low OUR, it was decided to shut down and clean the system thoroughly. Previous experience has shown that complete recovery from bulking conditions is rare especially when operating laboratory scale activated sludge systems and the advisable alternative was to shut down, clean and restart with fresh seed inoculum.

The laboratory scale process was started afresh with seed inoculum from Darvil and referred to as phase 4 (A-E). The primary objective of this phase was to optimize the process for maximum carbon removal with periodic increase in influent COD concentrations, until full strength effluent from industry is treated (i.e. pre-treated effluent without dilution at a concentration of ±1800 mg/L COD). During phase 4 A the influent COD was increased to 800 mg/L. As observed in the previous phase there was the initial acclimation period at the beginning of the phase which allows the microorganisms time to acclimatize to the effluent prior to biodegradation of the organic matter. Excellent COD removal efficiencies were observed within 13 days, with approximately 90 % COD removal (Fig 4.12) and 722 mg/L concentration removed (Fig 4.13). Although the influent COD(Sti) was not fractionated, the above findings clearly indicate that at least 90% of the edible oil effluent is biodegradable (Sbi). The Sbi value could be higher if the process efficiency was not 100 %. The mean OUR followed a similar trend as the COD removal and was much higher than the previous phase reaching a maximum of 32mgO/L.h. (Fig 4.14). The slight decrease in process performance on day 35 (Fig's 4.12 &4.13) and the associated drop in the OUR during this period (Fig 4.14) could be as a result of experimental error since the error bars reflect high standard deviations or alternately toxic shock caused by some constituent of the influent. However, the system rectified itself within a few days and resumed above 90% removal capacity and relatively high OUR's indicating that the process had reached steady state performance. The latter correction of the system is also indicative of the resilient nature and buffering capacity of the activated sludge treatment process to toxic shock loads especially from industrial effluents (Eckenfelder and Musterman, 1995).

Since nitrogen was a limiting nutrient in the effluent from industry it had to be supplemented in the influent feed at a C:N:P ratio of 100:10:1. This resulted in relatively high FSA and nitrate concentrations in the effluent. The problem was addressed by including an anoxic zone (MLE configuration) and reducing the supplementation of N ratio to 100:5:1. This resulted in immediately reducing the effluent TKN value to suitable discharge standards. Current research maintained a low TKN/COD ratio across the phases. During phase 4A the ratio was 0.01. and this allowed for excess COD becoming available to allow for complete denitrification (Pitman, 1982). Effluent TKN values for the entire duration of the research centred around a mean value of *ca* 5.7 mgN/L, except for some periods of process perturbations which were negated in the above calculation. The effluent TKN consist of free and saline ammonia, biodegradable soluble organic N, unbiodegradable soluble organic N and TKN in the effluent volatile solids (Marias and Ekama, 1984).

During phase 4 B the influent feed concentration was increased to 1200 mg/L. The system responded immediately with an increase in the COD removal efficiency. The performance of the system was typical of steady state with the percent COD removed peaking at 92 %(Fig 4.15) and concentration removed at 1142 mg/L(Fig 4.16). The OUR remained high throughout the phase indicating a high level of metabolic activity by the microorganisms (Fig 4.17). Previous research by Dueholm *et al.*(2001) investigating transformation of lipids in activated sludge processes recorded OUR's in the range of 7.0-10.7 mgO_2/gVSS. However, these findings were based on activated sludge treatment of single carbon source e.g. oleic acids and not triacylglycerides which are present in the oil effluents. The mean MLVSS/MLSS ratio was very consistent during phase 4 B, ranging from 0.8 to 0.9 mg VSS/mgTSS.(Fig 4.18). The normal range of this ratio for activated sludge is 0.77 when treating domestic wastewater, and Wentzel *et al.* (1988) were able to obtain ratios as low as 0.46 mgVSS/mg/TSS when developing the EBPR mechanism. Current findings displayed slightly higher ratios. The percentage of biological solids in the aeration basin also varies with the amount and nature of industrial wastewater contribution. Eckenfelder and Musterman, (1995) further declared that increasing the sludge age(current R_s =16 days) also increased the biomass percentage, as influent VSS undergoes degradation and synthesis. Similarly, addition of soluble industrial wastewater increases the biomass percentage in the activated sludge, possibly accounting for the slightly elevated ratios produced during phase 4 B. Phase 4 B when compared to other phases during the investigation, undoubtedly showed that the process had reached steady state and the results were consistent. In addition the COD removal efficiency and OUR were relatively good. It should be noted that steady state conditions were reached within the phase which encompassed common operating parameters.

There was a disruption in the supply of effluent and the process was without fresh effluent for 10 days of phase 4 C. The final effluent had to be recycled and remaining COD (low concentration) served as influent feed in order to sustain the system. During this period, the COD removal efficiency was diminished as expected. However, the increase reflected in the percentage COD removal graph for days 84 to 91 (Fig 4.19)should not be compared to phase 4 B since the actual concentration of COD removed was low. Once again, this perturbation in the system during this period was reflected in the decrease in the OUR (Fig 4.20). This substantiated findings of the previous phases where reduction in COD removal capacity was also reflected in the diminished OUR's by the activated sludge microflora. With the decrease in the influent feed COD and the MLSS remaining constant, the F/M ratio decreased with parallel decrease in OUR. The current findings were consistent with earlier work by Dueholm *et al.* (2001) which confirmed that with a decrease in the F/M ratio, the OUR's also decreased when using activated sludge to treat lipid wastewater's. However, on resumption of fresh effluent feed, the process responded with increase in COD removal and gradual increase in the OUR(Figs 4.19& 4.20).

Phase 4 D reflected the end of phase 4 C but reaching steady state conditions with consistent COD percentage removal efficiency (Fig 4.21). Maximum COD concentration removed exceeded 1200 mg/L COD at the beginning of the phase. However, there was a slight decrease in the concentration removed as the phase proceeded. (Fig 4.22). The OUR's recorded were also high and consistent indicating a high level of metabolic activity (Fig 4.23). The VSS/MLSS ratio was similar to phase 4 B and consistent throughout the phase (Fig 4.24).

Phase 4 E was initiated with the influent COD concentration increased to 1500 mg/L. However, the system responded with a sharp decrease in COD removal efficiency (Fig's 4.25 & 4.26) which was matched with a decrease in the OUR during the same period (Fig 4.27). The reason for the above behavior could be associated with one of various factors. Analyzing this trend which showed recovery in COD removal capacity within a few days of the episode, the most likely cause could have been the presence of inhibitory compounds in the influent which when dissipated within a few days allowed the system to return to normal performance. The latter theory was supported with the parallel response of the OUR which also increased and normalized (Fig 4.27), reflecting increased metabolic activity of the microorganisms. On recovery of the process, the influent COD concentration was increased to 1800 mg/L which was basically the pre-treated effluent from industry without any dilution i.e. full strength. There were odd occasions after pretreatment, that the concentrations were lower or much higher depending of the effluent batch, but these were more exceptions than the rule and the influent COD concentrations were adjusted accordingly. As the influent COD increased, the concentration removed also increased with the highest being 1 550 mg/L COD. The concentrations removed during the mid to latter portions of this phase were high averaging greater 1 000 mg/L COD(Fig 4.26) and slightly erratic (Fig 4.25). However, towards the end of the phase the COD removal efficiency decreased and so did the OUR(Fig 4.27) indicating that the system was experiencing a certain degree of stress.

During the current investigation, the maximum or threshold potential of the adapted activated sludge system to treat edible oil effluent was not determined since :

- The effluent from industry had to be pre-treated and the maximum concentration achieved after pretreatment was 1 800 mg/L
- at the above concentration the system showed good removal potential *ca* 80 % and therefore could very well remove higher concentrations if exposed to. However , this could not be

determined.

Ozturk *et al.* (1990)using pilot scale activated sludge processes to treat DAF pre-treated oil effluents (Influent concentration 2500 mg/L COD) achieved removal rates of 72 % i.e 1 800 mg/L COD. Therefore by comparison, since at steady state the current laboratory scale activated sludge process was achieving greater than 80 % removal rates when exposed to influent COD of 1800 mg/L, one could correlate and assume that the process would achieve removal concentrations of 1800 mg/L or higher if exposed to higher influent COD concentrations. Major disruptions at the industry resulted in suspension of effluent supply for approximately 5 weeks. The lab scale process was terminated during this period. However, the MLSS was maintained using feed influent from another oil company and remained viable since high OUR's were recorded during this period. This MLSS biomass served as seed sludge when restarting the process during the next phase (5).

On resumption of effluent supply from industry, the treatment process was initiated(phase 5A) with the seed sludge used from phase 4 E with a MLSS concentration of 2542 mg/L. However, the seed sludge was mixed with fresh Darvil sludge at a 1:1 ratio. The system responded immediately producing high COD removal, (Fig 4.28) since the seed sludge used was pre-exposed to the effluent previously and there was no need for an acclimation period. Once again, the strength of the influent COD was dictated to by the organic strength of the industrial effluent and final concentration after pretreatment which during this phase was 1542 mg/L COD,and weaker than the influent COD during phase 4E. Comparatively good COD removal efficiencies were observed, *ca* 85 % but there was a gradual decrease as the phase progressed (Fig 4.28). During the first 5 days high concentration of COD were removed peaking at 1345 mg/L COD. However, the concentrations decreased and reached a plateau as the phase progressed (Fig 4.29). Although the recorded OUR's were erratic during the phase, the overall rates were high with the maximum at 42 mgO/L.h. (Fig 4.30)indicating high rate of metabolic activity by the activated sludge microflora.

Rohbrechtbuck and Sekoulov, (1990) in order to treat high COD edible oil refinery wastewater with fluctuating quality and quantity adopted a multi -stage treatment system. The first stage was an equalization basin which served to absorb and buffer the system directly from inhibitory substances present in the effluent that cause toxic shock. Therefore, variable quality influent affecting the performance of biological treatment systems and causing problems were experienced by other researchers as well. Phase 5 B, once again produced low strength effluent obtained from industry and after pretreatment, influent concentrations of 701 mg/L were achieved which was much lower than the previous phase. As expected high percentage removal rates were obtained reaching 88 % and consistent throughout the phase(Fig 4.31). Due to low influent feed concentrations, the COD concentrations removed were also low (Fig 4.32). However, as high as 88 % or 680 mg/L COD was removed, indicating a relatively large portion of the oil effluent was biodegradable.

The next batch of effluent after pretreatment once again presented elevated influent feed COD concentration > 1 400 mg/L . The process efficiency was high recording 90% removal rates (Fig 4.33) and COD concentrations removed at 1324 mg/L COD (Fig 4.34). The performance of the system during the phase was typical of steady state behavior with consistent removal rates, except towards the end (day 72). An observation that required mentioning was that with change in effluent batch and increase in influent COD concentration, the process reacted with immediate higher COD concentration removed e.g. 5 B (701 mg/L) and 5 C (1465 mg/L) with 88 and 90% removed, respectively. This trend was evident also during phase 4. Possible justification for the above behavior could be that the capacity of the activated sludge system to remove oil effluent COD exceeded the pre-treated influent COD concentrations during the current research. Ozturk *et al.* (1990) showed higher maximum removal rates of 1 800 mg/L COD. Erratic OUR's recorded could be due to experimental error, but the overall rates were high (Fig 4.35). As expected , the VSS/MLSS ratios were similar to other phases at 0.8 to 0.9 mgVSS/mg/TSS(Fig 4.36) indicating that a large composition of the total suspended solids was biomass. The above ratio has been consistent throughout this investigation.

During phase 5 D, a new batch of effluent from industry after pretreatment resulted in influent feed concentration of 915 mg/L COD, which was substantially lower than the previous phase. Towards the end of the previous phase, there was a decrease in COD removed. However, within the first five days this trend was reversed with 90 % COD removal rates being achieved and consistent throughout the phase (Fig 4.37). As the phase progressed the influent COD concentration increase to 1050 mg/L and the maximum concentration removed was 937 mg/L COD (Fig 4.38). Although the same batch was used, the slight increase in the influent COD could be attributed one of many factors, possibly addition of some external source of organic matter (oil) or resident grease on the wall of the feed tank that added to the soluble COD. The OUR's were in the normal range and the MLVSS/MLSS ratio was similar to the previous phase. With the next batch of effluent, the influent COD further decreased to 712 mg/L on initiation and was referred to as phase 5E. As the phase progressed, there was a gradual decrease in the process efficiency (Fig's 4.39 and 4.40). Although the OUR's recorded during this period were erratic but above average comparatively(Fig 4.41), the trend reflected followed a similar pattern to the COD removal graph. As previously shown in the last few phases, the MLVSS/MLSS ratio ranged between 0.8 and 0.9.and consistent throughout the phase (Fig 4.42).

Phase 5 F showed good COD removal with a maximum of 93 % (Fig 4.43). In addition, compare to phase 5E, higher concentrations of COD was removed with a maximum of 1 200mg/L (Fig 4.44). This was due to higher influent COD concentration due to a new batch. The system responded in the same manner as previous phase by increasing the COD removed and the behavior was typical of steady state performance. The COD removal rates were consistent throughout the phase and was supported by consistent OUR's with a maximum of 28 mgO/L.h.(Fig 4.45). The MLVSS/MLSS ratio was consistent throughout the phase (Fig 4.46). The high ratio(0.84 -0.87) was indicative of the high active portion of the of the MLSS.

During phase 5 G the influent COD strength was reduced due to a new batch. The efficiency of the process fluctuated during this phase. There was a gradual decrease in the percentage removed, but a rapid decline on day 172 (Fig 4.47)which was subsequently corrected. Throughout the phase there appeared to be a stepwise decline and immediate recovery. This phenomenon was repeated thrice during the phase (Fig 4.48). The OUR's also gradually decreased during the phase from 31 to 19mgO/L.h. Eckenfelder and Musterman, (1995) demonstrated the effects of lipid soluble organic compounds in "uncoupling" oxidative phosphorylation. The result of this uncoupling effect is uncontrolled respiration and oxidation of primary substrates and intracellular metabolites. At low concentrations, uncoupling was evidenced by highly elevated OUR's but no effect on cell growth or substrate removal. At higher concentrations, inhibition and toxicity are demonstrated by dramatic reductions in both the OUR's and cell growth. During the current phase, it appears that the latter theory is more plausible than the former. However, the immediate recovery of the process could be attributed to the inhibitory compounds being flushed out in the final effluent. The recurrence of the episode 3 times could be associated with re-introduction of the inhibitory compound in the influent. However, identification of the actual cause was not confirmed during the current research. The VSS/MLSS ratio was similar to the previous phase.

Having achieved the desired objectives and reproducibility of the system results to changing parameters, the process was terminated.

One of the primary objectives of the research was to ascertain the response of the system to an increase in the influent COD(organic loading). As explained previously, the treatment process was partitioned into phases based on varying operational conditions, one of which was the increase in S_{ti} The phases, periods and progressive increase in the S_{ti} across phases is shown in Table 4.4. in addition to the mean F/M ratios. The results indicating increase in the S_{ti} were recorded over a period of approximately 1 year starting at 500 mg/L COD(diluted) to 1800 mg/L (undiluted). The overall trend was clearly evident that with the phasic increase in the S_{ti}, the concentration of COD removed by the treatment process also increased (Fig 4.49). It should be noted that these observations were made across phases where the operational parameters were not always the same. Therefore Fig 4.49 was useful for observing the overall trend. However, minor deviations did occur e.g. there was a slight decrease in the COD concentration removed during PH 4E1 compared to PH 4 B, although the concentration was higher. A similar pattern was observed during PH 5C and PH4 E2. However, the magnitude of difference was relatively small. Although graphically it appears that the system had reached a plateau phase from PH4 E 1 onwards, it was not exposed to higher concentrations than 1800 mg/L COD (Fig 4.49). Analyzing the response of the system to increase in COD, it was highly likely that if S_{ti} of greater than 1 800 mg/L COD was used, the system would have removed higher concentrations of COD. Current findings substantiate research by other workers in the field with

Ozturk *et al.* (1990) achieving removal capacity of 1 800 mg/L COD using S_{ti} of 2 500mg/L. Mkhize,(2002) showed average COD removal capacity of 1976 mg/LCOD when exposed to oil effluent with S_{ti} of 2 600 mg/L. Abdel-Gawad and Abdel-Shafy, (2002) used a two prong approach treating refinery effluent. These included in-plant modification and end of pipe biological treatment. The combined process efficiency was 99.2 % COD removal with a S_{ti} of 9 945 mg/L. The concentrations reflected in Fig 4.49 are not the actual concentrations removed during the phases, since the means were used to construct the graph. Maximum values absorbed were reflected in earlier graphs and are closer to the findings of above researchers.

The F/M ratio is an important parameter for the operation of activated sludge processes and describes the degree of starvation and potential food availability to the microbial population (Droste, 1997).The F/M ratio, sludge age and SVI are all interrelated and impact on the process performance and the ratios reflected in the current investigation varied from 0.27 to 1.33 mgCOD/mgMLSS.d. Theoretically, an increase in the S_{ti} should result in an increase in the F/M ratio, providing that the MLSS remains constant. Finding's do not conform to and were inconsistent with the latter, whereby increase in the S_{ti} from 500 to 800 mg/L COD resulted in a decrease in the ratio from 0.54 to 0.27 (Table 4.4). This was attributed primarily to an increase in the MLSS during Ph 4A. A similar trend was noticed when the F/M ratio decreased from 1.33 to 0.93mg COD/mg MLSS.d. Mkhize, (2002)showed a reduction in the COD removal efficiency and attributed it to the increased operational F/M ratio that was maintained at 1.5 kgCOD/kgMLSS/d. However, increasing the F/M ratio of the system is reported to have an overloading effect to the biomass, which results in the overall poor system substrate metabolism performance (Casey *et al.*, 1995).

Ozturk et al., (1990) using a lab scale activated sludge process to treat edible oil refinery, found that with the F/M ratio range between 0.36 and 0.57, the percentage efficiency increased marginally with an decrease in the ratio e.g. 0.57 produced 88 % efficiency and 0.36 produced 93%, respectively. Current findings can be compared favorably with the latter since a low F/M ratio 0.27 (PH 4A) produced a maximum removal efficiency of *ca* 93% (Fig 4.12) and slightly higher ratio of 0.54 (PH 3) produced a maximum efficiency of 80 % COD removal (Fig 4.8). With reference to Table

4.4and Fig 4.49, the maximum COD removed appeared at a F/M ratio of 0.93 mg COD/mgMLSS.d. However, the COD removal efficiencies although related to the F/M ratios, cannot be solely attributed to the manipulation of these ratios since other operational parameters such as sludge age, pH, temperature, nutrient supplementation would also impact. Dueholm *et al.* (2001) investigating OUR measurements of different substrates i.e. fatty acids using activated sludge process showed a decrease in the OUR as the F/M ratio decreased. However, the latter investigation focused on single carbon source as opposed to complex oil effluents. The F/M ratio is an alternative control parameter to sludge age and one which is widely used in practice(Horan, 1990).

The sludge age (R_s) describes the residence time of sludge in the system whereby the biomass assimilates the substrate and reproduces. If the sludge is not able to reproduce itself before being washed out of the system, failure will result. Also, the R_s is related to the F/M ratio describing the relative state of starvation of the of the microorganisms (Droste, 1997). Previous research by Ozturk *et al.*(1990) operating a lab scale process treating oil effluent investigated the effect of different sludge ages (R_s - 10, 15,20 and 30 days) on process performance. They found that decrease in the R_s from 30 to 15 d had minimum impact in terms of the COD removal efficiency with a reduction from 93 to 91 %, respectively. During the current research, the R_s was set at 16 days and the process performance at steady state also reached > 90 % COD removal. However, it is known in activated sludge systems, higher sludge ages cause the sludge to undergo more endogenous decay. This has an effect on the settleability of the sludge as well as on the total amount of sludge produced in the system(Droste, 1997). When selecting R_s for optimum treatment of especially industrial effluents, it was crucial to be cautious and select a R_s that is long enough to allow complete nitrification and sustain the ideal MLSS and active fraction allowing maximum degradation, at the same time not to long as to allow the sludge to undergo endogenous decay. The R_s adopted i.e.16 d during the current research appeared to produce the desired process efficiency.

During the current investigation, the ratio of MLVSS/MLSS under steady state conditions were very constant and relatively high, ranging from 0.8 to 0.9 mgVSS/mg/TSS during Phases 4B&D and C,D,E &F, respectively. This indicated that the acclimated biomass (active portion) or MLVSS of the MLSS was relatively high and the concentrations consistent throughout the phases. These results were possibly indicative of a highly specialized bacterial community (discussed in Chapter 5). Previous research by Atkinson (1999) showed steady decline in the VSS/TSS ratio from 0.77 to 0.53mgVSS/mgTSS and attributed the decrease to enhanced culture development. Typically the ratio of VSS to TSS in the mixed liquor for conventional activated sludge systems ranges from 0.75 to 0.80 mgVSS/mgTSS (Droste, 1997).

Another parameter that is intimately related to the VSS and TSS is the sludge volume index (SVI)which is a measure of the settleability and compatibility of sludge in a lab column settling test and is defined as the volume in mL occupied by 1g of sludge after it has settled for a specified period of time. A low SVI is indicative of sludge that settles well. The F/M ratio and R_s is related to the SVI and therefore impacts on the settleability of the sludge. In addition, the SVI is a function of the F/M ratio. During the current investigation, the SVI was not routinely measured and only enforced when operational malfunction symptoms were manifested. During phase 3 (day 15 onwards) there was a gradual decrease in the MLSS (Fig 4.11). However, approximately 2 weeks later an erroneous setting in the OUR meter further compounded the problem resulting high SVI being recorded i.e. 172 ml/g. This was clear evidence of a bulking problem. Most activated sludge plants suffer sporadic incidences of bulking when the SVI increases from an average value of < 80 to > 120 ml/g. The actual cause of the bulking could be attributed to various reasons. It is known that the dominant group of organisms dictate the settling properties of the sludge and their predominance is dependent on the relative abundance of substrate in the reactor and the prevailing dissolved oxygen concentration (Horan, 1990). The gradual loss of MLSS from the reactor possibly increased the organic loading rate. This could have produced changes in the R_s with increases loading giving rise to a younger sludge as a result of increased filamentous growth during this period (discussed in chapter 5). This perturbation could have been further compounded by the over-aeration resulting in abnormal DO conditions and increase in filamentous growth, resulting in full blown bulking conditions. Scruggs and Randall,(1997) showed that the growth of *Norcardia* which is associated with bulking in systems associated with industrial wastewater treatment, was stimulated with increase in the DO with a near linear relationship of up to 5 mg O_2/L. In addition, the F/M ratio in the lab systems during this period was also relatively low i.e. 0.54 mgCOD/mgMLSS and these conditions are known to support the abundance of certain filamentous bacteria (Blackall, 1999) which exacerbated into a bulking condition. The bulking conditions resulted in premature termination of the process.

The color of the mixed liquor in activated sludge treatment processes treating domestic effluent and operated optimally is generally dark brown (chocolate brown) (Fig 4.50 A). (Lilley *et al.*, 1997). However, the effect of the oil effluent on the color of the sludge was expected and resulted in a transition from the original brown to gradually becoming cream with time (Fig 4.50 B-D). During the course of the investigation, there were periodic incidences of foaming which was localized more on the surface of the aerobic reactors. However, the problem was purely aesthetic and foam was removed regularly and did not impact on the process performance or quality of the final effluent. The presence of high levels of emulsifiable fatty material such as oils and greases in industrial effluents has often been associated with foaming in activated sludge systems (Jenkens *et al.*, 1993; Franz and Matsche, 1994). The substrate treated during the current research consisted of large portions of emulsified fatty material. Although much of it was supposedly removed during pretreatment, the resident fats, oils and greases was present in the influent and therefore it was highly probable that it supported the growth and proliferation of filamentous bacteria (see Chapter 5) and foam former's that contributed to the foaming incidence witnessed.

The topography and ultrastructural details of the activated sludge samples comprising of flocs were observed using an environmental scanning electron microscope (ESEM). This approach had substantial advantages, one of which was the sample was studied in it's natural state without having to be subjected to damaging preparation techniques. The samples from the lab scale unit were also subjected to treatment using a non-polar solvent in order to remove the oil film surrounding the floc and reveal further ultrastructural detail. The sludge that was used to seed the system was used as a control and for comparative purposes.

Microbial flocs are principally composed of microbial cells, bio-organic (cellular debris and extracellular polymeric substances (EPS)) and inorganic material in a complex matrix (Droppo *et al.*, 1996). The ESEM images of the Darvil (control seed sludge) at lower magnification revealed an abundance of microorganism which are characteristic of a healthy floc structure (Fig 4.51 A). At higher magnification, the EPS forms a matrix in which the bacteria and other microorganisms are embedded. The bacterial diversity reveals a multitude of cocci and bacilli shaped cells that are interconnected by a range of filaments (Fig 4.51 B-D). Compared to the Darvil floc, the topographical structure of the oil floc (lab scale reactor) at lower magnification revealed markedly different detail. The flocs appeared to coalesce together possibly due to the flocs imbibing some of the oil from the effluent (Figs 4.52 A&B).

The presence of the oily layer contributed to the hydrophobic nature of the floc. This hydrophobic interactions may be fundamental in the cohesiveness and properties of the bacterial aggregates (Jorand *et al.*, 1997). In addition, a more hydrophobic surface could also be attributed to a higher R_s (16-20 d) as in the current investigation and provide stronger van der Waals and /or hydrophobic interactions that govern the stability of the sludge floc (Liss *et al.*, 2002). Magnusson, (1980) described the "hydrophobic effect" to result from the behavior of entities, in this case oil (particles or molecules) incapable of interacting electrostatically or establishing hydrogen bonds with water and are therefore drawn together when plunged in an aqueous phase. Even at higher magnification (X 2 500) the coalesced structure without any floc ultrastructural detail is visible (Figs 4.52 C lower &D). In addition a crawling ciliate is visible and intimately associated and possible feeding on the floc surface and feeding on bacteria (Fig 4.52 C Top). In addition, the presence of protozoa clearly indicates the presence of a healthy sludge and a lack of toxic inhibitory substances. The above observations were made when the system was functioning optimally.

At higher magnification more ultrastructural detail of the floc can was observed with a dense microbial mat of the cells visible. However, when comparing the Darvil floc (Fig 4.51 B) to the oil covered floc (Fig 4.52 F) at the same magnification (X10 000) the surface is less diffuse and although cells can be seen protruding there are many more flat and smooth areas of highly condensed and less hydrated material. The same is also visible in Fig 4.52E and more evident at a lower magnification (Fig 4.52 D). The bacterial diversity is also visible with cocci and filaments evident (Fig 4.52F).

With the latter microscopic evidence revealing the floc being covered with a layer of oil or EPS, the question arises on the mechanism of diffusion of oxygen into the floc. Taking into relatively high OUR's being recorded when the process was operating optimally, the microorganisms had adapted and had developed a mechanism to obtain oxygen to satisfy their metabolic needs . Current findings therefore substantiated previous research by Hrudey, (1981) who demonstrated that mixed liquor oxygen consumption rate was independent of lipid loading. Also, the specific soluble substrate utilization rate (g BOD utilized/g MLSS) was not adversely affected by high lipid loadings and could not be distinguished from the response obtained for equivalent BOD loading administered with sucrose. The majority of fat's are triglycerides (glycerol substituted fatty acids) and their hydrolysis is carried out by various lipases outside the cell. This results in the formation of glycerol and fatty acids which then undergo β- oxidation, resulting in the continual removal of Acetyl-CoA (C_2) units until the saturated fatty acids is completely converted to C_2 units which is then used in the tricarboxylic acid (TCA) cycle (Doelle, 1994).

The treatment of the oil sample with the non-polar solvent which removed the oily layer, presented bacterial cells with their structures more clearly visible. The shapes of the cells were more defined with cocci, short rods and interconnecting filaments evident and partially embedded in the loose EPS matrix (Fig 4.53). The fact that a non-polar solvent resulted in clearer microcopy, was proof that the layer surrounding the floc was oil which was removed by the treatment. The latter observation also confirmed and provided *in situ* evidence that the floc comprised of an abundance of microorganisms

EDAX analysis of the microbial floc of two samples at different stages of treatment clearly indicated that C and O were the major elements in both samples investigated (Fig's 4.54A and B). In addition the % weight of these elements between the two samples did not vary substantially (Table 4.5). The microbial floc comprised of bacteria and other organisms, and whose composition and activity changes with sludge age. The flocs are held together by a polymeric matrix (EPS) whose chemical composition is highly variable (Kerley and Forster, 1995; Frolund *et al.*,1996). The spectra shown (Fig;s 4.54A &B) indicated that C and O which are the building blocks of organic material predominated. In the current studies, most of the organic material would be in the form of lipids due to the wastewater being treated. The amount of lipids in the EPS matrix has been reported to be in the range of 13-89 % of the total EPS content (Goodwin and Forster, 1985). However, high concentrations of lipid in the EPS is also common with high sludge ages of > 16 d (Liss *et al.*, 2002) as in the current research. The minor differences in the composition of the elements in both floc samples (Table 4.5) could be attributed to sludge characteristics at the time of sampling such as exact R_s and the effect of influent feed composition.

The capacity of microorganisms to degrade industrial effluents containing toxic organics is well known; but, in practice very few processes are efficient (Buitron *et al.*, 1997). However, with regards to edible oil effluent there is much evidence to support effective activated sludge treatment (Eroglu *et al.*,1990; Rohbrechtbuck and Sekoulov, 1990; Mkhize, 2002). As mentioned, previous researchers and current findings refer to the organic removal in terms of COD. An added component of the current research focused on a more detailed analysis concentrating on the chemical composition of the COD in the influent and COD removed. The latter detailed chemical investigation was conducted using a GCMS. The results provides clear evidence showing 18 out of the 20 compounds were removed biologically after activated sludge treatment (Table 4.6). The chemical composition of the influent feed (flocculated effluent) consisted of a variety of organic compounds, primarily essential oils. Rohbrechtbuck and Sekoulov, (1990) attributed the purpose of refining as to eliminate as far as possible the extraneous substances present in crude oils used, such as seed particles,dirt, phosphatides, carbohydrates, proteins, mucins, free fatty acids, dyes, waxes and substances imparting a characteristic taste or smell. Many of these compounds end up in the wastewater's. In addition, these effluents also contain substances which are formed during processing due to chemical reactions or under the influence of heat e.g. products of oxidation of fatty acids, polycyclic aromatic hydrocarbons and sodium sulphate or chloride. However the composition of these wastewater's is highly variable with each batch refined and subjected to specific process used including temperatures and pressures and on the raw material used and how often the oil is changed (Rohbrechtbuck and Sekoulov, 1990).

As is evident, the results shown and the compounds present in the influent comprise primarily of essential oils and aromatic hydrocarbons and no free fatty acids as expected and shown by other researchers. Andreozzi *et al*, (1998)investigating olive oil mill effluents showed high concentrations of oleic and nonanoic acid. Sunflower oils do consist of long chain fatty acids such as oleic and linoleic in proportion. However, with the refinery process employed at the industry (Company X), it appeared that none of the fatty acids eluded in the effluent However, the essential oils such as that were present in the industrial effluent(Table 4.6) had high commercial value and could be extracted and purified as a value added bi-product. The raw effluent comprised of 28 compounds and the essential oil alpha- pinene was more pronounce (22.05%) than the other compounds. The quantifying of the compounds (actual concentrations) would require relevant standards and the area percentage simply represents the comparative predominance of the compound in the sample. Some of the more common essential oils in the flocculated effluent were cymene, limonene and cineole.

Since 90 % of the compounds were removed during the activated sludge process (Table 4.6), this is clear evidence supporting the hypothesis of the biodegradability of the oil effluent. The majority of the oil industries are compatible with biological treatment, with or without the pretreatment step and activated sludge is the preferred treatment scheme (Orhon *et al.*, 1999). With specific reference to the essential oils, the results show that some compounds that were present in the raw effluent have been either precipitated out with the flocculent or chemically transformed in the flocculated effluent. However, subsequent to biological treatment the two compounds that remain , namely cyclohexene and methyl benzene could be derivatives of the various microbial bio-transformation pathways e.g cymene is a colorless benzene derivative. However, it should be emphasized that the percentage probability of the compounds identified from the search library was low i.e. 59 and 72 %, respectively. This was much lower than those for other compounds identified and therefore the identification of the 2 compounds could not be confirmed.

There have been various studies regarding microbial-mediated biotranformation pathways. The toluene degrading strain *Rhodococcus opacus* PWD4 was found to hydroxylate D-limonene to pure trans-carveol and traces of carvone. Another toluene degrader *(Rhodococcus globerulus)* oxidized most of the trans-carveol to carvone (Duetz *et al.*, 2001). Cymene which is one of the compounds identified in the tested oil samples is a aromatic hydrocarbon and can be degraded by *Pseudomonas putida* by oxidizing the benzylic methyl group to yield ρ-*cumic* alcohol (Koo and Oh, 2000). Alpha-pinene is a monoterpene and a major component of tea-tree oils. Most of the latter bio-transformation pathways are enzymatically mediated. Alpha-pinene is transformed to limonene by *Bacillus pallidus* and subsequently to carveol using the enzyme limonene dehydrogenase (Wong and Li, 2001). Current research findings show that most of these compounds that were present in the flocculated effluent (influent feed) were completely biodegraded, possibly using the above pathways and was non-existent in the final effluent (Table 4.6).

Most of the research conducted in the field of biological treatment of oil effluent focused on assessing the removal of the organic substrate using a collective parameter such as COD or BOD (Eroglu *et al.*, 1990; Mkhize, 2002). However, the current research further investigated the specific compounds that comprised the organic fraction (COD). Other researchers have found that assessment of the organics is not so simple since wastewater contains a great variety of organics which cannot be identified individually on a routine basis (Orhon *et al.*,1999). In addition, not much has been mentioned on the actual mechanism of transformation of the triacylglycerides (TAG) which is the major component of the oil effluent, in activated sludge. The major part of lipids are present as TAG and a minor part as free LCFA (Quemeneur and Marty, 1994). As the lipids are generally hydrophobic , they are adsorbed to particles and only to a minor extent soluble. The high fraction of TAG in the oil effluent meant that hydrolysis of the substrate by lipases outside the cell had to occur prior to conversion in the biomass. In addition researchers showed that hydrolysis of lipids to free fatty acids was a potential rate limiting step in the metabolism of lipids, and only low levels of lipase activity have been observed in the EPS matrix accounting for high concentrations of lipids in the EPS, resulting in the increased hydrophobic nature of a high R_s floc compare to a low one. (Frolund *et al.*, 1995). The above explanation could justify the decrease in process performance at the beginning of some phases with change in industrial effluent batches. The new batches could have variable lipid composition, resulting in the decrease in the hydrolysis rate which is corrected within a short time. However, the lipase concentration of the floc was not measured currently and will be the scope for future research. LCFA is assumed to be taken up easily by many bacteria in activated sludge under aerobic and anoxic conditions. According to the hypothesis put forward by Andreasen and Nielsen,(2000), the LCFA that are taken up by the microorganisms are assumed to be oxidized or stored.

Lipid adsorption studies demonstrated that emulsified lipids are rapidly adsorbed when placed in contact with activated sludge solids previously acclimated to relatively low concentrations (Hrudey, 1982). Overall findings of the current research were consistent with the latter whereby the activated sludge was exposed to low concentrations of the substrate during the acclimation period (pre-Phase1) and the process started of initially with a low organic loading, i.e. 500 mg/L COD during phase 2 and was gradually increased in subsequent phases with the system efficiency responding accordingly. Because of the hydrophobic nature of the substrate, the mechanism of adsorption of lipids onto microbial flocs involves special mechanisms for the uptake and transport after hydrolysis. Some of the more common hypothesis that have been forwarded include :

- Incorporation of micro-droplets much smaller than the cell itself is the most common method used by unicellular microorganisms, and is often accompanied by production of biosurfactants to emulsify the free-phase hydrocarbons or oils and enhance substrate availability.
- Direct transport of macro-droplets into the cell is probably related to hydrophobic cell surfaces which allow the cells to contact or even move into the organic phase (Morgan and Watkinson, 1994).

A mentioned previously, the TAG is hydrolysed by lipases into glycerol and fatty acids which undergoes β-oxidation resulting in the removal of Acetyl-CoA(C_2) units until saturated fatty acids is completely converted to C_2 units which is then used in the TCA -cycle (Doelle,1994) However, the breakdown of aliphatic hydrocarbons is related to the breakdown of fatty acids, in that the *n* – alkanes are converted via an alcohol to the corresponding fatty acid and subsequently used as described above. This oxidation reaction requires significantly larger amounts of oxygen because of the conversion of a methyl group to a carboxyl group (Soddell,1999). Although no physiological investigations of the floc was conducted during the current research, the probability of the above explanation on the hydrolysis, uptake and metabolism of the TAG's in the oil effluent by the activated sludge micro-flora appears very high.

The hydrophobic oily layer covering the flocs appeared to be a common feature as observed using electron microscopy (discussed earlier) and did not pose as a limiting factor since COD was degraded and the mechanism explained above appears to be highly plausible. It should be noted that there were incidences during early phases were the oil effluent was not pre-treated and the system failed due to high FOG loading which super-saturated the floc with oil. However, no electron microscopy was conducted during this period to verify the physical structure of the flocs and the occurrence was temporary.

4.5 CONCLUSION

The conclusions that were derived from the laboratory scale treatment studies were as follows :

- The treatment process was conducted in phases which encompassed common operational parameters and therefore allowed accurate critical analysis of the data,
- large scale pretreatment achieved the desired objectives and removed sufficient COD including inhibitory compounds such as high concentration FOG's to facilitate biological treatment,
- effluent collected from industry varied in composition and strength amongst batches and therefore had to be corrected e.g N & P supplementation prior to serving as influent feed,
- trials to optimize biological phosphorus removal from the effluent were not successful due to limited time(restricted to phase 2) and P no longer being in excess in the effluent
- the COD parameter was used as a measurement of the organic removal capacity of the system, as has been common practice by other researchers in the field,
- the OUR served as a good measure of the metabolic activity of the process, reflecting similar trends as COD removal and process efficiency regularly,
- bulking of the process at the end of phase 3, signifying high SVI, low OUR's and MLSS

impacted on the process performance and the system was shut down,

- the influent COD concentration was gradually increased from 500 mg/L COD to 1 800 mg/L (undiluted) and the system responded accordingly with improved COD removal and maximum COD removed at 1 550 mg/L during steady state conditions (Phase 4 E),
- The mean MLVSS/MLSS ratio was consistent throughout most of the investigation and ranging between 0.8 and 0.9 mgVSS/mgTSS, reflecting low inert material concentration
- during the investigation, system perturbations were common occurrences e.g. phase 4 C, disruption in the supply of effluent necessitated the final effluent being recycled to sustain the biomass. However, on resumption of fresh influent, the system responded positively immediately. Disruptions in supply, caused the process to be non-functional for 5 weeks between phase 4 &5,
- the F/M ratio varied and ranged from 0.27 to 1.33 mgCOD/mgMLSS.d during the phases and the system responded accordingly,
- sludge age used was R_s =16 d. and produced the desired COD removal efficiency, ranging > 85 % at steady state. Maximum efficiency obtained was 92 %,
- the above confirmed that > 90 % of the influent COD was biodegradable,
- the infrequent foaming incidences was associated with the fatty material present in the substrate (oil effluent) that supported the growth of filamentous foam-formers, but did not effect process performance,
- compared to normal activated sludge floc, ESEM micrograph's of the floc that was exposed to oil effluent showed the flocs coalescing together at lower magnification,
- however, treatment with a non-polar solvent removed the oily layer surrounding the floc, thus confirming it's existence and revealed the ultrastructural detail comprising an abundance of microorganisms, dominated by prokaryotes,
- EDAX analysis of the microbial flocs investigated showed carbon and oxygen to be the major elements present, possibly representing the high lipid portion of the EPS,
- detailed chemical investigations showed that the substrate (oil effluent) comprised many essential oils with high commercial value with majority being biodegraded and not present in the final effluent,

- the mechanism of metabolism of the substrate within the floc macrostructure could be restricted to hydrolysis of the triacylglycerides by lipases to simpler forms such as fatty acids and uptake by the bacteria. Many theories have been forwarded regarding actual lipid adsorption

CHAPTER FIVE

MICROBIAL COMMUNITY ANALYSIS OF A LABORATORY SCALE ACTIVATED SLUDGE PROCESS

5.1 INTRODUCTION

Optimal performance of biological wastewater treatment processes requires the establishment of a microbial community that conforms to the metabolic type targeted by the process control strategy (Lui *et al.*,1998) The conditions in a reactor are manipulated to promote the presence and activity of desirable organisms. The design and maintenance of such systems is, in effect, engineering the bacterial populations and its activities (Curtis and Craine, 1997). The comparison of the bacterial diversity of activated sludge plants provides important information about the functions of the different groups of bacteria and could facilitate manipulation of these communities and enhance their functions. However, it is difficult to engineer processes which cannot be measured accurately. The limitations of the culture dependent based techniques in characterizing activated sludge microflora has been exposed repeatedly by various researchers (Amann *et al.*, 1990b; Mobarry *et al.*, 1996; Lindrea *et al.*, 1999). The advent of culture- independent techniques in the 1990's has provided researchers with additional and more accurate tools to conduct microbial community analyses investigations. Techniques such as 16S RNA approach, in particular whole cell hybridizations (Amann *et al.*, 1995),PCR and DGGE (Muyzer *et al.*, 1993) have recently been used extensively. These techniques exploit differences between the ribosomal gene sequence of different organisms.

The comparative analysis of rRNA molecules has revolutionized our view of microbial taxonomy and evolution (Woese,1987). Ribosomal RNA sequences are perfect targets for fluorescently labeled oligonucleotide probes, because they are highly conserved and naturally amplified, and can therefore be used in determinative studies in microbiology (Amann *et al.*, 1990b). By using selected regions within the larger rRNA molecules (16S and 23S like RNA) as hybridization targets for synthetic oligonucleotides, probe specificity to individual phyla or species, can be freely adjusted. In addition, the amount of a fluorescent rRNA -targeted probe that binds to cells should reflect growth rates and metabolic activities. Delong *et al.*(1989) also showed that probe binding varied with ribosomal content and reflected cell growth rate, viz metabolically active cells will produce intensified fluorescence, due to their increased rRNA content. A 'nested' approach adopted from Stahl (1986)can be used with probes being applied to parallel sub-samples in an ordered top to bottom manner. A variety of such probe sets have been developed starting with family level probes, followed by probes specific for genus and species.

The application of FISH for microbial community analysis of activated sludge processes could be considered a rather novel approach with a comparatively higher degree of success. Dual staining of samples with probe EUB and DAPI (Hicks *et al.*, 1992) gives an indication of not only the metabolic activity of the bacteria, but also that cells had sufficient rRNA for detection and were permeabilized for probes by the standard fixation procedure. However, some bacteria are less permeable to probes by normal fixation procedure. A high EUB/DAPI ratio in activated sludge would indicate a highly metabolically active bacterial population. Probing activated sludge with oligonucleotides revealed that the microbial consortia of various municipal plants being dominated by the class of Proteobacteria (Wagner *et al.*, 1993). Subsequent research report members of the beta-subclass as playing a major role in the microbial consortia of activated sludge plants and alpha and gamma sub-classes being less abundant (Wagner and Amann,1997)

Another molecular analytical tool that complemented the FISH approach was a DNA-based technique called denaturing gradient gel electrophoresis(DGGE), which was commonly used for rapid cross community comparisons of population complexity and similarity. The procedure is based on electrophoresis of PCR amplified 16S rDNA fragments in polyacrylamide gels containing a linearly increasing gradient of denaturants. DNA fragments of the same length but with different base pair sequences can be separated according to the melting property of the DNA (Muyzer *et al,* 1993). Best results can be achieved when these molecular methods are used together and enforce the credibility of findings concerning microbial community analysis of environmental samples.

In activated sludge microbiology, filamentous bacteria has attracted much attention since they are known to be the causative agents of phenomenon such as foaming and bulking which are cost-intensive malfunctions in wastewater treatment plants. Recently, increasing number of industrial wastewater treatment plants are regularly affected by these problems (Wanner *et al.*, 1998). The diversity of filamentous bacteria present in industrial wastewater treatment plants was analyzed by a combination of classical and molecular-biological approaches. Many unknown filamentous bacteria were observed in about 80 screened activated sludge samples from different industries with sometimes severe sludge bulking problems. A hardly cultivable filament called *Nostocoida limicola* was the focus of attention during these studies and believed to be associated with bulking conditions experienced in these plants(Snaidr *et al.*,2002) During the current investigation, characterization of filamentous bacteria was not within the scope of the investigation. However, they were studied due to the periodic bulking conditions experienced during the course of the laboratory scale treatment process.

The presence of protozoa in biological water treatment processes was noted almost as soon as each process was introduced and they feed by active predation on suspended particulate material including bacterial cells (Curds, 1992). Among the various groups of protozoa, the ciliated protozoans are usually considered to dominate the biomass, and thus have attracted the most attention. They are divided into a number of useful ecological groups on the basis of their feeding habits and their physical location within the biomass e.g free swimming ciliates, crawling ciliates which crawl over and through flocs, attached, sessile ciliates which are fixed to flocs through holdfasts and extend into bulk liquid and carnivorous ciliates which feed on other protozoa (Seviour, 1999). The composition of the ciliate community is known to vary with most of the plant operational conditions and a host of environmental factors (Abraham *et al.*, 1997). Most of the literature supports the view that the ciliate protozoa play a vital role in reducing the numbers of freely suspended and loosely attached bacterial cells in the bulk liquid by a process of grazing, and thus improve the quality and clarity of the final effluent leaving the plant. Furthermore, the effluent BOD and concentration of suspended solids and other factors decrease significantly in the absence of ciliated protozoans (Curds, 1992; Madoni, 1994). During the course of the current research, the analysis of the protozoan population in the process was subjected and restricted only to periods of system perturbations e.g settling problems, in order to draw possible correllation.

The primary objective of this aspect of the research was therefore to conduct a detailed microbial community analysis of the laboratory scale process using novel molecular techniques. For the current research, the approach that was used was qualitative. The aims were :

- Determine the community structure of the laboratory scale process using 16S rRNA *in situ* hybridization techniques
- apply a qualitative approach using DGGE to determine the species diversity of the process and complement the FISH analysis.
- investigate population shifts with gradual increase in the influent COD concentration using FISH
- correlate system perturbations to the characterization and presence/absence of filamentous bacteria and the protozoan population.

5.2 MATERIALS AND METHODS

5.2.1 Mixed liquor sampling

Grab samples of mixed liquor were collected from the aerobic and anoxic zones of the lab scale reactor throughout the course of the investigation. Samples were contained in polypropylene 50 mL bottles comprising of glass beads, which enhanced the desired disruption of the microbial floc and subsequently processed as follows.

5.2.2 Cell fixation and immobilization on microscope slides

For *in situ* identification of Gram-negative bacteria in the activated sludge, samples were fixed immediately by adding three volumes of fresh (not older than 24hours) 4% (w/v) paraformaldehyde solution to one volume of the sample (Appendix 15) and held at 4^0C for 1 h. For the detection of gram-positive bacteria in activated sludge, the samples were fixed by adding one volume of ice-cold absolute ethanol (Wagner *et al.*, 1994a), held at 4^0C for 1h and stored at -20^0C. Fixed cells were then centrifuged at 5000 × g and the pellet washed with phosphate buffered saline [PBS; 130 mM sodium chloride, 10 mM sodium phosphate buffer (pH 7.2)] and resuspended in PBS. One volume of ice cold ethanol (1:1, v/v) was added and the samples were be stored at -20^{0}C. The stored samples were then washed once in PBS and sonicated using a Virsonic 100 sonicator (United Scientific (Pty) Ltd.), for 8 min at 5 watts to facilitate dispersion of the cells bound within the floc structures (Appendix 16). In addition, a non-ionic detergent Igepal Ca-30 was mixed with the samples at 10% v/v concentration. Following sonication, the samples were diluted 10% in double distilled water, before being immobilized by spreading on poly-L-lysinated microscope slides and then left to air-dry (Appendix 17). The immobilized samples on the slides were then dehydrated by dipping through successive passages of 50, 80 and 98% ethanol.

5.2.3. Membrane filtration and DAPI staining for total cell counts

DAPI staining for total cell counts was carried out as described by Porter and Feig, (1980) (Appendix 18).

5.2.4 Oligonucleotide probes used

Oligonucleotide probes were synthesized and labeled with either rhodamine (red) or fluorescein (green) by Roche Molecular Biochemicals (Germany). Probes used are given in TABLE 5.1. In addition, representative bacterial strains from each of the classes, sub-classes and genera investigated and used to evaluate probe specificity are given in Table 5.2

TABLE 5.1 . Probe sequences and target sites for *in situ* hybridization.

Probe	**Sequence**	**Target site**	**Fluor**	**Reference**
EUB338[1]	5'-GCTGCCTCCCGTAGGAGT-3'	16S	Fluorescein	Amann *et al.* 1990
ALF1b[2]	5'-CGTTCG(C/T)TCTGAGCGAG-3'	16S	Rhodamine	Manz *et al.* 1992
BET42a[3]	5'-GCCTTCCCACTTCGTTT-3'	23S	Rhodamine	Manz *et al.* 1992
GAM42a[4]	5'-GCCTTCCCACATCGTTT-3'	23S	Fluorescein	Manz *et al.* 1992
HGC69a[5]	5'-TATAGTTACCACCGCCGT-3'	23S	Rhodamine	Roller *et al.* 1994

[1]EUB338 probe specific for all hitherto sequenced species affiliated to the kingdom *Bacteria* (previously referred to as Eubacteria); [2]ALF1b, specific for the alpha subclass of *Proteobacteria* which including most members of the phylum "spirochetes and relatives", and *Flexistipes sinusarabicus*; [3,4]BET42a and GAM42a, specific for the beta and gamma subclass of *Proteobacteria*, respectively; [5]HGC69a, specific for the *Actinobacteria.*

TABLE 5.2 Bacterial reference strains used to determine probe specificity.

Organism	**Source**	**Probe specificity**	**Synonym**
Acetobacter acetii	SABS	*Proteobacteria* - alpha	ALF
Alcaligenes faecalis	TN	*Proteobacteria* - beta	BET
Acinetobacter calcoaceticus ATCC - 14987	ATCC	*Proteobacteria* - gamma	GAM
Flavobacterium spp.	UCT	*Cytophaga-Flavobacterium*	CF
Corynebacterium glutamicum	SABS	GPBHGC	HGC

SABS - South African Bureau of Standards culture collection
TN - Technikon Natal Microbiology Department
ATCC - American Type Culture Collection
UCT - University of Cape Town Microbiolgy Department
GPBHGC - Gram Postive Bacteria with High G + C DNA content

5.2.5 Whole cell hybridization and DAPI staining

Samples were hybridized with probes according to Amann *et al.*, (1995), with 10 µl of probe solution containing 0.9 M NaCl, 0.01% sodium dodecyl sulphate, 20 mM Tris-HCl (pH 7.2), 50 ng of oligonucleotide probe. The formamide stringencies (v/v) for the different probes were; 20% for probes EUB338 and ALF1b, 25% for probe HGC69a and 35% for probes BET42a and GAM42a. Hybridization was carried out at 46°C, for 90 min in a sealed 50mL poly-propylene centrifuge tube and equilibrated with pre-warmed hybridization buffer to provide an isotonic environment. The hybridization was stopped by removing the probes from the slides with pre-warmed wash buffer containing 20mM Tris/HCl - pH 8.0, 0.01%SDS and the following concentrations of NaCl, 0.12 M for probes BET42a and GAM 42a, and 0.9 M NaCl for probes EUB338 and ALF1b. The slides were then immersed in a 50 mL polypropylene centrifuge tube containing pre-warmed wash buffer at 48°C for 20 min, then rinsed shortly with PBS and dried at room temperature.

After hybridization of the samples, 10 µl of DAPI (4',6-diamidino-2-phenylindole 0.25 µg/ml) was spotted onto the samples and stained in the dark for 10 min, before being washed with double-distilled water and left to dry at room temperature. To prevent fading of the bound probe, 1 drop of vectashield (Vector Laboratories Inc.) was spotted onto the sample before being covered with a 22 mm × 22 mm (Euroslip) coverslip, laminated with clear nail varnish and allowed to dry. Twenty fields were randomly selected for enumeration by image analysis. The cell count for each probe was determined by using the following equation:

$$\frac{\mathbf{n(Probe)}}{\mathbf{n(DAPI)}} \times \frac{\mathbf{100}}{\mathbf{1}}$$

Where; n(Probe) = average number of cells bearing probe conferred fluorescence
n(DAPI) = average number of cells bearing DAPI conferred fluorescence

5.2.6 Microscopy and image analysis

Visualization of fluorescence indicating the binding of the probe occurred using a Zeiss Axiolab microscope fitted for epifluorescence with a 50W high pressure mercury bulb and Zeiss filter sets specific for DAPI, tetramethylrhodamine-5-isothyocyanate (TRTC, Boehringer Mannheim, Germany) and carboxy-fluorescein-N-hydroxysuccinimide-ester (FLUOS, Boehringer Mannheim, Germany). In addition 30 randomly selected images per slide, were captured by Zeiss KS300 image analysis software and converted to TIFF files. There was a limitation with regards to quantification of the cells due to non-homogenous distribution of the bacteria. Therefore cell counts were carried out by using the above imaging system and creating computer macros and measuring the area of each signal (Appendix 19).

5.2.7 Nucleic acid extraction and PCR

The bacterial genomic DNA was obtained by the phenol extraction method at 55^{0}C and ethanol precipitation (Appendix 20). This DNA was further purified using a commercially available purification kit (Bio-Rad laboratories). These DNA preparations were then used as template DNA's in the PCR.

The variable V3 region of the 16S rDNA was enzymatically amplified using the PCR method. The nucleotide sequences of the primers were as follows : Primer 1,5'- CCTACGGGAGGCAGCAG-3'; primer 2,5'- ATTACCGCGGCTGCTGG-3' and primer 3,5'-CGCCCGCCGCGCCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3' (Muyzer *et al.*, 1993). Primer 3 contained the same sequence as primer 1 but had at it's 5' end an additional 40-nucleotide GC -rich sequence (GC clamp). A combination of primers 2 and 3 were used to amplify the 16S rDNA region. The PCR amplification was carried out using a Hybaid Thermal cycler in 0.5 mL reaction volumes. The reaction volumes contained : 10μl of 10x PCR buffer(100 mM Tris-HCl),10μl of 200μmol deoxynucleotide triphosphate solution, 65μl of distilled water, 2μl of a 50 μM stock solution of each primer and 10μl of 10ng/μl genomic DNA. The samples were first incubated at 94^{0}C for 5 min in order to denature the template DNA and subsequently cooled to 80^{0}C at which point 1 unit of Taq DNA polymerase was added. This hot start technique was used to minimize non-specific annealing primers to non-target DNA. This was followed by 30 cycles of denaturation (1 min at 94^{0}C), annealing (1 min at 53^{0}C) and extension (2 min at 72^{0}C) and a final extension at 72^{0}C for 4 min. The amplified DNA was verified by electrophoresis of 5 μl of the PCR product on a 1 % agarose gel in 1x TAE buffer (20 mM Tris acetate pH 7.4 , 10mM sodium acetate and 0.5 mM disodium EDTA).

5.2.8 Denaturing gradient gel electrophoresis (DGGE)

The PCR amplified DNA fragments was subjected to DGGE using the Bio-RAD D Gene^{TM} system. PCR samples were applied directly onto 7.5% (wt/vol) polyacrylamide gels in 0.5x TAE(20 mM Tris acetate [pH 7.4],10 mM sodium acetate , 0.5 mM Na_2-EDTA) with gradients which were formed with 8%(wt/vol) acrylamide stock solutions (acrylamide -*N,N'*- methylenebisacrylamide, 37:1) and which contained 30 and 60%, 30 and 70%, 30 and 80% denaturant (7M urea [Boehringer Mannheim]) and 40 % (vol/vol) formamide (Merck). Electrophoresis was performed at a constant voltage of 200V and a temperature of 60°C. The latter method was adapted from Muyzer *et al.*(1993). Some of the parameters were varied in order to optimize the conditions for DGGE. The parameters included gradually increasing the denaturant concentration gradient from 30/60% up to 30/80% denaturant. From the perpendicular gradient analysis, a defined gradient of 30 to 70% was selected based on different sequence variants and the migration patterns. The length of time of electrophoresis was set at 180 min. which was predetermined. Another parameter that was experimentally optimized was the sample concentration that was applied to the gel. The volume ranged from 10 µl to 50 µl with the latter producing better results. The success of the DGGE technique depended ultimately on obtaining the ideal blend of the latter process parameters in order to achieve the desired results.

After electrophoresis, the gels were removed and stained with 250 mL 1x TAE buffer and 10µl Ethidium bromide (10mg/mL) for 1-2 min. The gel was then transferred to another container containing 250 mL 1x TAE buffer where it was de-stained for 15 min and viewed using a UV transilluminator (Hoefer) and subsequently photographed.

5.2.9 Identification of filamentous bacteria

The observation and identification of filamentous bacteria in the lab scale process was not within the scope of the initial investigation. However, due to system failure which was attributed to bulking conditions, the investigation was restricted to this period only i.e. late phase 3.

5.2.9.1 Sample collection and handling

Grab samples of mixed liquor was obtained from the aerobic reactor 1 in the midst of the bulking conditions, at the end of phase3 (refer to Chapter 4). The samples were collected in 500 mL sterile Schott bottles. There was no need for specific storage procedures since examinations were conducted on -site in the laboratory and immediately.

5.2.9.2 Identification techniques

Activated sludge wet mounts and smears were prepared by spreading 1 drop of sample evenly over approximately 50 % of the area of a standard microscope slide and air dried. The wet mounts were studied under direct illumination at 1000x magnification to determine the morphological characteristics of the various filaments. In addition, smears were also stained using the Gram and Neisser staining techniques. Stained smears were examined under oil immersion and direct illumination at 1000x magnification. Various other test and procedures were concluded e.g. sulfur oxidation test, polyhydroxybutyrate (PHB)stain, crystal violet sheath stain and others in accordance to the format outlined by Jenkins *et al.* (1986) for filamentous organism identification (Appendix 21). Initially, the samples were scanned at 100x magnification using phase contrast to ascertain the number of different types of filamentous organisms present.

The size of the filamentous population can vary considerably and therefore important for the population to be quantified. There are various filament quantification methods currently available, but the most commonly applied methods are the Filament index which is a scoring from 0 to 5 (none to very many) (Eikelboom, 2000) and the individual abundance level of each filament was determined using the subjective scoring system as outlined by Jenkins *et al.* (1986) and used during the current investigation. Each filament was scored on a scale between 0 and 6 (none to excessive).
The system rated filament abundance on an average ‘ per floc’ basis. Filamentous bacteria with individual abundance levels of 4 or more were classified dominant while those with individual abundance levels of 3 or less were classified as secondary.

5.2.10 **Identification of Protozoa**

The determination of the protozoan population during the current investigation was limited to periods of poor performance i.e periods of bulking and the first portion of phase 5. The Protozoa were observed microscopically under 100x and increased to 400x magnification when required. Wet mounts included obtaining 50 µl mixed liquor samples from the aerobic reactor which was placed on a microscope slide and observed in accordance to established techniques (Madoni, 1994). It is an accepted fact that the type of locomotion is the basis for classification of protozoa (Bitton, 1999). The current identification techniques followed the approach used by using morphologically distinctive forms observed during microscopic investigations and identifying and enumerating them using established reference text (Curds, 1992; Madoni 1994; Seviour, 1999 and Eikelboom, 2000).

5.3 RESULTS

5.3.1 Biodiversity of the laboratory scale process using FISH

The investigation using the FISH technique to conduct a detailed microbial community analysis was partitioned into 2 phases. Phase A covered the period of investigation spanning from 25-5-2000 up to 01-01-01 encompassing phase 2 to 4 of the lab scale process and phase B covered the period from 05-03-01 up to 04-10-01 encompassing phase 5 of the lab scale process(refer to chapter 4).

The composition and distribution of the bacterial population as per the phylogenetic classification system is presented in Table 5.3. The results are expressed as percentages and represent averages that were calculated by dividing the number of probe specific counts by the number of DAPI counts for 30 randomly selected microscopic fields (400x magnification). Total probe percentages were calculated through summation of the average probe percentages for each respective sample. The microbial community profile of the seed inoculum is represented by sample 1 (obtained from the aerobic zone of Darvil wastewater works) and served as a control to determine possible population shifts that could have occurred with increase in the influent organic strength and during the operation of the lab scale process. Samples 2 to 6 represented grab samples obtained from the process at progressive time intervals during phase A of the process (Table 5.3).

TABLE 5.3 Probe specific counts (*in situ*) expressed as a percentage of total cells stained with DAPI and total cell counts as obtained by membrane filtration for Phase A of the lab-scale MLE process .

	Darvill WWW	**Phase A of Lab-scale Modified MLE Process**				
Sample number	1.	2.	3.	4.	5.	6.
Date	25.05.00	27.08.00	25.10.00	11.12.00	23.01.01	01.02.01
DAPI (%)	100	100	100	100	100	100
α (%)	18(6)	35(4)	33(3)	21(4)	34(2)	30(3)
β (%)	35(8)	21(4)	17(2)	30(7)	16(3)	23(8)
γ (%)	14(4)	8(5)	9(2)	6(3)	9(5)	11(6)
HGC (%)	13(4)	7(2)	5(3)	6(1)	2(1)	4(3)
Total (%)	80	71	64	63	61	68
EUB338 (%)	81(6)	74(9)	69(2)	65(7)	65(3)	67(8)
Total Cell Count (Cells/mL)	4.9×10^9	3.7×10^9	3.1×10^9	3.0×10^9	2.0×10^9	2.3×10^9

- Sample standard deviations are presented in parenthesis

Application of the probe EUB 338 to sample 1 revealed a 81 % EUB/DAPI ratio. There was a slight decrease with probing samples from the process whereby the ratio decreased and ranged from 74-65 %, respectively. However, these results still confirmed that majority of the fixed cells were from the domain *Bacteria.* Total cell counts (DAPI) of the seed sludge was 4.9×10^9cells/mL and process samples ranged from 2.0×10^9 cell/mL to 3.7×10^9 cells/mL and details are given in Table 5.3. When compared to the EUB probe (81%), the total percentage of probes that conferred signals and were bound to target sites was 80% for the seed sludge. This suggested that only 1 % of the bacteria were unaccounted for i.e. possibly belonging to another subclass such as CF. Similarly, for samples 2 to5 the total percentage of probes bound were slightly lower than the percentage EUB for the respective samples. However, for sample 6 the total percentage bound was 1% higher (68%) than the total bacterial population represented by EUB 338 probe (67%) (Table 5.3). This anomaly could be attributed to non-specific binding of one of the group specific probes.

Community structure profile of the seed sludge (sample 1) indicated the beta-subclass of *Proteobacteria* to be numerically predominant i.e. 35% (Beta 42/DAPI) followed by alpha at 18%(Alf1b/DAPI),gamma 14% (Gam42a/DAPI) of the subclass *Proteobacteria* and *Actinobacteria* at 13% (HGC69a/DAPI) respectively, of the DAPI population. Figure 5.1 serves as a graphical representation of probe specific counts shown in Table 5.3 and reflects the percentage specific probe bound in relation to DAPI. Comparing the community profiles of samples 2 to 6 (progressive samples obtained at intervals during phase 2-4 of the lab scale process), there is clear evidence that as the phase progressed there was a gradual decrease in the domain *Bacteria* counts (EUB 338) (Table 5. 3 & Fig.5.1).

The community profiles will be presented in correlation with the performance of the process during the respective phases with reference to Table 5.3 and Figure 5.1. The alpha-*Proteobacteria* population increased from 18% (sample 1 ,seed sludge) to 35 % of DAPI in sample 2. Correlation to the process efficiency during this period revealed optimum performance with 90 % COD removal (Fig 4.12). There was a marginal decrease in the alpha-*proteobacteria* population in sample 3. During this period there was a short disruption in influent feed supply, negatively impacting on COD removal and a slight decrease in the OUR. The influent COD concentration was increased from 800 to 1200 mg/L. Sample 4 was obtained when the influent feed concentration was increased to 1500 mg/L. The process was performing optimally during this period. However, during this period there was a notable decrease in the alpha-*Proteobacteria* numbers. During sampling periods 5 &6 the process performance was poor with decreased COD removal efficiencies and OUR. The influent feed COD concentration was increased to 1800 mg/L. The alpha-*Proteobacteria* population recovered with 34 and 30% predominance during this period.

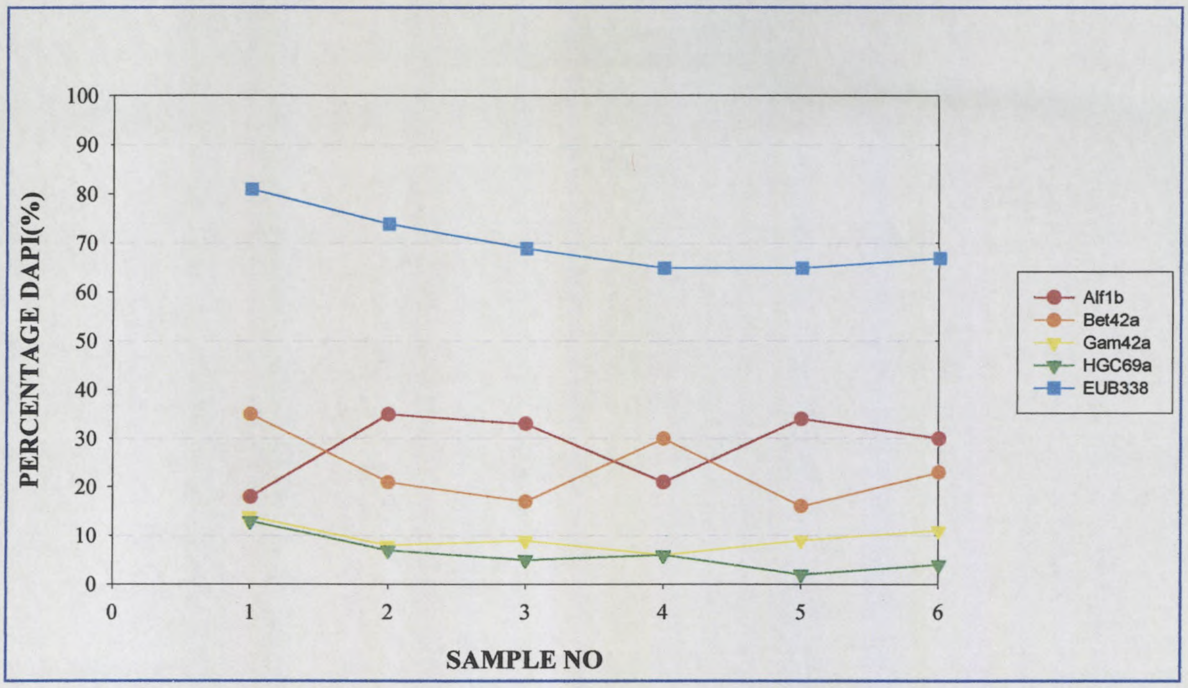


FIGURE 5.1 Graph representing the distribution of bacteria in relation to DAPI during phase A of the lab scale process

The distribution of the beta-subclass of *Proteobacteria* was in direct contrast with the alpha subclass and decreased from 35% in the sample 1 (seed sludge) to 17% in sample 3. There was a increase in the COD from 800 to 1200 mg/L during this period and the process performance was poor due to disruption in influent feed supply. In sample 4. in contrast to the alpha, the beta-*Proteobacteria* numbers increased to 30%. The system was performing optimally during this period and influent COD was increased to 1500 mg/L. The population decreased to 16% in sample 5 and increased slightly to 23 % in sample 6. During these periods the influent COD was increased 1800 mg/L and the latter portion of the phase during the sampling periods was characterized with system perturbations. The gamma -*Proteobacteria* also showed a decrease from the seed sludge (sample1) at 14 % to 8% in sample 2 and the population remained relatively stable during the rest of the phase. The *Actinobacteria* (HGC) followed a similar trend as the gamma subclass. It should be noted that when compared to the seed sludge (sample 1) there was a overall decrease in the percentages of the respective subclasses of *Proteobacteria*. The dominance of alpha and beta-subclasses in both the seed sludge and the lab scale process samples were pronounce (Fig 5.1 &Table 5.3).

On resuming operation of the lab scale process during phase B, the seed inoculum comprised of a mixture of MLSS that was maintained from phase A during the suspension period and fresh Darvil sludge at a ratio of 1:1. The microbial community profile is presented in Table 5.4.

TABLE 5.4 Probe specific counts (*in situ*) expressed as a percentage of total cells stained with DAPI and total cell counts as obtained by membrane filtration for Phase B. of the pilot-scale MLE process .

	Darvill/ A.MLSS	**Phase B of Lab-scale Modified MLE Process**				
Sample number	7.	8.	9.	10.	11.	12.
Date	05.03.01	09.05.01	21.06.01	24.07.01	28.08.01	04.10.01
DAPI (%)	100	100	100	100	100	100
α (%)	16(5)	37(5)	33(1)	35(9)	38(3)	29(7)
β (%)	36(9)	19(3)	16(5)	11(1)	13(3)	16(3)
γ (%)	12(8)	10(3)	16(4)	2(1)	6(4)	3(1)
HGC (%)	7(4)	3(3)	<5	5(2)	1(1)	<5
Total (%)	71	69	65	53	58	48
EUB338 (%)	74(4)	72(7)	68(2)	57(5)	60(7)	49(8)
Total Cell Count (Cells/mL)	7×10^9	6.5×10^9	5.1×10^9	2.2×10^9	4.6×10^9	1.3×10^9

° Sample standard deviations presented in parenthesis

Sample 7 represented the seed inoculum (combined MLSS) and samples 8-12 represented grab samples obtained periodically during phase B of the operation of the lab scale process. Although the mixed liquor of the starter culture was combined, the results revealed a similar community profile as sample 1 (Darvil seed sample) with the beta subclass of *Proteobacteria* predominating with an average of 36% followed by the alpha and gamma subclasses with averages of 16 and 12 %, respectively. *Actinobacteria* (HGC) was the lowest at 7%. The high beta subclass presence in the Darvil sludge appears to have sustained any effect on altering the community (sample 7) from the mixed liquor obtained from phase A and predominated as in sample 1 (Table 5.4).

Application of probe EUB 338 to sample 7 showed that 74% of the microorganisms belonged the domain *Bacteria* (EUB/DAPI ratio). The latter results were similar to sample 1 and once again confirmed that majority of the fixed cells were bacteria. When compared to the seed sludge, the EUB/DAPI ratio of the samples (8-12) obtained from the process decreased as the phase (B) progressed and ranged from 72% (sample 8) to 49% (sample 12) respectively (Fig 5.2), thus following a similar trend as phase A. The seed sludge (sample 7) recorded a DAPI total cell count of 7 x 10^9 cell/mL. The DAPI total cell counts of the process samples ranged from 6.5 x 10^9 (sample 8) and decreased to 1.3 x 10^9 (sample 12). In comparison to the EUB 338 probe (74%), a total probe percentage of 71% was calculated for sample 7 indicating that 3 % of the bacteria were not detected using the selected probes. This phenomenon was repeated when subsequent samples (8-12) obtained from the process also presented slightly lower total percentage of probes bound when compared to their respective EUB 338 counts (Table 5.4).

The community profile of the seed sludge (sample 7) which comprised partial Darvil sludge reinforced findings of phase A (sample 1) and showed beta *Proteobacteria* to be predominant at 36% followed by alpha, gamma and HGC of the total DAPI population (Table 5.4). Figure 5.2 graphically presents the probe specific counts shown in Table 5.4 and reflects the percentage specific bound in relation to DAPI.. The profiling of the microbial community will be presented in correlation with the performance of the process and the operational conditions that persisted during the respective phases and with reference to Table 5.4 and Figure 5.2. Similar to the population shift witnessed in phase A, the alpha *Proteobacteria* population increased from 16% (sample 7, seed sludge) to 37% of DAPI in sample 8. During this period the process was at steady state and performing optimally. The mean influent COD was 1465 mg/L and removal efficiency was averaging 90% (Fig 4.33). There was a slight decrease in the alpha subclass in sample 9 at 33% and in relation the system was experiencing low influent COD feed during the period with a decreased COD removal efficiency and a corresponding drop in the OUR. However, in sample 10 there was a marginal increase in the alpha subclass (35%) although the system perturbations and decreasing efficiency continued (Figs 4.39& 4.41). The marginal increase in the alpha population continued in sample 11 i.e. 38% regardless of the low influent feed concentration and the corresponding poor removal efficiency during the period.

Sample 12 was obtained at the end of the same phase (5G) and at the end of the lab scale process. During this period the system efficiency was poor(Fig 4.47) andthere was a substantial decrease in the OUR from 31 to 19 mgO/L.h. However, the alpha *Proteobacteria* decreased substantially during this period and was recorded at 29 %.

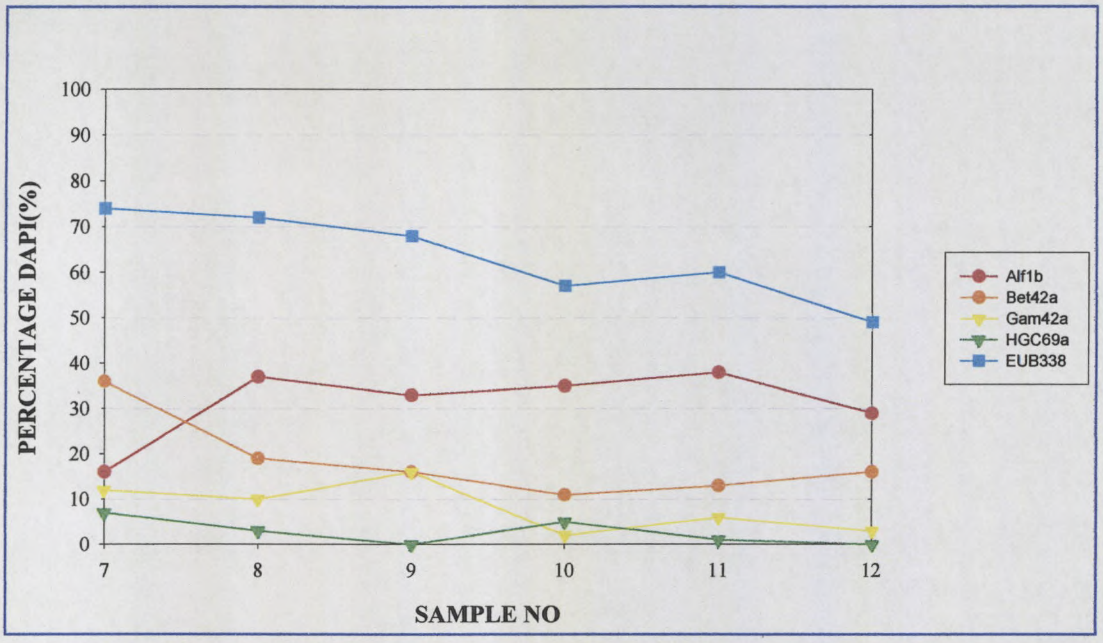


FIGURE 5.2 Graph representing the distribution of bacteria in relation to DAPI during phase B of the lab scale process

Contrary to the alpha subclass and following the same trend as in phaseA, the beta subclass of *Proteobacteria* in sample 7 (seed sludge) which was at 36% decreased to 19% in sample 8. The process was operating optimally during this period. with COD concentration removed being 1324 mg/L. The community profile of samples 9 & 10 showed a continuous decrease in the beta subclass recording 16 and 11 %, respectively. During this period the influent COD concentration was comparatively low and the system performance was relatively poor (Fig 4.39). However, the beta subclass population began to increase gradually in sample 11 and 12 and calculated at 13 and 16%, respectively. The samples (11 &12) were obtained during phase 5 G which was the last phase of the process with lower influent COD concentrations and diminished performance especially during sample

12 isolation.

The gamma subclass population in the seed sludge (sample 7) was12 % and marginally decreased to 10 % of DAPI in sample 8 with optimal process performance. However, the gamma population increased substantially in sample 9 (16%) during a period of process perturbation with decreasing COD removal efficiency. In sample 10 there was a substantial drop to 2%. Sample 9 and 10 were isolated at the beginning and end of the same phase (5E). The erratic population variation of the gamma subclass continued with sample 11 recording a slight increase at 6% during a period of decreased process efficiency, and sample 12 was obtained at the end of the lab scale process and once again recording a decrease in the gamma subclass at 3% of DAPI.. The *Actinobacteria* (HGC) population was comparatively smaller and the seed sludge sample 7 was recorded at 7% and decreased in samples isolated from the process, ranging from 3-5% of DAPI (Table 5.4 & Fig 5.2)

Some of the more common and pronounce features when comparing phase A and B were as follows:

- In both phases the seed sludge recorded higher EUB/DAPI ratios when compared to process samples and the ratio decreased as the process progressed,
- beta *Proteobacteria* predominated in the seed sludge and the proceeding sample (2 &8) from the process showed a substantial increase in the alpha population and a corresponding decrease in the beta subclass,
- the alpha subclass was the dominant community, followed by the beta subclass in the lab scale process,
- compared to the seed sludge (samples 1&7) there was a overall decrease in the total percentages of the respective subclasses of *Proteobacteria* and there was a continued decline as the process progressed,
- in both phases (A & B) the bacteria that were not detected when comparing the total percentages bound by group specific probes to the EUB 338 probe was relatively low,
- the DAPI total cell count of the seed sludge (sample 1& 7) was higher than the process samples and the trend showed an overall decrease as the lab scale process progressed.

The decrease in the bacterial population as depicted by the decrease in the EUB/DAPI ratio in phase A was not reflected by a decrease in the VSS during the respective period of operation (Phase 3 &4). The similar trend was reflected in phase B with a decrease in the EUB population not supported with a corresponding decrease in the MLVSS. One of the aims of the research was to identify possible shifts in the microbial community profile as the influent COD concentration was increased i.e. 800 mg/L to 1800 mg/L. During phase A the periodic increase in influent COD concentrations for samples 2-6 were established at 800, 1200, 1500, 1800 and 1800 mg/L, respectively. During phase B, due to dependency on the substrate organic strength from industry, the process was initiated with an influent COD concentration averaging at 1800 mg/L but decreasing gradually as the phase progressed. Therefore, emphasis on the population shifts will be restricted to phase A. With reference to Table 5.3 and Fig 5.1, the bacterial population (EUB/DAPI) decreased as phase A progressed (sample 2-5), with a marginal increase in sample 6. The alpha subclass presented an overall increase and was the dominant population as the COD increased. The beta subclass showed an overall decrease in the population, although the shift was erratic and not consistent as the COD increased. Sample 4 performed contrarily to the above trends. Comparatively, the gamma and HGC population also showed an overall decrease as the COD increased. In phase B and reference to Fig 5.2, a similar pattern in the population shift as in phase A was observed, although the COD did not increase throughout the phase. Therefore it was likely that in the current investigation, the influent organic strength (COD) was not primarily responsible for determining the population shifts in the lab scale process. In phase B the influent COD concentration decreased as opposed to phase A and regardless, the population profile trends remained relatively the same. Other operational parameters such as sludge age, HRT, DO concentration and also the influent chemical quality could play a role in determining the population. Micrographs representing DAPI staining and *in situ* hybridization using group specific probes for the seed sludge and samples isolated from the process are given in Figs 5.3 to 5.11 with details in the legends.

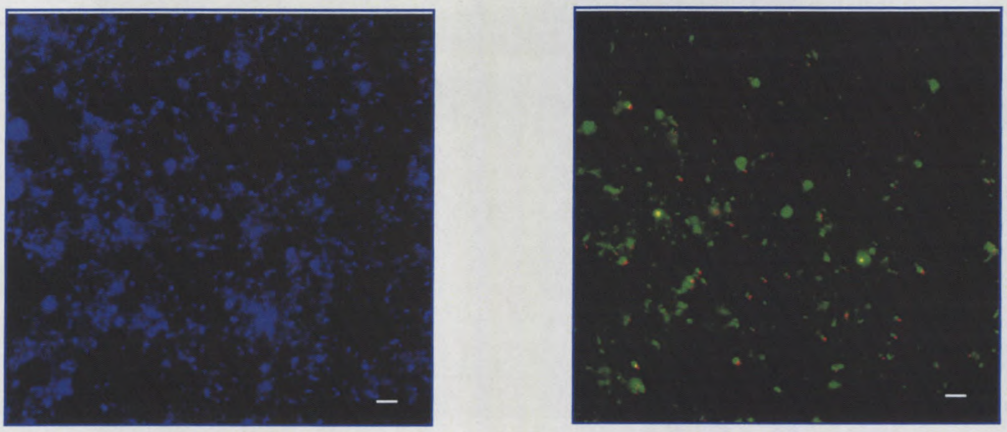


FIGURE 5.3 Micrograph of Sample 1 (Darvill seed sludge) after DAPI staining (LHS) and *in situ* hybridization with fluorescein (green) probe EUB338 specific for the kingdom *Bacteria* and rhodamine (red) probe ALF1b specific for alpha *Proteobacteria* (RHS) for the same microscopic field (400x magnification; scale bar indicates 10μm).

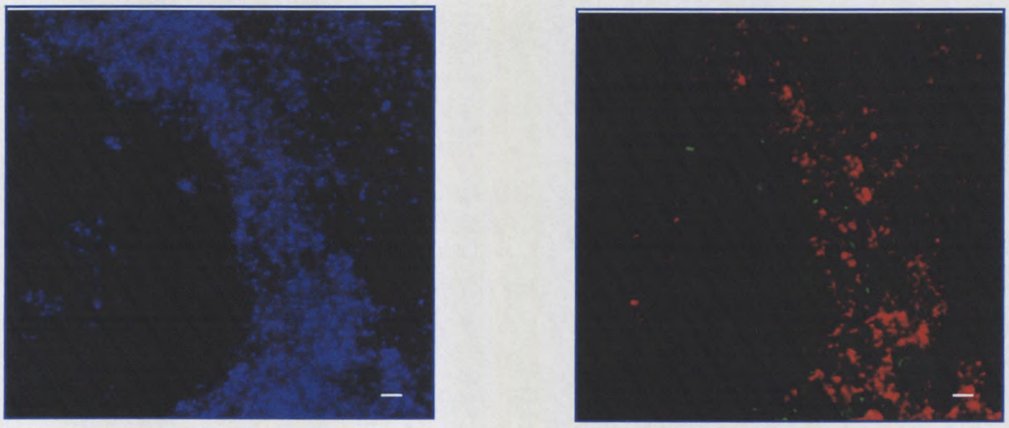


FIGURE 5.4 Micrograph of Sample 1 after DAPI staining (LHS) and *in situ* hybridization with rhodamine (red) probe BET42a specific for beta *Proteobacteria* and fluorescein (yellow) probe GAM42a specific for gamma *Proteobacteia* (RHS) for the same microscopic field (400x magnification; scale bar indicates 10μm).

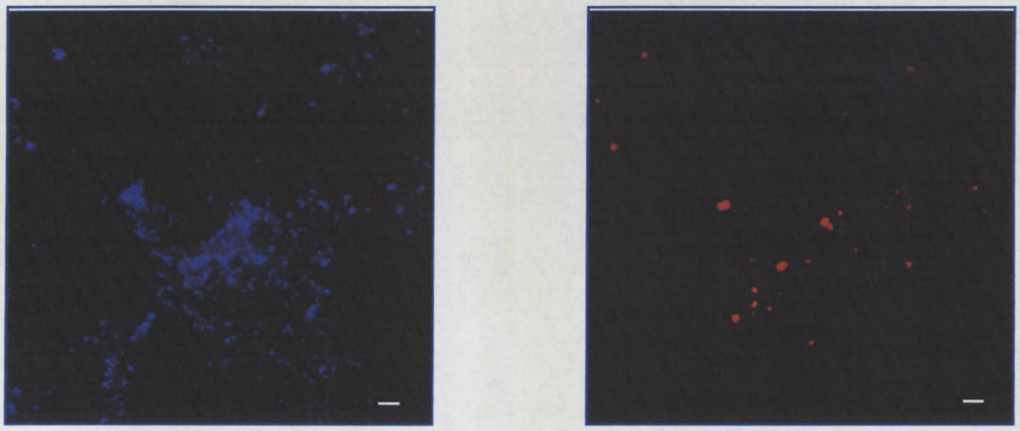


FIGURE 5.5 Micrograph of Sample1 stained with DAPI (LHS) and displaying probe conferred signal after hybridization with probe HGC69a (RHS) for the same field (400x magnification; scale bar indicates 10μm).

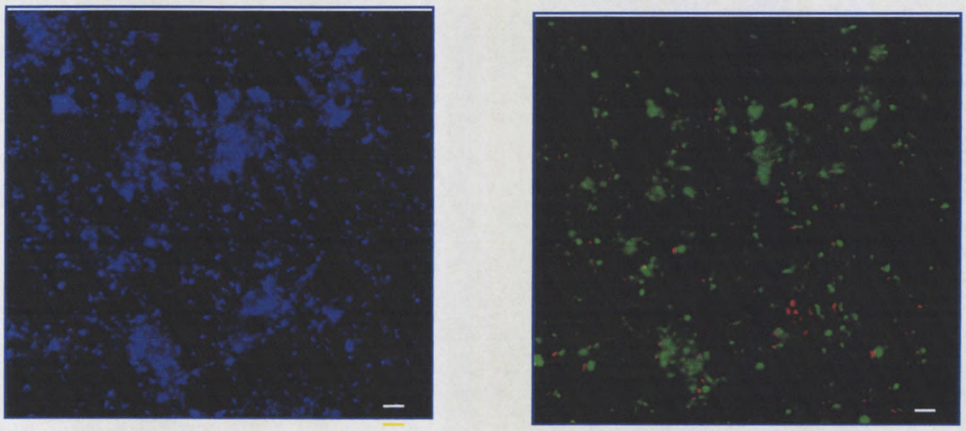


FIGURE 5.6 Micrograph of Sample 5 after DAPI staining (LHS) and *in situ* hybridization with fluorescein probe EUB338 and rhodamine probe ALF1b (RHS) for the same microscopic field (400x magnification; scale bar indicates 10μm).

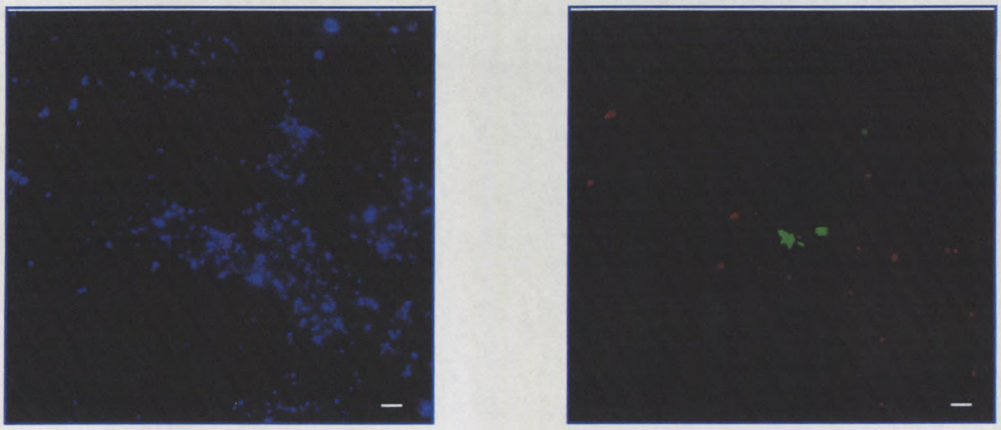


FIGURE 5.7 Micrograph of Sample 5 after DAPI staining (LHS) and *in situ* hybridization with rhodamine probe BET42a and fluorescein probe GAM42a (RHS) for the same microscopic field (400x magnification; scale bar indicates 10μm).

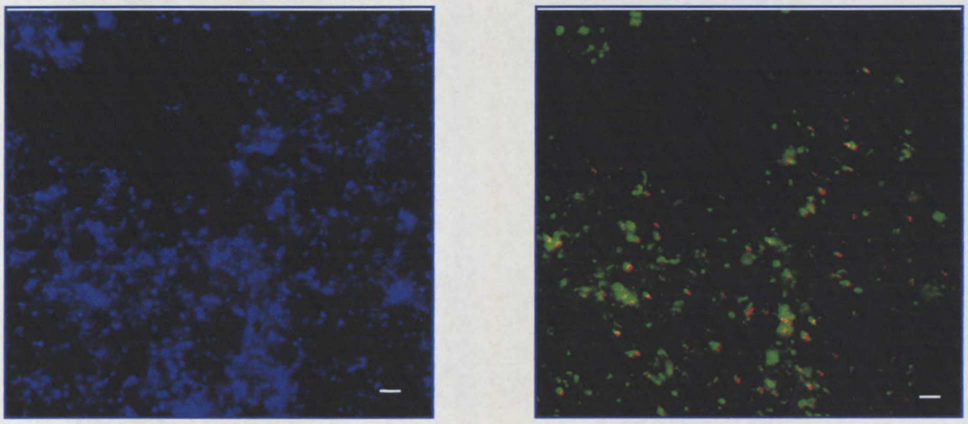


FIGURE 5.8 Micrograph of Sample 7 after DAPI staining (LHS) and *in situ* hybridization with fluorescein probe EUB338 and rhodamine probe ALF1b (RHS) for the same microscopic field (400x magnification; scale bar indicates 10μm).

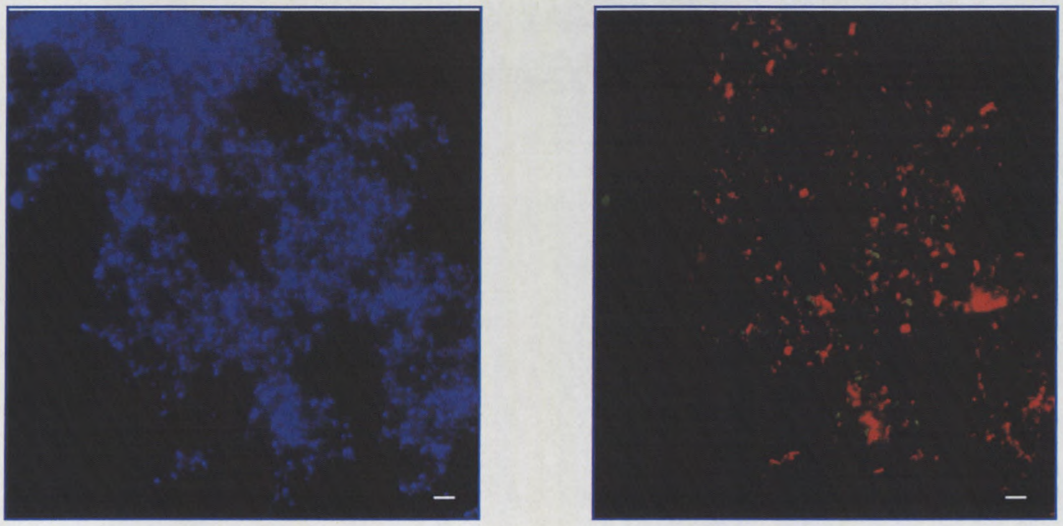


FIGURE 5.9 Micrograph of Sample 7 after DAPI staining (LHS) and *in situ* hybridization with rhodamine probe BET42a and fluorescein probe GAM42a (RHS) for the same microscopic field (400x magnification; scale bar indicates 10μm).

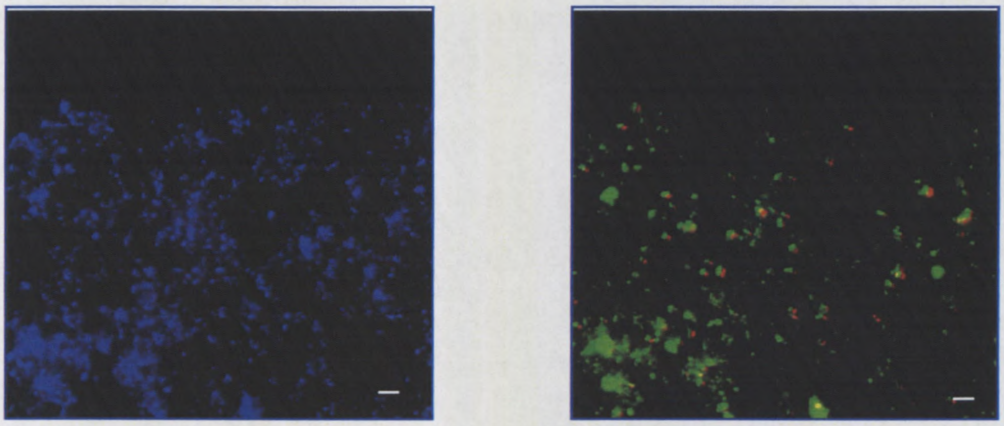


FIGURE 5.10 Micrograph of Sample 11 after DAPI staining (LHS) and *in situ* hybridization with fluorescein probe EUB338 and rhodamine probe ALF1b (RHS) for the same microscopic field (400x magnification; scale bar indicates 10μm).

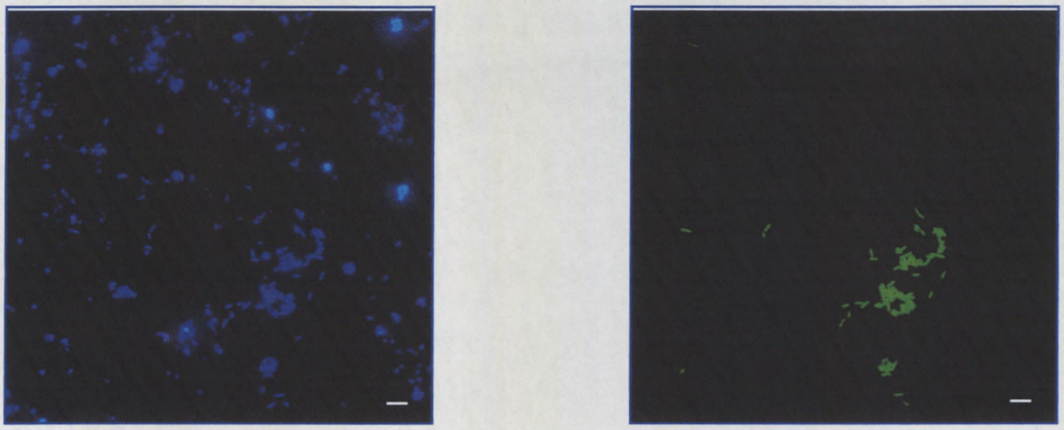


FIGURE 5.11 Micrograph of Sample 11 after DAPI staining (LHS) and *in situ* hybridization with fluorescein probe GAM42a (RHS) for the same microscopic field (400x magnification; scale bar indicates 10µm).

5.3.2 Determination of bacterial diversity using PCR-DGGE

Three sets of samples were obtained during the operation of phase B (05/01– 10/01). The samples comprised of nucleic acid obtained from the MLSS of the aerobic 1, aerobic 2 and anoxic zones of the lab scale process. Details are shown in Table 5.5

TABLE 5.5 Period of isolation, zone of reactor and lane occupied in the gel for the DGGE analysis

Date	Zone	Lane occupied in gel(L-R)	Sample (Set)
09/05/01	Aerobic 1	1	1
09/05/01	Aerobic 2	2	1
09/05/01	Anoxic	3	1
21/05/01	Aerobic 1	4	2
21/05/01	Aerobic 2	5	2
21/05/01	Anoxic	6	2
03/08/01	Aerobic 1	7	3
03/08/01	Anoxic	8	3
05/03/01	Aerobic	9	seed

In order to determine the optimal conditions for DGGE of the PCR-amplified gene fragments to characterize the microbial population, various experimental protocols were investigated. The method used was according to Muyzer *et al.*, 1993 and adapted as follows :

- The volume of sample used varied from 10 to 50 μl and the latter volume produced the best results
- the denaturant gradient was linearly increased using 30:60, 30 :70 and 30:80% and the best results were obtained using at the denaturant concentration of 30:70 %.
- to determine the length of time of electrophoresis for the maximum resolution, various time

intervals (2-6 h) were investigated and the fragments did not migrate much further in the gel after 160 min. To be on the safe side 180 min was selected as the optimum time for electrophoresis

The results of the PCR-DGGE analysis is shown in Figure 5.12. The bands formed in each well reflects the migration of the DNA molecule, each with a unique melting temperature. The bands represent different base- pair sequences and affiliated with different phylogenetic groups. Since the purpose of the PCR-DGGE analysis during the current investigation was simply to be used as a tool for rapid cross community comparisons of population complexity and similarity by focusing on identical banding patterns, the distinctive bands were not sequenced to determine the base pair profiles for phylogenetic classification. Determination of the sequences for identification and creation of a phylogenetic tree was not within the scope of the current investigation and due to time limitation.

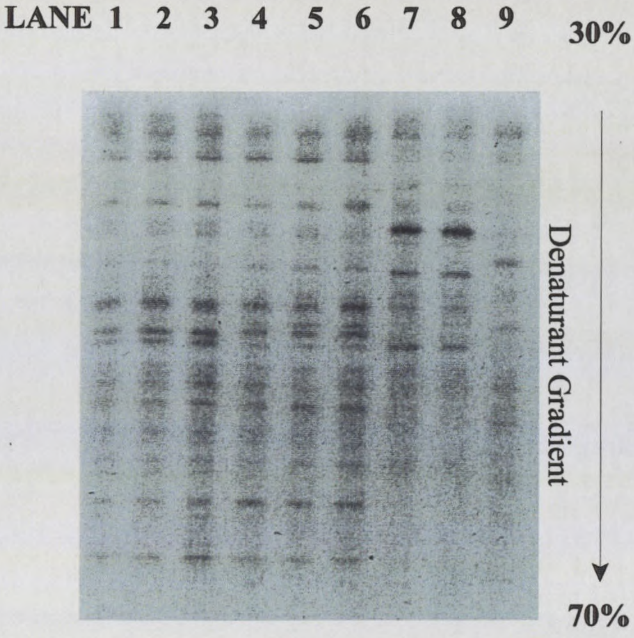


Figure 5.12 PCR-DGGE analysis of seed sludge (Lane 9) and process samples (Lane 1-8). Lanes are numbered from left (1) to right(9). Details of sampling conditions are in Table 5.5 Denaturant concentration was 30:70 % and electrophoresis for 180 min using 50 μl sample volume and set at 200V.

Critical analysis of the PCR-DGGE results was restricted to visual comparison of the migration patterns of the bands amongst the 9 samples and no similarity analysis was conducted. The community structure using DGGE revealed the following. The banding pattern of the seed sludge (lane 9) did not show much similarity with the 3 sets of process samples (lane 1-8) and surprisingly not even close to first set of process samples(9-05-01) at lane 1-3. There were few predominant bands. The process sample sets 1 (lane 1-3) and 2 (lane 4-6) were obtained during different times (11 days lapse) of the same phase (5 C) of the lab scale process. Comparative analysis show the migration and patterns of the bands are very similar except for a few distinctive low intensity bands that differ. However, the latter indicated that although there was a small lapse in sampling period (11 d), there were minor changes in the community profile. In order to correlate the process conditions to the population profile, during samples set 1 the process was at steady state with good process efficiency. Samples set 2 showed there was a comparative decrease in the influent COD concentration and process performance In addition, there were large number of predominant bands in sample sets 1&2, indicating a comparatively substantial species diversity. In samples set 3 which was obtained approximately 75 d later, there was a noticeable difference in the banding pattern when compared to sets 1 & 2 with fewer bands being evident. However, the relative intensity of the predominant band which was common in both samples (lane 7&8) was quite pronounce (Fig 5.12). The latter indicated a decrease in the species diversity as the process progressed. During this period (phase 5 F) the process performance was good (> 90%) with the introduction of a new batch of influent.

Comparative analysis of the 3 sets of samples clearly indicated that the population profile as indicated by the banding patterns was identical amongst the 3 zones (aerobic 1, aerobic 2 and anoxic). This trend was reflected in all 3 sets of samples. The latter findings substantiated that the community profile i.e bacterial species composition was exactly the same in the aerobic and anoxic zones of the reactor. Since only PCR-DGGE was used without following the sequencing procedure, interpretation of the data presented by the gels do present some inherent limitations and caution has to be exercised. Bands that are very close together due to minor base pair differences can be mistaken as a single band (single species). In addition, low intensity bands could be ignored completely due to poor visualization, resulting in inaccurate community profiling. Although the sequencing procedure would have alleviated some of the latter problems, during the current investigation, reproducibility of results to confirm accurate profiling was done by improvising and running many gels.

5.3.3 Survey of filamentous bacteria during bulking

The identification and predominance of filamentous bacteria was restricted to periods of bulking experienced by the process at the end of phase 3. The results are given in Table 5.6.

TABLE 5.6 Morphological staining characteristics of filamentous bacteria observed in the laboratory scale process

FT	GS	NS	PHB	TL	TLL	CS	I	SH	AG	CL/S	RANKING
S. natans	-	-	+	E	**>500**	+	+	+	-	**rods 1x1-2**	**Dominant**
H. hydrossis	-	-	-	E/F	**20-100**	-	-	+	+	**variable**	**Dominant**
Type 1701	-	-	+	E	**20-80**	+	+	+	+	**round rods 0.8 x 1.2**	**Dominant**
M. parvicella	+	+	+	IN	**100-400**	-	-	-	-	**variable**	**Secondary**
Type 0675	+/-	+/-	-	IN	**60-160**	+	-	+	++	**squares 1x1**	**Secondary**

Ranking in order of prevelance

FT= filament type; GS= gram stain; NS= neisser stain; PHB= presence (+) or absence (-) of polyhydroxy butyrate; TL= trichome location; TLL= trichome length (μm); CS= cell septa present (+) or absent (-); I= indentation present (+) or absent (-); SH= sheath present (+) or absent (-); AG= attached growth present (+) or absent (-); CL & S= cell lenght and shape; E= extended growth; IN= interbridging; ++= excessive attached growth.

The identification of the filamentous types was based on characteristic morphological features such as cell trichome size and shape, staining etc. as per identification keys (Eikelboom, 2000). However, the morphology and staining reactions can vary significantly depending on cultivation conditions and wastewater conditions. Sludges from industrial treatment plants often display large variations in the morphology of the flocs and cannot be classified into a few groups (Muyima *et al.*, 1997).

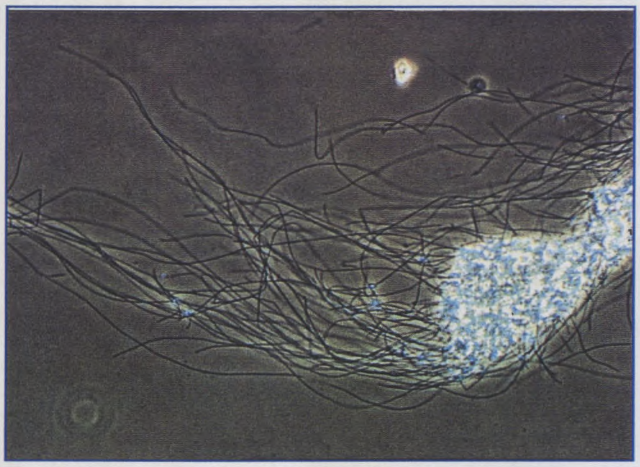


FIGURE 5.13 Gross morphology of the floc from the process indicating excessive abundance of filaments using the subjective scoring system (Jenkins *et al*, 1986).Filaments growing in high abundance into bulk solution (*x1000* magnification).

As shown in Fig 5.13 the predominance of filamentous organisms is evident with the extended filaments protruding into the bulk liquid. In addition, these filaments appear to be growing in profusion, both inside and outside the floc. These observations are characteristic of filamentous bulking activated sludges. The floc macrostructure is also typical of "bulking conditions" with an imbalance of filaments to floc and growing in profusion beyond the confines of the activated sludge floc and an irregular shaped floc. It should also be noted that the above floc characteristics are common in sludges in industrial treatment plants (Eikelboom ,2000). With reference to Table 5.6, filamentous bacteria that were positively identified during the bulking period included *S. natans, H. hydrossis*, Type 1701, M. *parvicella* and Type 0675. The morphological and staining characteristics are given in Table 5.6. The latter identification were confirmed with MLSS samples sent to a world expert in the field Dr Eikelboom, Netherlands. The above filamentous bacteria are most common types occurring in bulking sludges globally (Blackall, 1999), and therefore the findings concur with the bulking conditions experienced in the process.

Quantification of the filamentous population was conducted using the subjective scoring system by Jenkens *et al.*, (1986). Filament abundance was reflected in terms of the ranking of the bacteria in the category of 'dominant' or 'secondary'. The results are presented in Table 5.6 and the ranking is in 'order of prevalence'. *S. natans* showed the highest level of abundance followed by *H. hydrossis* and Type 1701. The less abundant bulking filaments included *M. parvicella* and Type 0675. Figure 5.14 presents the filaments on the periphery of the floc with *S. natans* showing straight to slightly bowed filaments with the sheath and septa clearly visible. *H. hydrossis* can be seen with typical straight filaments protruding from the floc and no visible septa.

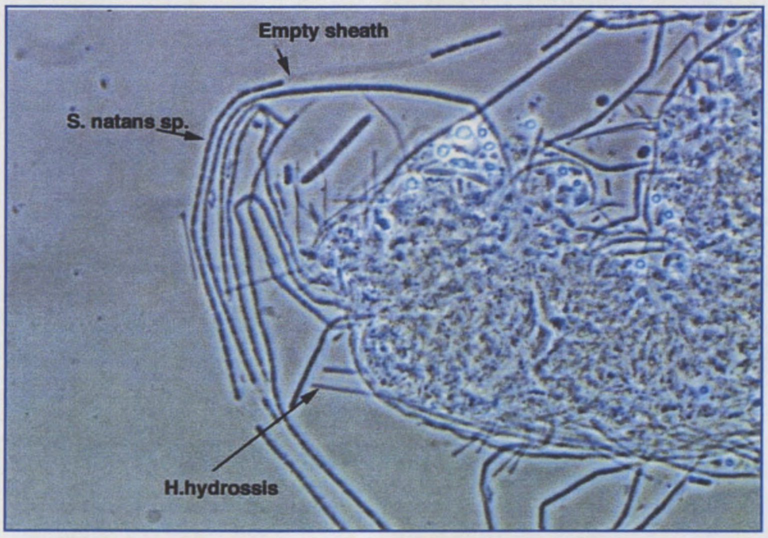


FIGURE 5.14 Micrograph of *S. natans* showing typical empty sheath and *H. hydrossis* protruding from the floc. Filaments are extending into bulk solution (*x1000* magnification)

5.3.4 Survey of Protozoa

The period of observation and quantification of the protozoan population was restricted to phase 5 and the results were correlated to process conditions during the respective period. Data on the metazoan (rotifers and nematodes) population has been presented for comparison purpose only. The population profile is presented in Figure 5.15.

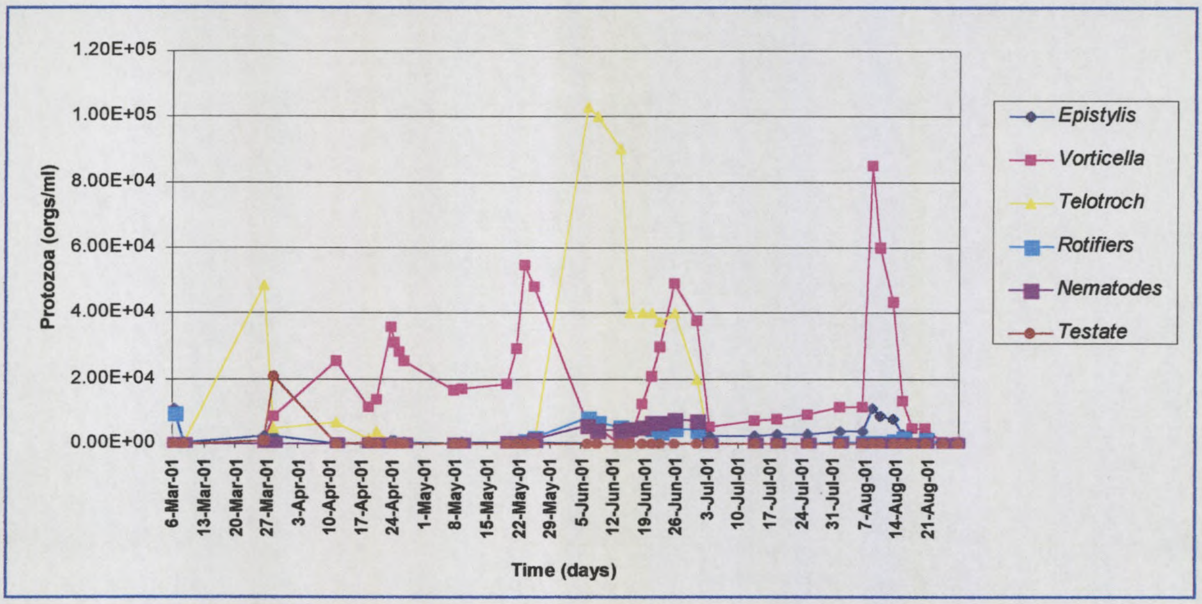


FIGURE 5.15 Protozoan and metazoan population profile during phase 5 of the lab scale process

With reference to Fig 5.15, the results clearly indicate that the common protozoan representatives that occur in activated sludge plants were present in the process. The ciliates (stalked and crawling) which are known to occur in high abundance prevailed through most of the process. Testate amoebae were also present throughout the process but in low concentrations. Representatives of the stalked ciliates (*Vorticella sp*) and *Telotroch* sp predominated in the process with distinctive high and low peaks as the process progressed. As expected the seed sludge comprised a substantial population but when exposed to the feed oil influent, there was a noticeable decline in the protozoan numbers(March 01), possibly due toxic shock from initial exposure to edible oil effluent and the presence of residue flocculent that was carried into the influent. The protozoan population gradually began to establish itself as the process progressed. Previous research showed that the quality of wastewater entering the plant and the organic load were both particularly important in determining both ciliate numbers and their diversity (Eikelboom, 2000).

During the current research the population diversity was tracked in correlation with process conditions during the respective periods. Approximately 1 week after the commencement of the process, the testate amoeba and *Telotroch* sp were the first protozoan communities to establish themselves. During this period the organic load i.e.F/M ratio was 0.4 gCOD/gMLSS.d. and is known to support the growth of these organisms. At the end of March 2001, the stalked ciliate *Vorticella* sp began to establish itself coinciding with a period of low organic loading (mean F/M ratio 0.21 gCOD/g MLSS.d. The *Vorticella* sp numbers peaked in the latter portion of May 01 and once again matched with a low F/M ratio (0.19 gCOD/gMLSS.d) due to introduction of a new batch of effluent from industry. Due to system perturbations caused by excess flocculent being introduced into the system towards the end of May and into June there was a drastic decrease in the *Vorticella* sp numbers, but on correction of the process conditions the numbers began to increase towards the latter part of June. On the 26 June 01 with the introduction of a new batch of effluent, the *Vorticella* sp numbers began to decline to low concentrations and persisted at these numbers until 7 Aug 01 when there was a sudden increase in the numbers peaking at > 8×10^4 orgs/mL, being the highest numbers recorded during the process. The latter dominance only prevailed for an short time as the numbers declined again. Evaluating the predominance of *Vorticella* sp it is clearly evident that the population prevailed in large numbers for relatively short periods during the process.

The *Telotroch* sp having dominated during the start up process of the phase was not evident in large numbers until the period of system perturbation caused by excess flocculent in the feed source. During this period (29 May-05 June 01) and in direct contrast to the population dynamics of *Vorticella* sp, there was a substantial increase in the *Telotroch* sp numbers recording the highest single species dominance of the process. However, there was a rapid decline in the population 12-19 June 01 and the organism was non-existent in the process after the 03 July 01. Surprisingly, as the *Telotroch* sp dwindled and disappeared, the stalked ciliate *Epistylis* sp, although at comparatively low numbers, immediately became evident. The protozoan is common in activated sludge, especially at sludge loading levels of 0.1 - 0.2 g BOD/gMLSS.d. When using the theoretical conversion factor of BOD to COD, the organic load of the process during the respective period was within the above limit to support the growth of *Epistylis.* sp. A noticeable factor when evaluating the population dynamics of *Epistylis* sp was the concurrent increase in the numbers between 7 Aug 01 and decreasing on the 14 Aug 01, creating a mirror image of the behavior of *Vorticella* sp, although the actual counts were much lower. Both organism were stalked ciliates. The population profiles of the protozoa in the process clearly supports a succession theory.

The metazoa including the rotifers and nematodes were present in relatively low numbers and had no significant effect on the system and are known to play a subsidiary role in activated sludge treatment.

5.4 DISCUSSION

During the current investigation the primary purpose for applying novel molecular techniques such as FISH and PCR-DGGE to accurately determine the microbial community profiles of complex environments such as activated sludge, was due to the inherent limitations of culture dependent techniques (Wagner *et al.*, 1993; Amann *et al.*, 1996).

5.4.1 Community analysis using FISH

Although quantitative FISH has provided novel insights into the structure and dynamics of microbial communities, it suffers certain limitations including limited accuracy for samples containing densely aggregated cells such as activated sludge. The latter problem can in part be ameliorated by the use of confocal laser scanning microscopy (CLSM) for the detection of probe- labeled cells. However, even if CLSM is used, it is not feasible to manually count a sufficient number of cells in each hybridization experiment in a reasonable time period to obtain statistically reliable results (Daims *et al.*, 2001). Therefore, during the current investigation to overcome the limitations of the FISH technique, although CLSM was not used, a non-ionic detergent (Igapel Ca-30) was mixed with the samples and facilitated improved cell dispersion during the sonication process, thereby greatly enhancing the accuracy of the cell counts. In order to further ensure accuracy of the cell counts, samples were sonicated prior to hybridization to release floc bound cells. However, the sonication process needed to be optimized since excess sonication was found to decrease probe conferred signals during hybridization due to disruption of cells and subsequent release of the cellular content including the ribosomal material. In order to overcome the limitation of the manual cell counting procedures, digital image analysis software was used to accurately quantify fluorescently labeled bacteria.

In addition to accurately profiling the microbial community of the lab scale process, the FISH technique was used to ascertain any population shifts that would have occurred as the phases progressed with increasing COD concentrations and variable operating conditions. The technique was useful since the determined communities could be correlated to process performance, and accurately target bacteria that played a predominant role in the edible oil effluent degradation. It has been well established that on various taxonomic levels phylogenetic groups are characterized by rRNA sequence idiosyncrasies, their so called signatures (Woese *et al.*, 1985). Oligonucleotide probes are more suitable for routine screening of such signatures than sequencing (Stahl *et al.*, 1988). Such probes have been designed for the highest taxonomic levels, the domains *Archaea, Bacteria* and *Eucarya* (Delong *et al.*, 1989; Amann *et al.*, 1990a); for intermediate levels, e.g., the alpha, beta and gamma subclasses of *Proteobacteria* (Manz *et al.*, 1992), the flavobacterium-cytophaga and bacteroides clusters of the CFB phylum (Amann *et al.*, 1995); and for lower taxa (genus-, species-, and subspecies-specific probes) to list a few(Stahl *et al.*, 1988; Amann *et al.*, 1992; Devereux *et al.*, 1992). In addition, as an alternative to sequencing, probing of important phylogenetic groups in a top to bottom approach allows a more directed and rapid, yet less detailed, characterization of the microbial community structure (Wagner and Amann, 1997). However, previous research probing activated sludge to describe the microbial community structure favored the use of oligonucleotide probes specific for the *proteobacteria* since results showed that activated sludge samples were dominated by *proteobacteria* from the alpha, beta or gamma subclass (Wagner *et al.*, 1993). Therefore during the current investigation, the probes used were restricted to and complementary to conserved regions of the rRNA of the alpha, beta and gamma subclasses of *proteobacteria*, HGC

(*Actinobacteria*) and of all bacteria.. In addition group specific cell counts were determined by FISH and expressed as a percentage of DAPI.

Since rRNA content has been found to be directly proportional to growth rates in bacteria (Delong *et al.*, 1989), it can therefore be assumed that bacterial cells present in the activated sludge biomass producing conferred fluorescence are therefore metabolically active. In both the seed sludge samples, 81 % (sample 1) and 74% (sample 7) of the cells visualised by DAPI were detected by the bacterial probe EUB 338 (Tables 5.3 &5.4). These results indicated that majority of the fixed cells belonged to the domain *Bacteria*. In addition, the high EUB/DAPI ratio indicated that most bacteria in activated sludge were growing and metabolically active (Wagner and Amann, 1997). Previous research also showed similar findings i.e. 79 ±6 with respect to the EUB/DAPI ratio of the same seed sludge (Darvil) (Mudaly, 2001). However, an overall decrease in the EUB:DAPI ratio occurred during both phases with a decrease from 81 % in sample 1 to 67% in sample 6 (TABLE 5.3) during phase A, and decrease from 74%(sample 7) to 49%(sample 12)(TABLE 5.4) during phase B. The latter clearly indicated a decrease in the bacterial population (decrease in the metabolic activity of the bacterial biomass) which was common in both phases. However, the latter decrease in the bacterial domain was not matched with a equal decrease in the MLVSS as expected during the respective period of process operation (phase 4 &5). The consistency in the MLVSS regardless of the decrease in the EUB (bacterial population) could be explained by a possible corresponding increase in some other biotic component (fungi etc.) of the biomass, thus negating any impact on the MLVSS. Similar results were obtained by Stoffels *et al.* (1998) investigating the bacterial community dynamics of a trickle -bed bioreactor treating aromatic compounds, whereby there was a decrease in the EUB/DAPI ratio when comparing the seed inoculum to the process sample i.e 76% to 68%, respectively. The continued decrease in the ratio as the process progressed indicated a decrease in the bacterial population and the metabolic activity imparted by the bacterial population. The latter phenomenon can undoubtedly be attributed to inhibitory compounds present in the feed influent (edible oil effluent) which caused possible limited toxicity to the bacterial biomass. The impact was noticeable in the process with decrease in the EUB/DAPI ratio. Another possible explanation could be that proposed in a model by Cloete, 1997 which correlates species diversity with various microbial habitats which are encountered and categorizes the communities.

Category C represents a stable community where both the number of species and cell numbers of the species are high. Activated sludge (seed sludge sample 1&7) are examples of category C which are suited to overcome environmental stress i.e. toxic shock in which case the sludge is likely to initially to move to category A (species diversity and cell numbers are low) community. After the acclimation period there is generally a shift towards category D which indicates an extreme environment where a few species with a high number of representative individuals exist. The latter was the most likely scenario during the current investigation and could justify that although there was a decrease in the EUB population, there was a overall shift towards the development of a acclimatized 'specialized' community that adapted to oil effluent as a substrate. The latter theory could also justify the accompanied overall decrease in the DAPI total cell counts from 4.9 x 10^9 cells/mL (sample 1 seed sludge) to 2.3 x 10^9 cells/mL (sample 6, phase A) and 7x10^9 cells/mL (sample 7, seed sludge) to 1.3 x 10^9 (sample 12, phase B).

Detailed analysis of the microbial community profile of the seed sludges used during both phases A &B (sample 1 &7) which was obtained from the aerobic zone of Darvil waste water works (EBPR process) revealed a predominance of members of the beta subclass of *Proteobacteria,* followed by alpha, gamma and *Actinobacteria (*HGC). The comparatively low frequency of *Actinobacteria* in the Darvil seed sludge (8% detected by FISH) was also shown by Mudaly, 2001. Although the order of predominance was the same in both phases, there were minor variations in the actual numbers of the specific groups (details in Tables 5.3 & 5.4). The latter could be attributed to the samples being obtained at different times. The current findings substantiate previous studies on EBPR sludges indicating the predominance of the beta-*Proteobacteria* and/*or Actinobacteria* (Kampfer *et al.*, 1996, Snaidr *et al.,* 1997; Bond *et al.*, 1999; Mudaly *et al.*, 2000). Mino *et al.*, (1998), who surveyed twenty full scale activated sludge plants operating in Japan showed the beta-*Proteobacteria* to be the most predominant, followed by the alpha subclass and the HGC group third.

The microbial community profile of the process samples in phase A & B revealed a close correlation in the order of predominance of the bacterial groups present. A distinct population shift was observed in both phases i.e. there was a shift in the population from predominance of the beta subclass in the seed sludge to a community dominated by the alpha subclass of *Proteobacteria* in the process samples 2 &8. (Tables 5.3 &5.4). While conversely, there was a decrease in the beta subclass population for the same samples. During the period of sampling the process was performing optimally. The shift to the alpha subclass could be attributed to the response of the microflora to a different substrate (edible oil effluent) or new pollutant load. Similar population shifts were observed in previous research by Stoffels *et al.*, 1998 using a trickle -bed bioreactor to treat aeromatic compounds whereby the seed inoculum comprising of the gamma subclass when inoculated into the fermentor, resulted in a population shift and predominance of members of the alpha and beta subclass. As witnessed in the current research, the response to pollutant adaptation resulted in the system selecting the appropriate bacteria to degrade the substrate thereby enforcing the population shift towards the alpha subclass. The decrease in the beta subclass could be attributed toxicity of the substrate to the bacteria within the subclass. Substrate uptake is a very important phenomena in the selection of microbial populations in activated sludge and determines dominance of one group over the other. Microbes that are not adapted to metabolize the substrates are usually washed out of the system (Satoh *et al.*, 1998), explaining the decrease in the beta population in relation to the alpha subclass. The overall comparative predominance of alpha subclass in both phases throughout the process further supported the theory of acclimation i.e. members of the subclass had adapted to the substrate. However, a detailed literature search resulted in no information or previous studies on specifically microbial community analysis of a activated sludge process treating edible oil effluents using the novel nucleic acid methods, to verify current findings.

During the current research, although the microbial communities were similar in phase A & B, it became essential to analyze them separately to determine individual trends since process parameters differed somewhat during the respective phases. A significant finding by Kaewpipat and Grady, 2002 showed that the bacterial communities in identically operated activated sludge reactors became significantly different over time, even though they started from a common community. In spite of differences in the community composition, the COD removal efficiencies in two reactors were similar, demonstrating that different microbial communities can be functionally similar. Researchers also found that when studying population dynamics of functionally stable bioreactors, the bacterial community structure was highly dynamic, and even suggested that it followed a chaotic pattern. They observed rapid shifts in the species composition of the bacterial community, whereas the archaeal community remained relatively stable over a two year period (Fernandez *et al.*, 1999; Zumstein *et al.*, 2000). They suggested that the evolution of the microbial community seemed to be induced by inherent parameters, which could be biotic, such as phages, predation, etc. However, as mentioned previously the overall trend in both phases of the lab scale process were very similar with regards to the respective dominance patterns of the subclasses of *Proteobacteria* and *Actinobacteria.*

The alpha *Proteobacteria* population peaked in sample 2, which coincided with optimum process performance. There was a marginal decrease in the population in sample 3 and could be attributed to disruption in influent supply and also reflected in a decrease in the OUR and COD removal efficiency. The depleted OUR reflected stress on the population as depicted by the decrease in the alpha subclass which was the dominant population throughout the process. However, sample 4 showed a further and relatively substantial decrease in the alpha *Proteobacteria*, although the process was performing optimally with a further increase in influent COD. Surprisingly, the population profile of the same sample (4) showed a corresponding increase in the beta subclass. With no evident abnormal process symptoms such as decrease in OUR, VSS etc, during this period, the direct opposite dominance pattern of the alpha and beta subclass one of the possibilities could be attributed to some constituent in the influent feed at that time, that although did not negatively impact on the COD removal, but stimulated a population shift towards the beta sub-group. Another possible explanation points to the high F/M ratio(1.5) in the process during the period that negatively impacted on the alpha subclass. Wagner *et al.*(1993) also observed that the alpha *Proteobacteria* were a dominant group (together with beta subclass) in an activated sludge basin with a relatively low F/M ratio . In the high load basin (F/M ratio 1.8) only 10% of the alpha *Proteobacteria* (ALF1b/EUB 338) was observed. In the low load basin their numbers increased to 37% (ALF1b/EUB 338). Therefore focusing on the current results, it is likely that the high F/M ratio of 1.5 during the period caused the decline in the alpha population ,and the subsequent recovery of the alpha subclass i.e 34 and 30 %(ALF 1b/DAPI) in samples 5 and 6, respectively as the F/M ratio once again declined to 0.93 (mean) (Table 4.4). It appears that the alpha was more sensitive to high F/M ratios than the beta subclass during the sample 4 period and the susceptibility of the alpha subclass resulted in the proliferation of the beta subclass possibly due to lack of competition for substrate. On restoration of the lower F/M ratio of the process (sample 5 & 6), the balance was restored with the alpha subclass of *Proteobacteria* once again showing overall domination followed by beta subclass.. Compared to the other groups (gamma and HGC) the alpha and beta subclasses of *Proteobacteria* predominated in phases A(Fig 5.1) and B (Fig 5.2)possibly due to the overall low and favorable F/M ratios experienced in the process (Table 4.4).

Current findings was also in agreement with research by Layton *et al.* (2000), who conducted a detailed microbial community profile of a activated sludge system treating wastewater from a chemical manufacturing process and on acclimation, showed the % 16S r DNA cloned sequences with representatives of the alpha predominating followed by beta and gamma subdivisions of *Proteobacteria*. The microbial community of a dairy wastewater plant was shown to be numerically dominated by spindle-shaped bacteria belonging to the ' Flavobacter- Bacteroides' phylum (Manz *et al.*, 1994). These findings reiterate that the microbial community profile of municipal wastewater plants treating domestic waste and comprising predominantly beta subclass (Wagner *et al.*, 1993) certainly differ from those treating industrial effluents. Population shifts become evident once the seed sludges are exposed to the industrial wastewater (influent) and can be attributed to substantial differences in the chemical composition of the waste streams. Industrial wastewater's generally have a much higher total organic carbon load when compared to municipal (Layton *et al.*, 2000). With reference to the current research, the edible oil effluent was a complex substrate comprising of a range of lipids that necessitated initial hydrolysis by the bacteria before the substrate was metabolized. However, the organic content of the substrate was substantial. *In situ* analysis confirmed that the gamma and HGC estimates were numerically low in the seed sludge and trend repeated itself throughout the process in phase A & B (Fig 5.1 & 5.2). Previous research has shown that members of the gamma subclass were less abundant in municipal activated sludge (Wagner *et al.*, 1993; Manz *et al.*, 1994). *In situ* studies of a plant treating dairy waste showed a proliferation of HGC from 9 % in the seed inoculum to 25 % on acclimation (Manz *et al.*, 1994). High HGC abundance levels were also observed in municipal plants with EBPR (Wagner *et al.*, 1994a; Wagner, 2000).
The above findings reiterate that the microbial community profiles are also dependent on the composition of the wastewater being treated.

When compared to the seed sludge samples 1 & 7 during phase A & B, it was evident that there was a overall decrease in the total percentages of the respective subclasses of *Proteobacteria*, following a pattern parallel to the EUB/DAPI ratio (Tables 5.3 & 5.4). Researchers have shown that probing activated sludge of municipal treatment plants reveals the dominance of *Proteobacteria* (Wagner *et* *al.*, 1993). The latter was also the case in the current investigation. The latter phenomenon could be attributed to development of a "specialized community" amongst members of the alpha subclass and possible washout from the system of 'irrelevant' bacteria, reflected in the decrease in the overall *Proteobacteria* population.

The population profile in phase B with regards to EUB/DAPI ratio and total cell counts (Table 5.4) was similar to phase A. The dominance patterns of the subgroups of *Proteobacteria* in the seed sludge was also same as phase A and also the increase of alpha subclass and corresponding decrease of the beta subclass in the first process sample (8). The overall reproducibility of the results in Phase B served to add legitimacy to the determination of the microbial community profile of the lab scale process. However, it should be remembered that identification of the microorganisms was limited to the subclass level only and not to genus and species level, which would have provided more detailed information for further comparison of the population within the phases and the identification of the specialized community. Probing at the genus/species level was not within the scope of the current research.

The detailed profiling of the community in correlation with the process performance during phase B (Fig 5.2) showed similar behavior as phase A. The alpha subclass of *Proteobacteria* predominated throughout phase B, followed by beta, gamma and HGC. On peaking (sample 8), the alpha subclass population reached a plateau phase until the latter part of the process(sample 12). During the end of the phase (sample 12), there was a noticeable decrease in the process performance and a corresponding decrease in the OUR. During the same period, there was a drop in the abundance of bacteria(EUB338/DAPI) and matched by a parallel decrease in the alpha subclass. Since the decrease in the bacterial population was reflected by a similar decrease in the alpha subclass only, and not the other groups, one could therefore postulate that the corresponding decrease in the process performance could be attributed to reduction in the abundance of the alpha subclass. The latter group could be playing a major role in the degradation of the substrate. However, during the same period (sample 12) the beta subclass increased in abundance (Fig 5.2). Similar to phase A, a decrease in the abundance level of the alpha, was matched by a corresponding increase in the beta population (sample 12). One could possibly attribute the reduction in the alpha population to the sudden increase in the

F/M ratio i.e. 1.5 during the period, since the alpha population is known to favor lower F/M ratios. The corresponding increase in abundance of the beta group could be attributed to lack of competition for substrate.

Once again and similar to phase A, the abundance levels of the gamma subclass of *Proteobacteria* featured after the alpha and beta (Fig 5.2). The latter findings supported previous research by Wagner *et al.* (1993) who also showed the abundance level of gamma, following beta and alpha. However, in sample 9 there was a relatively noticeable increase in the gamma population i.e. 16 % of DAPI and in contrast to the decrease in the abundance levels of the other groups. In sample 10 the gamma population decreased substantially and remained low until the end of the process. During the respective period (sample 9), the influent organic strength (COD) was relatively low and could have accounted for the proliferation of the gamma subclass. However, in sample 10 the influent strength increased and could have favored the other groups, thereby restoring the original balance. Similar to phase A, the HGC group did not feature prominently, except if sample 10 where the abundance levels marginally surpassed the gamma subclass (Fig 5.2). Although the HGC group were noticeable i.e. 25 %, in a process treating dairy wastewater (Manz *et al*, 1994), during the current research it appears that the substrate (oil effluent) did not favor proliferation of this group. In addition, it is known that activated sludge processes promoting biological phosphorus removal, supports the proliferation of representatives of the HGC (Wagner *et al.*, 1994a). Since the current research did not necessitate the implementation of the EBPR mechanism due to low influent P concentration, the conditions did not favor high abundance levels of members of the HGC group.

With the probes that were used during the current investigation, findings show that majority of the bacteria were classified within the subclasses of *Proteobacteria* and HGC group. The total probe percentages for each sample was calculated by adding together the average probe specific percentages obtained for each group and respective samples. Comparing the total probe percentages to the probe EUB 338 in the respective samples during both phase A & B, it is clear that most of the bacteria were classified. However, there were some unaccounted bacteria in both the seed sludge (samples 1&7) and process samples (2-6, phase A) and (8-12, phase B) (Tables 5.3 & 5.4). A noticeable observation was that in sample 1, only 1% (80 out of 81%{ EUB/DAPI})were not classified, supporting previous findings that microbial consortia in activated sludge municipal treatment plants were dominated by members of the *Proteobacteria* and to a lesser extent HGC (Wagner *et al.*, 1993). The unaccounted (by probes) bacteria e.g. 4% in sample 5 (phase A) and 3% in sample 8 (phase B) could belong to other divisions such as CFB phylum (Kampfer *et al.*, 1996), and low G+C Gram positive bacteria (Snaidr *et al.*, 1997). They may even represent some yet undiscovered divisions.

One of the aims of the research was to identify any population shift in the microbial community as the COD increased. Due to operational limitations this aspect of the investigation was limited to phase A. Some of the more pronounce observations during phase A included decrease in the EUB/DAPI ratio as the phase progressed, overall predominance in the alpha subclass of *Proteobacteria*, followed by the beta subclass (Fig 5.1) and a decrease in the total cell counts (DAPI) as the phase progressed. However, none of these events occurred specifically during periods of increase in the influent COD concentration and the above trends were repeated also in phase B, where there was no increase in influent COD as the phase progressed. Therefore it was very likely, if not certain that the organic strength (COD) of the wastewater did not play a major role in determining the population shifts in the lab scale process. Multiple factors including operational conditions such as F/M ratios, DO concentration, system parameters such as HRT and sludge age, system perturbations and the all important influent (substrate) quality could have determined the population profiles and shifts in the lab scale process.

The overall trends in the community structure in both phases were similar and served to enhance the reproducibility of the current findings using the novel *in situ* hybridization techniques.

5.4.2 Community diversity using PCR-DGGE

Denaturing gradient gel electrophoresis (DGGE) of PCR amplified 16S rRNA fragments was used as a rapid tool for cross community comparisons of population complexity and similarity. However, during the current investigation, similarities and comparisons of the microbial communities was limited to visual observations of the electrophoretic banding patterns, and the nucleic acid from the bands were not sequenced to determine the phylogenetic affiliations of the community representatives. The technique helped to complement FISH analysis in determining population shifts in the community during the operation of the lab scale process. The usefulness of DGGE in the analysis of microbial communities rested on the assumption that different sequences will migrate to different positions in the polyacrylamide gels based on their melting behavior (Muyzer *et al.*, 1993) and simulations show that 95% of single base sequence differences will be detected by this method (Myers *et al.*, 1985). In a previous study, it was observed that two sequences that co-migrated in DGGE gels differed in 5% of their base pairs, although they did share the same GC content, which could account for their similar migration (Jackson *et al.*, 1998). The latter could be considered a limitation of the technique.

In order to obtain accurate results, various experimental protocols for DGGE were investigated (data not shown) and optimized. The sample volume of 50 µl increased the intensity of all the bands, thus improving the resolution and interpretation of the data. The denaturant gradient concentration varied with the different investigations and depended on best results obtained. Muyzer *et al.*(1993) defined a gradient of 15 to 55% as a starting point to resolve different sequence variants in parallel DGGE gels. Jackson *et al.* (2000) used a much smaller denaturing gradient set at 30 - 55% aiming to improve separation of similarly migrating sequences. However the results showed that the smaller gradients (<15 -20%) tended to reduce the clarity of the bands, making the examination of samples containing multiple DNA sequences difficult. Murray *et al.* (1996) obtained best separation using a larger gradient i.e. 40-70%. During the current investigations. the gradients were linearly increased using 30:60, 30:70 and 30:80 % (results not presented). The gradient that produced the best separation was the 30:70 % and used for DGGE.

Another parameter that was important for optimum selection of running conditions was the length of time of electrophoresis that would provide maximum resolution. The time intervals that were tried were between 2 to 6 h with 1 h intervals. It was crucial to optimize the electrophoresis running time in order to allow sufficient time for migration of all the bands especially in environments that have

complex microbial diversities such as activated sludge. The results showed that the fragments did not migrate further after 160 min but to be safe an extended running time of 180 min was selected for subsequent analysis. Current research showed that the optimum conditions for DGGE are very specific for samples investigated and can only be determined by trial and error. However, conditions set by other researchers can be used as a guideline and adapted accordingly. A good example to support the latter included an optimum running time of 210 min, which was determined as optimum for DGGE in order to analyze the microbial diversity of EBPR systems (Nielsen *et al.*, 1999). Muyzer *et al.*(1993) profiling complex microbial populations using DGGE, obtained maximum resolution and migration on the gel after 120 min and chose 150 min for electrophoresis.
The above researchers selected times at either side of the range as that selected for the current investigation i.e. 180 min.

With reference to Fig 5.12, large number of bands were visible providing a good estimate of species richness representing different samples of activated sludge. The bands represented different base pair sequences and provided a profile of the populations in that the relative intensity of each band and its position most likely represented the relative abundance of a particular species in the population. Although informative, this approach had a limitation in that only qualitative information about the population profile could be determined, and in the current research the interpretation of the migration patterns of the bands was limited to comparing species diversity and determining population shifts visually. No similarity index, sequencing or cluster analysis was determined. Sometimes DGGE analysis may have some specific limitation such as the detection of heteroduplex molecules (when two similar but different strands are joined together) and one bacterium having more than one rDNA sequence producing two bands (Luxmy *et al.*, 2000). Fortunately this phenomenon is thought to be relatively rare in bacteria and heteroduplex molecules are not commonly found (Curtis and Craine, 1998; Nubel *et al.*, 1999).

It should be noted that the samples were obtained from phase B of the lab scale process. The banding pattern of the seed sludge (lane 9) was noticeably different from the process samples (lane 1-8). The seed sludge was obtained on the 05/03/01 and the 1st set of process samples 1 (lane 1-3) on the 09/05/01. Surprisingly, there was little similarity between the two sets of samples (Fig 5.12), indicating that the process samples microbial community had changed substantially during the lapse in the sampling period (2 months). However, the transition of the population inferred by the PCR-DGGE analysis of sample 1 was well supported by FISH analysis of the same sample (sample 8, phase B,. Table 5.4) that showed a change in the population profile dominance from beta (seed sludge, sample 7) to alpha subclass of *Proteobacteria* (sample 8). Therefore, correlating the FISH results to DGGE, it can be inferred that the high intensity bands (3) which were common in the representatives of sample set 1 (centre of lane 1-3) indicating high abundance levels belonged to the alpha subclass. The relative intensity of each band provided a rough estimate of the relative abundance of each species (Nasu *et al.*, 2000).

The process sample set 2 (lane 4-6) was obtained 11 d after sample 1 (TABLE 5.5). Comparison of the banding pattern between sample 1 (lane 1-3) and sample 2 shows much similarity, except for a few distinctive low intensity bands that differed. Due to the low intensity of the bands, it could be concluded that the respective species that their represent were not predominant. Abundance based on intensity of the bands plays a role in the assessments of diversity because it is likely that bacteria occurring in low numbers will not be detected (Curtis and Craine, 1998). The close similarity in the banding pattern reflecting similar population profile was expected, since the lapse in the sampling period was only 11 d between sample 1 &2. However, the minor differences shown by low intensity bands indicated possibly the start of a transition in the population as the phase progressed. The high intensity bands (centre of lane 4-6) in sample 2 was similar to sample 1, with a addition of a low intensity 4th band in the region (Fig 5.12). As inferred in sample 1, it is likely that the band represented members of the alpha subclass of *Proteobacteria.*

Sample set 3 was obtained approximately 75 d after sample 2 and there was a marked difference in the banding pattern compared to sample 1 &2.. Fewer bands were evident in sample 3. However, the relative intensity of the predominant band (lane 7&8, Fig 5.12) was comparatively higher than any other bands in the gel. Correlating the sampling period (3/08/02) to the corresponding period during FISH analysis, the latter presented the alpha subclass to be predominant. The fewer bands of higher intensity clearly indicated fewer species but of higher abundance. The transition in the population profile pointed towards the establishment of a 'specialized community' and supported the FISH analysis whereby a decrease in the EUB/DAPI ratio and total cell counts also showed a community structure depicting a specialized community and not impacting on process performance. The results could also be explained by Cloete 's model on species diversity (1997) showing a shift towards category D, indicating an extreme environment (oil effluent) where a few species with a high number of individuals exist. The PCR -DGGE findings supported the FISH analysis of the lab scale process indicating a established community with a low species diversity.

With regards to correlation of PCR-DGGE analysis on the population profile to process performance during the respective periods in phase B (phase 5) of the lab scale process, it is clear that there appears to be no definite pattern. The population profiles indicated by the banding pattern of sample set 1 and 2 appear to be similar with possibly the alpha subclass of *Proteobacteria* dominating. However, the process efficiency during the respective periods when samples 1&2 were obtained were markedly different. During sample 1 the process was at steady state and performed optimally with approximately 90% COD removal. During sample 2 the influent COD was lower and the process performance was comparatively less efficient, yet the banding patterns indicating the community structure were very similar to sample 1. In sample 3 the species diversity was much lower than the other 2 samples and the process performed optimally with good COD removal efficiency i.e. > 90%. Although DGGE analysis could not present a trend effectively correlating process performance to the population profile, it served to ratify the results from FISH and showed a transition in the community structure from a high species diversity (richness) (seed sludge, sample set 1&2) to a low species diversity and high abundance (sample set 3), thus supporting the theory on the establishment of a specialized community.

DGGE analysis of the 3 sets of samples clearly show that the banding patterns depicting community structure were identical amongst the 3 zones(aerobic 1, aerobic 2, anoxic) of each sample (TABLE 5.5 and Fig 5.12) e.g. lane 1-3 (sample set 1) showing samples obtained from aerobic 1&2 and anoxic zones of the lab scale process having the same banding pattern. This trend was repeated in all 3 sets of samples (Fig 5.12), reinforcing the assumption that the bacterial community structure of the different zones in the lab scale reactor at any one time were the same. The latter findings conferred with previous research by Curtis and Craine, 1998 using DGGE and conducting comparison of community structure between sub-samples of MLSS from a activated sludge plant. Within site spatial comparison showed the banding pattern of samples obtained simultaneously from different locations in the aeration and nitrification basin of a municipal treatment plant were identical. Current findings also confirmed that the community structure of the aerobic and anoxic zones of the lab scale reactor at one time were also the same.

In summary, although the analysis of 16S rDNA fragments by DGGE has great value in environmental microbiology, it does have limitations (Jackson *et al.*, 2000). To overcome some of these during the current investigation, conditions form PCR- DGGE were optimized and experiments repeated to improve the reproducibility and accuracy of the results. As shown in the current research, perhaps the best use of DGGE in the examination of microbial communities is as an initial means of comparing two or more environmental samples. If the samples show different banding patterns, then the bacterial communities they contain are undeniably different. However, if they show a similar banding pattern, then they may or may not contain similar bacterial communities and further analysis is needed. Although the bands were not excised and sequenced for additional phylogenetic classification during the current research, the PCR-DGGE technique served to achieve the desired objectives by determining the gross community structure in the reactors, similarities in the different zones of the basin, determining the population shifts as the lab scale process progressed and helped to substantiate findings from *in situ* hybridization analysis.

5.4.3 Survey of filamentous bacteria during bulking incidence

It is accepted that filamentous bacteria form the backbone of activated sludge flocs and prevail in most activated sludge processes. However, for the current investigation, focus on the identification of filamentous bacteria was limited to episodes of bulking encountered in the lab scale process towards the end of phase 3. The reason bulking of activated sludge occurs is because of high numbers of filamentous microbes in the mixed liquor (Jenkins *et al.*, 1986; Eikelboom, 2000), thus impacting on settling characteristics and a poor quality final effluent. Globally, research has shown that about 10 filament types account for at least 90 % of all bulking episodes recorded, although the relative frequency of occurrence or importance of the dominant individual filamentous microbes varies significantly between geographical regions (Jenkins *et al.*, 1986; Chua and Lee, 1994). These differences in filament populations of plants in various countries may be explained partly in terms of differences in influent characteristics, like wastewater composition and strength , and in treatment plant design and operating conditions (Jenkins *et al.*, 1993).

During the bulking episodes the filaments were identified as per identification keys of Eikelboom, 2000, and the identifications were verified by sending samples to world expert (Dr Eikelboom) in the Netherlands. The identification was based on morphological and staining characteristics. However, researcher's have advised caution when using these techniques since many filaments undergo reversible morphological changes to unicellular rods or cocci in response to changes in culture or plant conditions (Wagner *et al.*, 1994b). However, the latter problem can be overcome with the use of novel DNA/RNA based molecular techniques. The floc structure observed during the bulking episodes were typical of bulking conditions with the floc being irregularly shaped with an overgrowth of filaments growing in profusion (Fig 5.13). In addition, during this period high SVI's were recorded, another process indicator of bulking conditions. However, Eikelboom, 2000 provided a few indicators with regards to observing flocs from industrial treatment plants and mentioned that sludges from industrial treatment plants often displayed large variations in the morphology of the floc and cannot be classified into a few groups. Some of the comments were :

- The floc in various industrial sectors (particularly those treating chemical wastewater) is not really robust and the flocs are small. The sludge often appears 'messy'.
- a growth *en masse* of filamentous bacteria, often results in large but markedly irregularly shaped flocs
- in fact, the 'normal' floc quality must be established individually for every industrial treatment plant on the basis of shape, structure, strength, size and any other characteristics.

Thus, the floc quality established can subsequently be used as a reference for the routine monitoring of the activated sludge in the respective plant. The current findings were in agreement with the above, since even out of the bulking episode, the floc characteristics observed microscopically in the process resembled to a great degree that mentioned i.e messy sludge and irregular floc shape.

The filamentous bacteria that were positively identified in the process during the bulking episodes included *S.natan*s, *H. hydrossis* (Fig 5.14), Type 1701, *M. parvicella* and Type 0675. The filaments identified in the current investigation were identified by other researchers as causative organisms of bulking conditions in activated sludge systems (Jenkins *et al.*, 1993; Blackall 1999). One of the postulated causes of bulking is known to be low F/M ratio in the plant (Blackall, 1999). During the period of bulking (end of phase 3) the mean F/M ratio was low at 0.55 and could have contributed to the bulking. Some other common causes were composition of wastewater (edible oil effluent), concentration of DO in the aeration tank (erratic at the end of phase 3), concentration of soluble substrate available to microbe and process operating conditions. Any one or more of the latter conditions could have promulgated in the bulking conditions experienced. Quantification of the filaments showed *S natans, H hydrossis* and Type 1701 to be dominant types in the process (TABLE 5.6). The latter type was comparatively less dominant than the former 2 filament types. The findings support previous research by Blackall, 1999 who also showed the above 3 filament types to be some of the more dominant filaments in bulking sludges. Typical *S. natans* and *H. hydrossis* morphotypes were seen protruding from the floc (Fig 5.14). However, *M. parvicella* and Type 0675which appeared to be secondary (ranking) in the current research were known be dominant types in other bulking sludges (Jenkins *et al.*, 1986). Therefore it can be concluded that the filaments present in bulking sludges are dependent on various factors that dictate their presence and abundance levels and it is important to study each environment (especially processes treating industrial wastewater) individually and not generalize filament occurrence.

The composition of the substrate in the influent wastewater is accepted as one of the parameters that determines the proliferation of filamentous types in a activated sludge process (Eikelboom, 2000). Although the filaments identified in the current investigation were known bulking organisms, the composition of the wastewater would have also supported their occurrence and abundance levels. Since the wastewater(edible oil effluent) was comprised primarily of hydrophobic substances such as wax, oils and greases, filaments such as *M. parvicella* which was a specialized lipid consumer(Nielsen *et al.*, 2002) had a competitive advantage and prevailed. *M. parvicella* consumes LCFA and due to the filamentous structure and relatively hydrophobic surface, much of the substrate would have reached the bacteria when the wastewater and sludge were mixed (Nielsen et al., 2002).

The organism accumulates the LCFA intracellularly in early stages of growth(up to 35% of the dry weight of the biomass) which presumably contributes to their hydrophobicity (Slijkhuis *et al.*, 1984) Recent studies also showed that *M. parvicella* preferred LCFA especially oleic acid (major LCFA in sunflower oil) compared to simpler substrates like glucose and acetate. Furthermore, oleic acid was taken up under aerobic and anoxic conditions(Andreasen and Nielsen,1997). The above characteristics certainly favored the growth of M. *parvicella* in the lab scale process. Although the organism was ranked secondary (lower abundance level) (Table 5.6) during the bulking period, it could have proliferated and been dominant under 'normal' plant process conditions. However, the survey of filamentous bacteria in the lab scale process was limited to the bulking period (end of phase 3) only. Due to its hydrophobic nature, *M parvicella* is also known to play a role in foaming. However, no problematic foaming incidences was observed in the lab scale process.

Previous research by Eikelboom and Geurkink, (2002) showed many unknown filamentous species, viz. morphotypes that were present in industrial sludges. However, those mentioned in the current investigation were commonly found in bulking sludges treating industrial wastewater's.

5.4.4 Survey of protozoa in the lab scale process

Protozoa, and frequently also metazoa, are almost always present in activated sludge. Some species are attached to the flocs whereas others are free in the water between the flocs. They mainly feed on bacterial cells which are present free in the liquid and those not firmly bound to the floc. In addition to producing a clear effluent, they are indispensable for a far reaching COD reduction and reducing sludge production (Eikelboom, 2000). The protozoans that were identified in the lab scale process (Fig 5.15) were similar to those present in activated sludge plants. During the current investigation, the sessile (stalked) ciliate *Vorticella* sp and *Telotroch* sp predominated throughout the process, although their abundance levels were erratic. The ciliates are known to graze on freely suspended and loosely attached bacterial cells (Madoni, 1994). With reference to FIGURE 5.15, it was evident that the resident protozoan population in the seed sludge declined on exposure to the feed oil effluent, possibly due to a toxic overloading caused by initial exposure and possibly resident flocculent that was carried into the influent. The demise of the protozoan population supported previous research by Eikelboom, 2000 who attributed the disappearance of protozoa and metazoa in a process to toxic components present in the influent. This can be followed within a few days by an explosive increase in the number of protozoa due to an increase in the numbers of free living bacterial cells, during their absence. Current research also supported the latter findings since there was a noticeable increase in the *Telotroch* sp numbers on acclimation of the process. In addition to maintaining good quality final effluent, the ciliated protozoans have been widely used as water quality indicators because they are simple to identify microscopically (Santana and Martinez, 2002).

Another distinguishing feature of the current research was the lack of diversity of protozoan types and species such as flagellates and other ciliated protozoa. Previous surveys of activated sludge plants revealed a substantial diversity of protozoa that were present (Curds, 1992; Madoni, 1994; Santana and Martinez, 2002). The low species diversity could partially be attributed to the composition of the wastewater (oil effluent) that selected for a reduced diversity of protozoa that acclimatized to the substrate without experiencing any toxicity. When evaluating diversity of protozoa in plants treating industrial wastewater,s, it is important to consider the wastewater quality and organic load (Eikelboom, 2000). Therefore, during the current investigation, protozoan population diversity was evaluated in correlation with process conditions during the respective period.

After 1 week on commencement of the process(phase 5), the *Telotroch* sp abundance levels were on the increase and the testate amoeba were also present but in lower numbers. They were the first protozoan groups to establish themselves. The organic load (F/M ratio) during the respective period was 0.4 gCOD/gMLSS.d. and within the range that supported the growth of these groups. At the end of March, the sessile ciliate *Vorticella* sp numbers began to increase and coincided with a period of low organic loading (mean F/M ratio of 0.21 gCOD/gMLSS.d. The delay in the onset of the stalked ciliates (*Vorticella* sp) produces interesting results. The ecological dynamics of activated sludge are such that response to a transient nutrient input should be reflected firstly in an increase in bacterial populations that feed directly on the nutrient and exhibit short generation times followed by delayed increase in scavenger organisms, such as stalked ciliates, which feed on bacteria and generally exhibit longer generation times (Hrudey, 1982). The latter could also explain the delayed onset and periodic dominance of *Vorticella* sp in the process. The *Vorticella* sp numbers peaked during sampling period 22 May and was once again matched with a low F/M ratio (0.19 gCOD/gMLSS.d), due to the introduction of a new batch of wastewater from industry. However, due to system operational problems caused by excess flocculent entering the feed at the end of May, the condition could have resulted in the decline of the stalked ciliate population. The *Vorticella* sp numbers began to recover throughout June. Correlation of the population to organic loading also showed high F/M ratios (mean 0.5) at the end of May associated with a decline in the population and a recovery in the *Vorticella* sp abundance throughout June when the F/M ratio was low (± 0.25). Once again, with the introduction of a new batch of wastewater (26 June), the stalked ciliate numbers dropped instantly. The latter trend 'lag period' (3 July– 31 July) was noticed among all the other protozoan groups as well and proceeded until 7 Aug (Fig 5.15). Just as the decline in the *Vorticella* sp population was instant, so was the sudden increase resulting in the highest organism counts being recorded. The latter phenomenon can undoubtedly be attributed to some toxic component in the influent feed (oil effluent), that once washed out of the system resulted in recovery of the population. The instantaneous nature of the recovery could be attributed to the abundance of food (bacteria) that prevailed and accumulated during the period when the stalked ciliate population was depleted (Hrudey, 1982). However the *Vorticella* sp numbers once again began to decline. Based on the above results and in agreement with previous research (Eikelboom, 2000), it can be concluded that the proliferation of the members of the stalked ciliates in activated sludge processes treating oil effluent depend on the composition of the wastewater and the organic loading of the process.

The *Telotroch* sp dominated at the start of the process and the sudden proliferation coincided with the period of system perturbation due to excess flocculent in the feed. During the same period there was a substantial decline in the *Vorticella* sp. There was a rapid decline in the *Telotroch* sp population (12-19 June) and was eliminated from the process by 3 July. The demise in the latter was proceeded immediately by an increase, although low comparatively, in the numbers of the stalked ciliate *Epistylis* sp. Being a stalked ciliate like *Vorticella* sp, *Epistylis* sp favored low organic loading and the population dynamics was a mirror image of *Vorticella* sp, although the numbers were lower, from the 3 July to 14 Aug 01 (Fig 5.15). The latter findings substantiated previous research where members within a groups of protozoa showed common behavioral patterns (Eikelboom, 2000). In addition, the connection between organic load (0.2-0.6 gCOD/gMLSS.d.) and the composition of the population was similar to previous research by Eikelboom, 2000. The trends on population dynamics of the different groups of protozoa during the current investigation can also be explained according to the model of temporal succession which advocates a succession of protozoan types during the maturation of activated sludge. (Curds, 1992). When a plant is first started, there is little sludge present and purification is partial. With time the sludge matures and effluent quality improves. As seen in the current investigation, a more or less predictable succession of protozoa appears in the plant during maturation. The flagellates are the first dominant protozoa to appear. As their numbers decrease, they are slowly replaced by free swimming ciliate which peak after about 3 weeks. They are replaced by crawling ciliates followed by stalked ciliates such as *Vorticella* sp and *Epistylis* sp (Curds, 1992). Although not exactly, the general trends on population dynamics during the current investigation do conform to the succession model.

Testate amoebae were present throughout the investigation at comparatively low numbers and the population was not effected by operational conditions (Fig 5.15). The desirable protozoa have been suggested to be the crawling and attached ciliates and the testate amoebae, while sessile ciliates like *Vorticella* sp are said to indicate poor effluent quality (Madoni, 1994). However. current findings are in contrast with the latter, since although *Vorticella* sp predominated, the final effluent emanating from the process was of relatively good quality. Another possible reason that could account for the proliferation of stalked ciliates during the current investigation could be that stalked ciliates are able to feed directly on the lipid emulsion (Hrudey, 1982). Since wastewater composition is known to impact on protozoan population selection (Eikelboom, 2000) and the substrate i.e. oil effluent comprised a large amount of emulsified lipids, the stalked ciliates would have enjoyed a competitive advantage over other protozoan groups and therefore dominated.

The metazoa were present in low numbers and although not investigated further in the current research, they are known to play a subsidiary role in wastewater treatment.

5.5 CONCLUSION

- Fish analysis showed that members of the domain *Bacteria* dominated both the seed sludge and the process samples.
- Both phases A & B showed substantial similarities with regards to their microbial community profiles.
- The EUB/DAPI ratios of the seed sludge were higher than process samples and decreased as the process progressed.
- The DAPI total cell counts were higher in the seed sludge than process samples and decreased with the continuation of the process.
- Beta subclass of *Proteobacteria* dominated the seed sludge and substantiated previous research.
- The alpha subclass of *Proteobacteria* was the dominant group in the lab scale process.
- The total percentages of the respective subclasses of *Proteobacteria* gradually decreased as the process progressed.
- The bacteria that were unaccounted for using the group specific probes in relation to the EUB 338 probe was relatively low.
- The conditions for DGGE were optimized at 50 μl sample size, denaturant concentration of 30:70% and gel running time of 180 min.
- PCR-DGGE analysis complemented *in situ* hybridization findings in showing differences between the microbial community of the seed sludge and process samples.
- PCR-DGGE analysis also showed a transition in the bacterial population as the lab scale process progressed towards a lower diversity indicating the possible development of a 'specialized community.'
- The banding pattern displayed a similarity in the microbial community amongst different zones in the reactor.
- The filaments that were ranked dominant were also associated with bulking sludges in other wastewater treatment processes.
- The floc structure was irregular and indicative of flocs associated with industrial wastewater treatment plants.
- Survey of Protozoa displayed a comparatively low diversity.

- Sessile ciliates such as *Vorticella* sp predominated in the process and were associated with periods of low organic loading (F/M ratio).
- The population dynamics of the protozoa in the process appears within the framework of the model of temporal succession.

CHAPTER SIX

GENERAL CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

Preliminary trials on edible oil effluent treatment using a lab scale activated sludge process was unsuccessful. The latter could undoubtedly be attributed to the high fats, oils and grease content of the effluent that inhibited biological activity. The approach then necessitated the development and optimization of appropriate pretreatment technology to remove the high FOG and prepare the effluent for biological treatment. Comparison of existing pretreatment technologies such as the use of coagulants including ferric chloride and alum combined with DAF to trials conducted using a commercial coagulant (C40), showed the latter coagulant to be more efficient, less labourious and space intensive such as DAF and cheaper. Although ferric chloride and alum combined with DAF were the technologies of choice for wastewater treatment in the edible oil sector, C40 was selected and a process optimized for pretreatment of the effluent obtained from industry, due to its relative advantages, during the ensuing lab scale activated sludge process. It should be noted that pretreatment was not within the original objectives of the research but included due to necessity. Therefore, a detailed chemical characterization of C 40 was not conducted.

Detailed chemical investigation of the oil effluent using GCMS analysis revealed 28 identifiable organic compounds in the raw effluent and twenty remained after flocculation. Of these, many were classified within the group of compounds called essential oils, which are known to have high commercial value. Semi-quantitative analysis reflected by area percentages on the chromatogram detailing organic compounds in the raw effluent, showed relatively high concentrations of the valuable essential oils. Although it was likely that some of these organic compounds would have precipitated out by the coagulant, chemical transformations attributed to the addition of the coagulant could possibly account for the presence of 'new' compounds such as "toluene" in the flocculated effluent. Higher concentrations of some original compounds e.g. "Furan" were present in the flocculated effluent. However, verification of the results for accurate quantification requiring the use of standards was not within the scope of this investigation. More importantly, the findings served to provide a qualitative analysis and gross chemical characterization of edible oil effluent.

Due to the inconsistent nature of the effluent (process influent feed), incremental increase in influent COD concentrations and variability in operational conditions, the lab scale treatment process was conducted in phases encompassing common operational parameters, therefore allowing accurate critical analysis of data. Biological phosphorus removal was not optimized due to P and N becoming limiting in the effluent obtained from industry, necessitating supplementation in the influent feed early in the investigation. After pretreatment, the influent feed COD concentration was increased incrementally and ranged from 500 to 1800 mg/L (undiluted) during the investigation and the system responded accordingly achieving a maximum COD removal of 1550 mg/L during phase 4 E. Steady state conditions (phase 4 D) showed high COD removal efficiency (> 80%) and the process showed potential of removing higher concentrations. The latter findings indicated the large portion of the oil effluent was biodegradable. However, the fluctuation of the process performance with regards to COD removal efficiencies could undoubtedly be attributed to variability in the organic strength of the effluent from industry and regular disruptions in supply. In addition system perturbations such as bulking (end of phase 3) also impacted on the performance. However, latter problems are common when applying activated sludge processes to treat industrial effluents. The MLVSS/MLSS ratio was consistent throughout most of the investigation ranging between 0.8 and 0.9 mg mgVSS/mgTSS, reflecting a low inert material concentration. The OUR which reflects the metabolic activity of a process mirrored COD removal efficiencies and was a good indicator of process performance.

Electron microscopy of the floc showed close association with oily material and the ultrastructural detail revealed an abundance of microorganisms dominated with bacteria. GCMS analysis of the final effluent showed that most of the organic compounds including essential oils that were present in the influent were biodegraded in the activated sludge process. The desired objective of the research was achieved with the optimization of a laboratory scale activated sludge process that successfully treated edible oil effluent. However, the research showed that appropriate remediation technology could only be developed and optimized on a case by case basis and not generalized for the entire edible oil sector due to the individual problems experienced. The operation of the activated sludge process necessitated continues attention to minimize system perturbation and drop in process efficiency.

The significance of conducting a detailed microbial community analysis of the process was aimed at accurately profiling and creating a better understanding of the microbial communities involved in the treatment of edible oil effluent. Population profiles of filamentous bacteria and protozoa was conducted to determine their association with bulking conditions and process performance. Not much information is currently available on microbiological investigations of plants treating industrial wastewaters. Novel molecular techniques such as FISH and PCR-DGGE served to provide accurate profiling of the microbial communities present in the process. *In situ* hybridization analysis showed members of the domain *Bacteria* predominated (EUB/DAPI ratios) in the seed sludge, however decreasing as the process progressed. Similar trend was observed with the DAPI total cell counts supporting the theory of development of a 'specialized community' as the lab scale process progressed. The organic strength and incremental increase of the influent COD concentration did not appear to determine the population dynamics as shown by the relatively similar bacterial community profile between phase A & B, although the operational conditions differed. However, a population shift was clearly evident in both phases with beta subclass dominating the seed sludge and alpha subclass of *Proteobacteria* dominating the process samples. Compared to the gamma *Proteobacteria* and HGC, the alpha and beta subclass dominated the process. Current research reiterated previous findings showing the *Proteobacteria* group dominating activated sludge processes. Majority of the bacteria were classified using the predetermined group specific probes in relation to the EUB 338 probe with the unaccounted bacteria possibly belonging to some undetermined groups. Therefore, it can be concluded with FISH analysis that the community structure of activated sludge processes treating industrial effluents such as edible oil effluent differ from municipal activated sludge processes with the substrate being the determining parameter.

The PCR-DGGE analysis served not only as a rapid tool for cross community comparisons of the population complexity but verified some interesting finding presented by FISH such as :

- differences in the microbial community profile of the seed sludge and process sample with possibly alpha subclass of *Proteobacteria* dominating the process
- a transition or population shift as the process progressed reflected by fewer bands of higher intensity (sample set 3) indicating a shift towards a lower diversity where a few species with a high number of individual exist i.e development of a 'specialized community'. The latter appears plausible since edible oil effluent could be regarded as an extreme environment.

Another important finding shown by DGGE was that the community profile of the different zones in the MLE process were similar. However, in order to obtain reproducible and accurate results the PCR-DGGR protocol had to be optimized by adapting standard methods to suit the current application.

The application of the novel molecular methods contributed to enhancing the understanding of the genetic diversity of complex microbial populations and satisfied the aims of the current research by achieving the desired results such as

- determined the community structure of the process by phylogenetic classification at a group level
- determined changes in the population profiles "shifts" as the process progressed and
- facilitated cross community analysis

However, further investigation focussing on sequencing and cluster analysis would provide more detailed phylogenetic affiliations of the community members and further aid cross community analysis.

Current finding reaffirmed previous research which associated abundance levels of certain filaments with bulking conditions. However, findings also added to the limited body of knowledge on filaments associated with bulking in industrial wastewater plants. The ranking showed *S. natans, H. hydrossis* and Type 1701 as dominant filaments. In contrast to previous research, the abundance levels of *M .parvicella* was comparatively low and ranked secondary, although it is known as a dominant bulking filament and a specialized lipid consumer. The floc structure was irregular with protruding filaments and typical of bulking sludges and treatment plants associated with industrial wastewater's.

A differentiating feature on the survey of protozoa in the process was the comparatively low species diversity compared to conventional activated sludge plants. This behaviour could be attributed to the composition of the substrate that selected for a reduced diversity and acclimation of a few species without experiencing any toxicity. The sessile ciliates e.g. *Vorticella* predominated in the process and are known to favour low organic loading rates (F/M ratio) as was prevalent in the process. Clear trends were evident showing patterns in the abundance levels and population dynamics of the different groups of protozoa in the process thus supporting the theory of succession in the community.

Some of the novel aspects of the research findings include :

- Detailed chemical characterization of edible oil effluents emphasizing the presence of valuable essential oils,
- development of suitable pretreatment technology and optimization of a biological process to successfully treat edible oil effluent,
- electron microscopy showing ultrastructural detail of an activated sludge floc treating edible oil effluent,
- accurate microbial community analysis of a lab scale process treating edible oil effluent using novel molecular techniques, and
- identification of filaments associated with bulking and protozoan population profile of a process treating industrial wastewater such as edible oil effluent.

6.2 RECOMMENDATIONS

- Conventional pretreatment technology for wastewater treatment in the edible oil sector has been limited to the use of ferric chloride, alum, other poly-electrolytes and more commonly used is DAF. Future studies should focus on the chemical characterization of the commercial flocculent C40 to further reduce the cost and enhance accessibility. The pretreatment technology developed during the current investigation using C40 far surpasses conventional technology for large scale application on site at industry since it has proven to be comparatively more efficient, cheaper and most important, it requires far less space than DAF and therefore more practical.

- Conventional production of essential oils which have high market value is very costly. These valuable oils can be derived from a waste product such as edible oil effluent and produced at a fraction of the cost. Future studies need to investigate novel technologies such as 'supercritical extraction' and adapt the technology for efficient extraction and purification of essential oils from oil effluent, thereby substantially reducing the cost for commercialization. In addition, these valuable essential oils could be extracted before treatment of the effluent stream, thereby supporting the waste minimization approach and reducing the load for 'end of pipe treatment'.

- The sludge that is produced from the pretreatment process comprises a mixture of coagulant and edible fats, oils and greases that could be used as a supplement to animal feed, thus further minimizing waste production and generating income.

- Current findings recommend that the technology of choice for the treatment of edible oil effluents should focus on an on site process applying a combination of chemical pretreatment using C40 or a generic equivalent with an activated sludge process, optimized to suit the specific wastewater treatment needs of the edible oil company. Parameters that have been optimized during the current investigation for the efficient performance of the lab scale process can be used as a guideline. The generation of acceptable quality final effluent from the optimized process can be recycled and used for non potable purposes at the industry such as use in the boiler and washing floors. This practice can be cost effective, save valuable water and reduce the volume of final effluent discharged, thus supporting effective waste minimization.

- Although activated sludge processes are known to have a comparatively higher threshold to failure by toxicity compared to other biological treatment systems, the inconsistent nature of wastewater produced by the edible oil industry can pose problems. Therefore, it is recommended that the process be monitored continuously during operation and to effectively execute troubleshooting when required.

- The information generated by novel *in situ* hybridization techniques to conduct a microbial community analysis of the process could be used to further optimize the process and minimize system perturbations. However, having ascertained the gross population profile and trends using group-specific probes, future studies should focus on FISH using genus and species-specific probes, to provide more detailed information on the community structure and more information on the specific genera/species comprising the adapted community of the activated sludge process treating edible oil effluent.

- PCR-DGGE analysis provided valuable information with reference to banding pattern, on cross community analysis and verified some of the findings FISH during the current investigation. Future research should focus on excising the bands from the gels, sequencing with reference to data banks to identify the phylogenetic affiliations of the community members and verify the population shifts shown by FISH analysis. Concurrent application of FISH and PCR-DGGE techniques could serve as an invaluable tool to accurately determine the microbial community structure of complex ecosystems such as the activated sludge process treating oil effluents.

- Current findings on the filaments that proliferated during bulking conditions need to be verified using rRNA oligonucleotide probes, thus overcoming the inherent limitations of the conventional identification procedure, which sometimes inaccurately identifies filaments producing variable cellular morphology (short rods)especially in processes treating industrial wastewater. Probes are commercially available from data banks or alternately can be designed from available sequences.

- FISH technology can be developed into a rapid diagnostic tool for the detection of bulking filaments, thus facilitating urgent measures to prevent bulking such as those experienced.. Collaboration with Dr Eikelboom (world expert on filaments) needs to be reinforced to create a database of filaments associated with industrial wastewater treatment globally, with many unknown filaments currently being discovered.

- Although the current research investigated the population dynamics, there is need to determine the toxicity threshold of protozoa to edible oil effluents in the activated sludge processes and the possibility of using the organisms to predetermine poor system performance.

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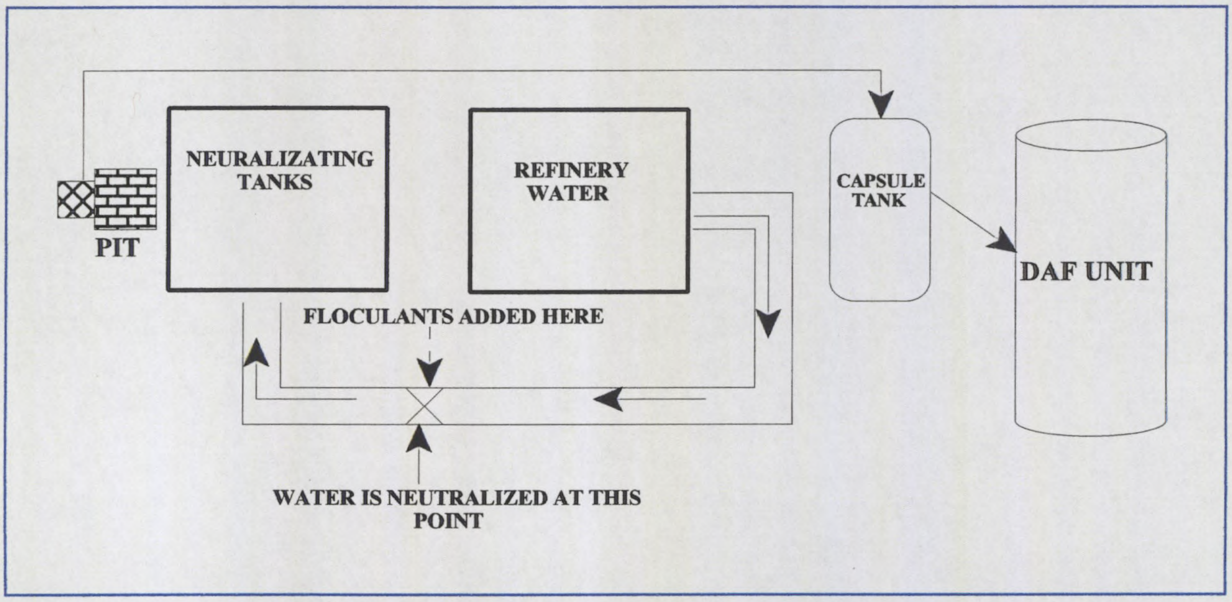
APPENDICES

APPENDIX 1

SCHEMATIC REPRESENTATION OF THE ON-SITE EFFLUENT TREATMENT PROCESS AT COMPANY X



APPENDIX 2

DETERMINATION OF CHEMICAL OXYGEN DEMAND (PHOTOMETER)

☐ Overview

The COD of wastewater is the measure of the oxygen equivalent of the organic matter content that can be oxidised by a strong chemical oxidant i.e. potassium dichromate. The sample is heated to boiling level with potassium dichromate and silver sulphate catalyst in strong sulphuric acid (Digestion procedure). The absorbance of the resultant coloured solution is measured using the Merck SQ 118 photometer.

☐ Method

- Add 3 mL of sample to a reaction cell.
- Add 0.3 mL of solution A and 2.3 mL of solution B to the reaction cell and mix.
- Place the reaction cell in the thermoreactor and heat at 100^0 C for 2 h.
- Remove the reaction cell and allow to cool to room temperature.
- The COD of the sample was measured using the Merck SQ 118 photometer.
- The Spectroquant analysis method 14541 was used for the range 100-1500 mg/L COD and 14555 for 500- 10000 mg/L COD.

APPENDIX 3

PREPARATION OF COAGULANTS

☐ FERRIC CHLORIDE

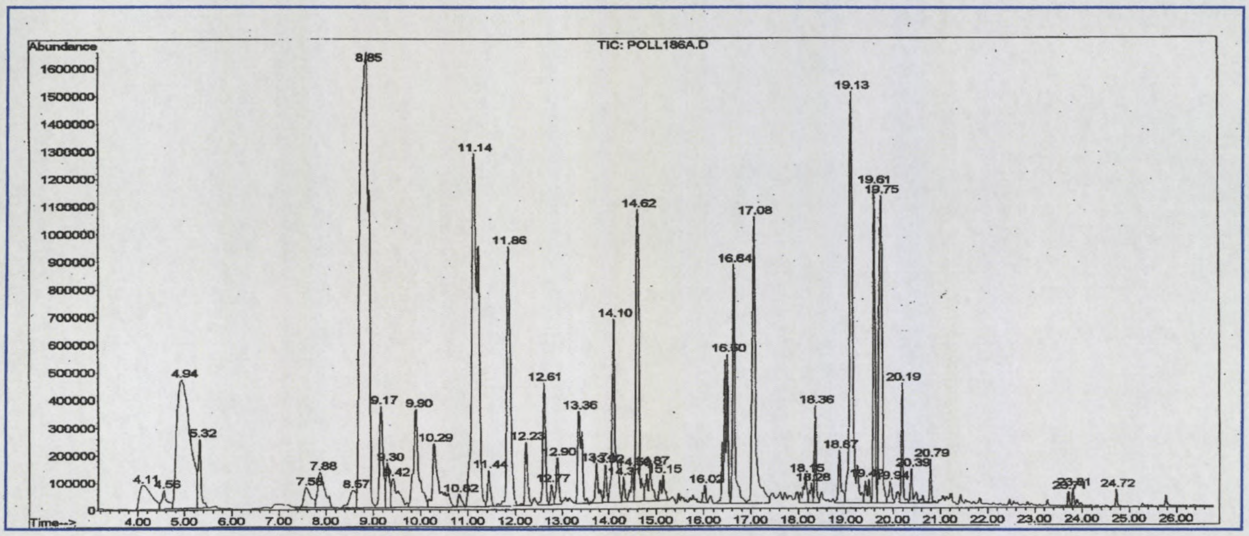
- A volume of 0.2 mL of 43% $FeCl_3$ was dispensed into a 100 mL volumetric flask and made up to 100 mL using deionized water.
- On adding to a 800 mL effluent sample, 1mL of the FeCl solution yielded a final concentration of 1ppm of the coagulant.
- Storage of the coagulant was problematic due to precipitation and therefore had to be prepared fresh when required.

☐ ALUMINIUM SULPHATE (ALUM)

- A volume of 4 mL of a 43.7% alum(density - 1.29) was dispensed into a 1000 mL volumetric flask and brought up to volume with deionized water.
- On addition to 800 mL effluent sample, the final concentration of alum in the solution was 2 ppm.

APPENDIX 4

GCMS CHROMATOGRAM OF RAW EFFLUENT (SAMPLE- 23 MAY 2001)



GC CONDITIONS		**FINAL TEMPERATURE**	**HOLDING TIME (MINS)**
Column Type	DB5-MS		
Oven Initial Temperature	50	50	5
Ramp 1	20	160	0
Ramp 2	6	250	0
Injector temperature	250		
Helium flow rate	**Constant velocity 40cm/sec**		
MS CONDITIONS			
Mass Range	50-450		
Transfer line temp	275		
MS Acquire start time	6		
Ion source temp	200		

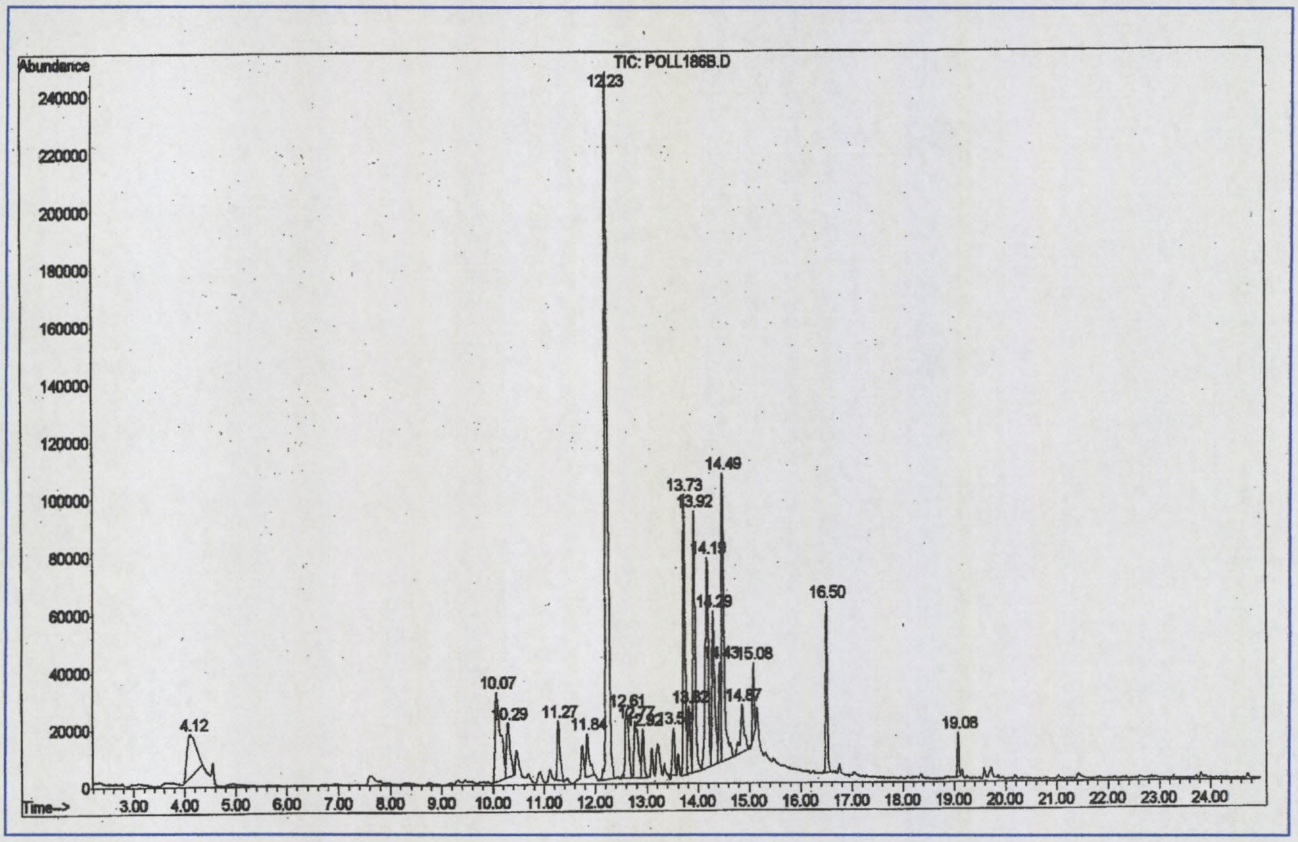
Ionisation type - electron impact ionisation

Type of ion resolution - ion trap

NIST library was used to identify peaks with FIT specifications

APPENDIX 5

GCMS CHROMATOGRAM OF FLOCCULATED EFFLUENT (SAMPLE - 23 MAY 2001)



APPENDIX 6

DETERMINATION OF FATS, OILS AND GREASE

❑ Overview

Groups of substances with similar physical characteristics are determined qualitatively on the basis of their common solubility in trichlorotrifluoroethane or diethyl ether. This method of FOG analysis is suitable for biological lipids and mineral hydrocarbons, and is also suitable for most industrial wastewater's or treated effluents containing these materials.

❑ Method

- Soluble metallic soaps are hydrolysed by acidification.
- Any oils and solids or viscous grease present are separated from the liquid samples by filtration.
- After extraction in Soxhlet apparatus with diethyl ether, the residue remaining after solvent evaporation is weighed to determine the fats, oils and grease content.
- Compounds volatilised at or below 103^0 C will be lost when the filter is dried.
- Diethyl ether was used as the non polar solvent and the analysis was conducted in duplicate.
- A volume of 100mL effluent sample was analysed according to the prescribed standard method (Reference No : 5520 D) (Standard methods, 1989).

APPENDIX 7

DETERMINATION OF TOTAL PHOSPHATE

☐ Overview

Total phosphorus (TP) determination of a wastewater sample includes all orthophosphate and polyphosphate, both dissolved and particulate , organic and inorganic. To release the organically bound phosphorus, a digestion and oxidation procedure is necessary. All forms of phosphorus are then converted to orthophosphate , the concentration of which is determined photometrically. Using the Merck phosphate cell test, the sample is mixed with reagents. In a solution acidified with sulfuric acid , the orthophosphate ions react with molybdate ions to form molybdophosphoric acid. Ascorbic acid reduces this to phosphomolybdenum blue which is then determined photometrically.

☐ Method

- Pipette 1mL of pretreated sample into the reaction cell and mix
- Heat the cell at 120^{0}C in the preheated thermoreactor for 30 min to digest sample.
- Allow the closed cell top cool to room temperature in the cell rack.
- Samples must be diluted with deionized water if required to achieve the desired concentration range.
- Spectroquant analysis method 14842 for the concentration 1.0- 30 mg/L PO_4-P was used to determine the total phosphorus as orthophosphate and the concentrations were obtained directly using the Merck SQ 118 photometer.

APPENDIX 8

DETERMINATION OF TOTAL NITROGEN (TKN)

☐ Overview

Nitrogen in wastewater appears both as oxidized nitrogen and reduced forms. Both the ammonium (free and saline) and the organic forms are present in wastewater and constitute the total nitrogen. Using the total nitrogen cell test, the organic and inorganic nitrogen compounds are transformed into nitrate according to Koroleff's method by treatment with an oxidizing agent in a thermoreactor. In concentrated sulfuric acid, this nitrate reacts with a benzoic acid derivative (Nitrospectral) to form a deep red nitro compound that is determined photometrically.

☐ Method

- Pipette a volume (usually 1 mL) of sample and distilled water to obtain the desired dilution factor into a cell containing reagent 1.
- Immediately close cell tightly and shake vigorously for 1 min.
- Place the cell in the preheated thermoreactor (for digestion procedure) and heat the cell at 120^0 C for 1 h.
- Allow the closed cell to cool to room temperature and shake the cell briefly after 10 min
- Pipette 1.5 mL of reagent 2 into the cell and leave the cell to stand for 10 min.
- This results in the development of a colour which is measured photometrically.
- The Spectroquant analysis method 14537 is used for concentration range 1.0-30.0 mg/L N and the concentration is determined using the Merck SQ 118 photometer.

APPENDIX 9

COLLECTION AND PRESERVATION OF SAMPLES

Samples were, as far as possible, collected and preserved according to Standard Methods (1989). As stated in Standard Methods, the primary objective of sampling is that relative proportions and concentrations of all pertinent components within the sample will be representative of the material been sampled and that no significant changes in composition before analysis.

During operation of the pilot plant, daily grab samples were considered adequate for analysis due to the fairly constant load entering the plant. All sampling bottles were triple rinsed with deionized water . The sampling bottles were 250 mL plastic honey jars. The following preservation techniques were used for the duration of the project:

Determinand	**Preservation technique**	**Maximum storage**
COD	H_2SO_4 addition to pH<2; refrigerate	24 h
Ammonia	H_2SO_4 addition to pH<2; refrigerate	24 h
Nitrate	H_2SO_4 addition to pH<2; refrigerate	24 h
Kjeldahl nitrogen	H_2SO_4 addition to pH<2; refrigerate	24 h
Total phosphorus	H_2SO_4 addition to pH<2; refrigerate	24 h
Soluble reactive phosphorus	immediate 0.45μm filtration; refrigerate	24 h
Solids	refrigerate	6 h
FOG	H_2SO_4 addition to pH<2; refrigerate	24 h

APPENDIX 10

DETERMINATION OF FREE AND SALINE AMMONIA (FSA)

☐ Overview

Free and saline ammonium nitrogen is readily available in wastewater as a nutrient that is primarily responsible for eutrophication. One of the products of the biodegradation of organic nitrogen in wastewater is FSA. Hence it is crucial to determine both the ammonium and organic nitrogen in wastewater.

☐ Method

- Ammonia is quantitatively separated from other forms of nitrogen in wastewater through sample distillation under alkaline conditions.
- The concentration of the ammonia in the wastewater is determined photometrically on addition of Nessler's reagent.
- The Merck SQ 118 photometer is used and the Spectroquant analysis method 14739 for the concentration range 0.01 - 2.0 mg/L NH_4-N.

APPENDIX 11

DETERMINATION OF NITRATE (NO_3-)

☐ Overview

Nitrate is the oxidized form of nitrogen. It is generally common in industrial wastewater rather than domestic sources. Industrial wastewater may contain appreciable amount of nitrates that result from the oxidation of the total nitrogen in wastewater due to chemical processes and harsh environmental conditions and other industrial and manufacturing processes. Since it is also a known polluting agent, it is important to test for nitrates in industrial wastewater's.

☐ Method

- A 1.5 mL volume of effluent sample is placed into a cell.
- Predetermined amounts of reagent A and a strong oxidizing acid is added to the mixture.
- The nitrate concentration of the wastewater is determined from the resultant coloured solution and measured against a blank using the Merck SQ 118 photometer.
- Spectroquant analysis method 14542 was used for the concentrations 2.0-80.0 mg/L NO_3^-.

APPENDIX 12

DETERMINATION OF MIXED LIQUOR SUSPENDED SOLIDS (MLSS) AND VOLATILE SUSPENDED SOLIDS (VSS)

☐ Overview

The MLSS is defined as the total amount of organic and mineral suspended solids present in the mixed liquor of the activated sludge reactor. The procedure of MLSS determination is according to Standard Methods (1989) (Method 2540 D &2540 E) and serves as a rapid indication of sludge biomass concentration. The organic portion of the MLSS is represented by mixed liquor volatile suspended solids which is comprised of non- microbial organic matter as well as dead and live microorganisms and cellular debris. MLVSS values are usually reported as a percentage of the MLSS and for the current investigation as a ratio of the MLSS.

☐ Method

Determination of MLSS

- Pipette 100 mL of wastewater sample into a centrifuge tube and centrifuge at 3000 rpm for 6 min.
- Discard the supernatant and transfer the sludge pellet into a pre-weighed crucible.
- Place crucible into a drying oven at 105^{0} C and leave overnight. Remove crucible and allow to cool in desiccator. Re-weigh crucible. MLSS is determined according to the following calculation :

$$\mathbf{MLSS\ (g/L) = \frac{mass\ of\ (crucible + sludge) - mass\ of\ (crucible)}{100\ mL} \times 10}$$

Determination of VSS

- Place pre-weighed crucible containing sludge from the MLSS determination into a muffle furnace.
- Allow the sludge to ash at 550^0C for 1 h.
- Remove the crucible and allow to cool in the desiccator and re-weigh.
- The VSS id generally expressed as a percentage of the MLSS and is determined according to the following calculation :

$$\text{VSS (\%)} = \frac{\text{mass of (crucible + sludge) - mass of (crucible + ash)}}{\text{mass of (crucible + sludge) - mass of (crucible)}} \times 100$$

* For the current research, the VSS component of the MLSS was expressed as a ratio.

APPENDIX 13

DETERMINATION OF SLUDGE VOLUME INDEX (SVI) AND DILUTED SVI (DSVI)

☐ Overview

The SVI and DSVI is used to monitor the settling characteristics of the mixed liquor in activated sludge treatment processes and determine if the sludge is bulking. The procedure involves settling a sludge sample (SVI) or diluted sludge sample (DSVI) in a measuring cylinder for a specific time period and recording the volume it occupies, in correlation with MLSS analysis. The DSVI is generally performed when the SVI sedimentation exceeds 200 mL/L.

☐ Method

- The method for SVI and DSVI require the same protocol and according to Standard Methods (1989) (Method 2710 D).
- Four 1 L measuring cylinder are required for the test. Fill the container with mixed liquor from the aerobic reactor (1or 2) and do not allow the sludge to settle. Fill the following volumes in the measuring cylinder :
  - * cylinder 1 --- fill to 1 000 mL mark
  - * cylinder 2 --- fill to 500 mL mark
  - * cylinder 3 --- fill to 250 mL mark
  - * cylinder 4 --- fill to 125 mL mark

- Cylinders 2,3 and 4 are then filled to the 1 L mark using clarified final effluent.
- The four vessels are then inverted 3 times to homogenise the sludge mixture.
- The cylinder are then placed on a flat surface and settling is allowed for 30 min.
- Subsequently, the volume occupied by the sludge in each cylinder is recorded.

Determination of SVI/DSVI

Cylinder 1 i.e., the cylinder containing only mixed liquor is used to calculate the SVI, defined by the following equation :

$$\text{SVI (mL/g)} = V_{30} / X$$

where V_{30} = Volume of settled sludge after 30 min of sedimentation
X = Concentration of activated sludge (g/L)

The DSVI is calculated from cylinder 2,3 or 4 which has a sludge volume of less than 200 mL after settling and is defined by the following equation :

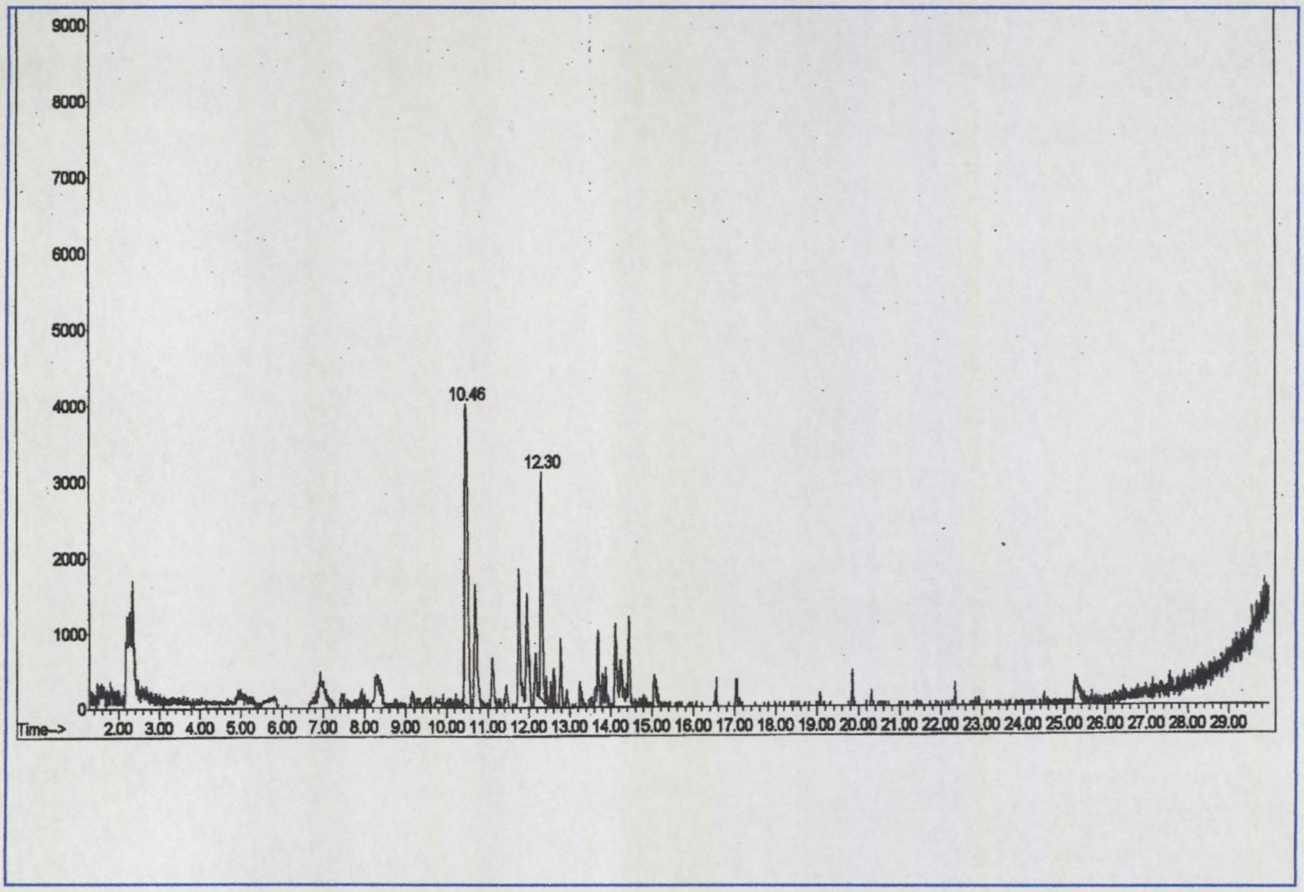
$$\text{DSVI} = \text{SV30/adjusted X}$$

where V30 = Volume of settled sludge after 30 min sedimentation
Adjusted X = Factor by which to divide the MLSS (see table below)

Cylinder number	**Dilution**	**Factor to divide MLSS for adjusted MLSS calculation**
1	Nil	1
2	50 %	2
3	25 %	4
4	12.5 %	8

APPENDIX 14

GCMS CHROMATOGRAM OF FINAL EFFLUENT (SAMPLE- 23 MAY 2001)



APPENDIX 15

CELL FIXATION WITH 4% PARAFORMALDEHYDE (Amann, 1995)

☐ Prepare the following solutions:

- 1 x phosphate buffered saline (PBS)
  - 130 mM sodium chloride.
  - 10 mM sodium phosphate buffer.
  - pH 7.2.
- 3 x PBS
  - 390 mM NaCl.
  - 30 mM sodium phosphate buffer.
  - pH 7.2.
- 4% paraformaldehyde (Merck, Germany) in PBS.
- 50%, 80%, 98% ethanol (Merck).

☐ Method

Preparation of 4% paraformaldehyde fixative:

- Heat 65 mL of ddH$_2$O to 60 °C.
- Add 4g of paraformaldehyde.
- Add one drop of 2M NaOH solution and stir rapidly until the solution has nearly clarified.
- Remove from heat source and add 33 ml of 3 x PBS.
- Adjust pH to 7.2 with HCl.
- Filter solution through a 0.2 μm filter.
- Quickly cool down to 4 °C and store in the refrigerator or on ice.

Paraformaldehyde fixation of cells:

- Add three volumes of paraformaldehyde fixative to one volume of sample and hold for 1 h at 4 °C.
- Pellet fixed cells by centrifugation (5000 x G) and remove fixative.
- Wash cells in 1x PBS and then resuspend in 1 x PBS to 50% of original sample volume.
- Add one volume of ice-cold ethanol and mix.
- Fixed cells may now be spotted on glass slides or may be stored (-20 °C) for several months.

APPENDIX 16

SONICATION OF ACTIVATED SLUDGE FOR CELL DISPERSION

☐ Overview

The activated sludge floc structure can sometimes be highly compact. In order to obtain accurate cell counts, it is often necessary to disperse the cells by sonication (Virsonic 100, Virtis, USA) at low frequency, after fixation (Mudaly, 2001).

☐ Method

- Place 1 mL of fixed activated sludge in a 2 mL micro test tube.
- Immerse the sonication probe into the micro test tube at least ¾ of the total volume.
- Cover the top of the tube surrounding the probe with parafilm.
- Sonicate at 5-10 watts for 10 min for an MLSS of 2500-3000 mg/L.

APPENDIX 17

IMMOBILIZATION OF FIXED CELLS ON MICROSCOPE SLIDES

Note: Eight well, teflon coated, diagnostic microscope slides (Merck, Germany) are routinely used.

☐ Method

Pretreatment of microscope slides (Sigma diagnostics; procedure no. P8920):

- Clean the slide surface by wiping with 70% ethanol and air dry.
- Place clean slides in diluted (1:10) Poly-L-Lysine solution (Sigma diagnostics, USA) for 10 min at room temperature (18-26 °C).
- Drain slides and dry in a 60 °C oven for 1 h or at room temperature overnight.

Immobilization of fixed cells on pre-treated microscope slides (Amann, 1995):

- Spread 10 μL of fixed cell suspension on the Poly-L-lysinated slide over a single well.
- Allow the smear to air dry.
- Dehydrate the cells by successive passages through 50, 80 and 98% ethanol washes (3 min each).
- Slides can be stored dry at room temperature indefinitely.

APPENDIX 18

TOTAL CELL COUNTS BY MEMBRANE FILTRATION AND STAINING WITH DAPI

☐ Method

Membrane filtration and DAPI staining (Porter and Feig, 1980):

- Counterstain cellulose acetate filters (pore size, 0.22 μm, Micron separations Inc.) with Sudan Black solution for 12 h.
- Add 900 μL of PBS (1x) to 10 μL activated sludge in a 2 mL micro-test tube.
- Add 100 μL of the non-ionic detergent (Igepal CA-30, Sigma Chemicals) and mix.
- Add 1 mL of DAPI (0.5 μg/mL) to 1 mL of the activated sludge mixture.
- Allow staining to proceed for ten minutes.
- Place the stained cellulose acetate filter and a 0.45 μm, backing filter at the base of a 15 mL filter tower and wet with sterile double distilled water.
- Quantitatively transfer the stained activated sludge mixture to the filter tower under a slight vacuum.
- After filtration, remove excess DAPI stain by washing the filter in the filtering device with sterile double distilled water.
- Mount the stained cellulose filter on one drop of glycerol:PBS mixture (95:5v/v) on a glass slide.
- Add one drop of an anti-fading mounting medium (VECTASHEILD®, Vector Laboratories, California) to the mounted filter surface before placing the cover slip.

- DAPI fluorescence was detected with a Zeiss Axiolab microscope (Zeiss, Germany) fitted for epifluorescence microscopy with a 50 W high-pressure mercury bulb and Zeiss filter set 01.

Determination of total cell counts:

- View the slide with the mounte dfilter under an epifluorescence (1000x magnification) microscope using an appropriate filter set for DAPI fluorescence (e.g., Zeiss filter set 01).
- Select twenty random fields for cell counts using the image analysis software (MACRO given in APPENDIX 5).
- Determine the mean total cell count using the following equation:

$$\mathbf{TCC = MTCC \times DF \times MF}$$

Where: TCC = Total cell count

MTCC = Mean total cell count for twenty fields

DF = Dilution Factor

MF = total number of Microscopic Fields on filter (55703 under 1000x magnification)

APPENDIX 19

IMAGE ANALYSIS MACRO FOR CELL ENUMERATION (Zeiss, KS300)

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Revisions: 6 March 1999 by Kurisu F (University of Tokyo)

4 May 1999 by Mudaly DD (Technikon Natal)

1 February 2001 by Degenaar AP (Durban Institute of Technology)

```
1. Imgdelete "*"

2. Gclear 0

3. !Scalint 2,3,59,255,0,255

4. median 3,4,3

5. !disdyn 4,5,11,-14,1

6. binscrap 5,6,0,10,0

7. binscrap 6,6,500,10000,0

8. Msmeasmask 6,1,"fitcarea", 1,2,10

9, !Msdrawmask 6,1

10. Imgdisplay 1

11. datalist "fitcarea",0,0
```

APPENDIX 20

EXTRACTION OF DNA

☐ Method

Phenol extraction of DNA (Roe *et al.*, 1996)

- A 1 mL volume of activated sludge sample is centrifuged at 12 000 rpm for 4 min at 4 C.
- The pellet is resuspended in 1 mL of TE-saturated phenol and vortexed for 15-30 seconds.
- The resuspended mixture is centrifuged at 12 000 rpm for 5 min.
- The uppermost aqueous layer is removed and placed into a clean tube.
- Equal volumes of TE-saturated phenol at a ratio of 1:1 is added to the extracted aqueous layer.

Ethanol precipitation of DNA

- At least 2.5 to 3 volumes of ethanol acetate is added to the extracted aqueous layer comprising the DNA.
- The tubes are repeatedly inverted to mix the contents and placed on ice for 10 min.
- This is followed by centrifugation at 12 000 rpm for 15 min at 4^0C.
- The supernatant is decanted and the tubes carefully drained on a paper towel.
- A 70% ethanol solution (corresponding to ~2 volumes of the original sample) is added.
- The mixture is then allowed to stand at room temperature for 5-10 min.
- The tubes are re-centrifuged at 12 000 rpm for 15 min at 4^0C.
- The supernatant is decanted and the tubes carefully drained.
- The pellet is then air dried for 5-10 min and resuspended in sterile distilled water.
- The sample containing the DNA material is stored at -4^0C until required.

APPENDIX 21

FORMAT FOR FILAMENTOUS ORGANISM IDENTIFICATION (Jenkins *et al.*, 1986)

No.______________ Sample____

COMMENTS:

OBSERVATION OF:

Protozoa:

Metazoa:

WET MOUNT OBSERVATION, 1000X, PHASE CONTRAST:

FILAMENT #	A	B	C	D
BRANCHING				
MOTILITY				
FILAMENT SHAPE				
COLOR				
LOCATION				
ATTACHED UNICELLS				
SHEATH				
CROSSWALLS				
FILAMENT DIAMETER				
LENGTH				
CELL SHAPE				
SIZE				
SULFUR DEPOSITS				
OTHER DEPOSITS				
COMMONNESS				
RANK				
STAINS, 1000X				
GRAM				
NEISSER				
I.D.				