MICROBIAL COMMUNITY ANALYSIS OF A LABORATORY-SCALE BIOLOGICAL PROCESS FOR THE TREATMENT OF VEGETABLE OIL EFFLUENT

Dissertation submitted in fulfilment with the requirements for the Masters Degree in Technology in the Department of Biotechnology and Food Technology, Durban University of Technology

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I hereby declare that this thesis represents my own work, unless stated to the contrary in the text, and that it has not been submitted in part, or in whole to any other Technikon/University

_____ _____

A. P. Degenaar Date

I hereby approve the final submission of the following dissertation for examination

_____ _____

Supervisor Date
Prof. F. Bux
(D.Tech: Biotechnology, DUT)

DEDICATION

This work is dedicated to my late mother... Dianna Anthea Degenaar for providing me with an aptitude for science.

ABSTRACT

Untreated vegetable oil effluents (VOEs) are known for creating shock-loading problems for the receiving wastewater treatment installations, resulting in poor quality final effluents being produced which do not satisfy municipal discharge standards. Onsite activated sludge treatment as an alternative has not been fully investigated. Hence, in this investigation biological treatment using the activated sludge process was chosen as the method for the treatment of VOE. The effect of VOE on measured process parameters was also determined. Novel molecular techniques such as fluorescent *in situ* hybridisation (FISH) and dot-blot hybridization have become powerful tools for the analysis of complex microbial communities that exist within activated sludge. The aim of this investigation was to evaluate biological treatment, optimize and apply FISH and dot-blot hybridization in order to analyze the microbial community implicated the biological treatment of VOE using probes EUBmix, ALF1b, BET42a, GAM42a and HGC69a. A laboratory-scale modified Ludzack-Ettinger (MLE) process setup and fed VOE with a COD (chemical oxygen demand) of ± 1000 mg/L. Daily monitoring of the process involved COD and TKN (total kjeldahl nitrogen) analysis of the influent and effluent as well as direct OUR (oxygen utilization rate) measurement and monitoring of the MLVSS (mixed liquor volatile suspended solids) concentration of the aerobic mixed liquor. The process exhibited overall COD and TKN removal capacities of 84% and 90% respectively. The aerobic mixed liquor had an OUR of 19 mgO/L.h and an average MLVSS concentration of 3000 mg/L. FISH results revealed that 72% of cells stained with 4', 6-diamidino-2-phenylindole (DAPI) within the aerobic mixed liquor bound to probe EUBmix, indicating a substantial Bacterial population within the laboratory-scale biological process. The alpha-*Proteobacteria* was identified as the dominant bacterial community comprising 31% of Bacterial cells, followed by the beta-*Proteobacteria* (17% of EUBmix), gamma-*Proteobacteria* (8% of EUBmix) and *Actinobacteria* (4% of EUBmix). Results of dot-blot hybridization were in agreement with FISH

results reiterating dominance of the alpha-*Proteobacteria*. This indicated that the class alpha-*Proteobacteria* could play a primary role in the biological degradation of VOE. This research will therefore aid in process design and retrofitting of biological processes treating VOE.

PREFACE

Aspects of the work covered in this thesis can be found in the following publications/proceedings:

Peer Refereed Articles

Degenaar, A. P., Ismail, A. & Bux, F. (2008). Comparative evaluation of the microbial community in biological processes treating industrial and domestic wastewaters *Journal of Applied Microbiology* **104** 353-363.

Conference Proceedings

Degenaar, A. P. & Bux, F. (2008). Microbial community analysis of a biological process treating edible oil effluent using flouresent *in situ* hybridization. Oral presentation. In *WISA 2008 - The Confluence of the Water Industry*. Sun City, South Africa. Detailed paper on CD.

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AOB	=	ammonia oxidizing bacteria
A-recycle	=	aerobic recycle
BCIP	=	5-bromo-4-chloro-3-indoylphospate
BOD	=	biological oxygen demand
BOD_5	=	5-day biological oxygen demand
BNR	=	biological nutrient removal
B_x	=	organic load rate
CFB	=	cytophaga-flavobacterium-bacteriodes
cfu	=	colony forming unit
COD	=	chemical oxygen demand
CSLM	=	confocal laser scanning microscopy
DAF	=	dissolved air flotation
DAPI	=	4’,6-diamidino-2-phenylindole
DIG	=	digoxigenin
DO	=	dissolved oxygen
DNA	=	deoxynucleic acid
DWAF	=	Department of Water Affairs and Forestry
EBPR	=	enhanced biological phosphorous removal
ECP	=	extracellular polysaccharide
EDTA	=	ethylenediaminetetraacetic acid
EUB	=	eubacteria
FA	=	formamide
FFA	=	free fatty acid
FISH	=	fluorescent *in situ* hybridization
FLUOS	=	5(6)-carboxyfluorescein-N-hydroxy succinimide
F/M ratio	=	food to microorganism ratio
FOG	=	fats, oils and greases
MLE	=	Modified Ludzack-Ettinger
MLSS	=	mixed liquor suspended solids
MLVSS	=	mixed liquor volatile suspended solids

MPN	=	most probable number
N	=	nitrogen
NBT	=	nitroblue tetrazolium
NOB	=	nitrite oxidizing bacteria
OME	=	olive mill effluent
OUR	=	oxygen utilization rate
P	=	phosphorous
PBS	=	phosphate buffered saline
PPK	=	polyphosphate kinase
Q_i	=	influent flow rate
rRNA	=	ribosomal ribonucleic acid
R_s	=	sludge age
RBOM	=	readily biodegradable organic matter
SBCOD	=	slowly biodegradable COD
S-recycle	=	sludge recycle
SWI	=	specific water intake
TKN	=	total Kjeldahl nitrogen
TP	=	total phosphorous
TRITC	=	tetramethylrhodamine-5-isothiocyanate
TSS	=	total suspended solids
UASB	=	upflow anaerobic sludge bed
VOE	=	vegetable oil effluent
VSS	=	volatile suspended solids
WWTP	=	wastewater treatment plants

CHAPTER 1
General Introduction

1.1 Background and motivation

Eutrophication is a natural process that is greatly aggravated by the action of man in the natural environment. The deterioration of South Africa's natural water resources results directly or indirectly from the discharge of industrial effluents rich in the nutrients nitrogen and phosphorous. Vegetable oil refineries in South Africa generally discharge poor quality effluent, causing a potential threat to natural water resources and wastewater treatment installations.

The vegetable oil industry has been identified as being amongst the 75 industrial groupings in South Africa. In total, there are 16 vegetable oil-processing plants, run by 10 separate groups. These industries refine and process approximately 300 000 tons of crude vegetable oil per year. This amount increases annually by about 3% (Steffen *et al.,* 1989). However, more recent information on the status of the industry has not been made available.

Vegetable oil effluent entering the sewer system consists mainly of fats, oils, greases (FOG), sulphate and phosphates resulting in both high inorganic and organic loadings for the receiving wastewater works. Often effluents from vegetable oil industries entering wastewater systems have been pretreated to remove most FOG, however due to their natural triglyceride structure not all FOG is removed. In this regard it is noted that previous studies have shown that fatty material within waste streams from food industries are readily biodegradable and therefore, it follows that these effluents are amendable to biological treatment (Eroglu *et al.*, 1990).

The biological treatment process design used in this study has been successfully applied to other research projects at the Centre for Water and

Wastewater Technology. The Modified Ludzack-Ettinger (MLE) process has been used in the treatment of both domestic and industrial effluents at pilot or laboratory-scale. Padayachee *et al*. (2006) used molecular techniques such as FISH and denaturing gradient gel electrophoresis (DGGE) to identify and evaluate the microbial community of a laboratory-scale MLE process treating domestic wastewater. Reddy *et al*. (2003) evaluated and determined the factors affecting the settleability of activated sludge in an MLE process also treating VOE.

1.2 The origins of vegetable oil effluent

The current investigation was limited to selecting a company within the local industry to serve as a case study. The process used to refine vegetable oil is common in the vegetable oil industry and therefore the wastewater generated could be regarded as similar in its chemical characteristics within the industry and a reflection of the broader industry in general. The industry that collaborated in this research was situated in Pietermaritzburg, in the Kwa Zulu-Natal midlands region. The name of the industry could not be revealed and will be referred to as Company X throughout this report. This company buys locally produced crude vegetable oil, and also imports crude vegetable oil to be refined on-site. Hence, the plant is mainly a refining factory; however, at the beginning of September 1999, milling was also introduced into the factory. Figure 1.1 illustrates the refinery process carried out at Company X. The main products that are produced include sunflower oil, soaps and candle wax. The factory also produces and sells acid oil, which is one of the by-products of soap production.

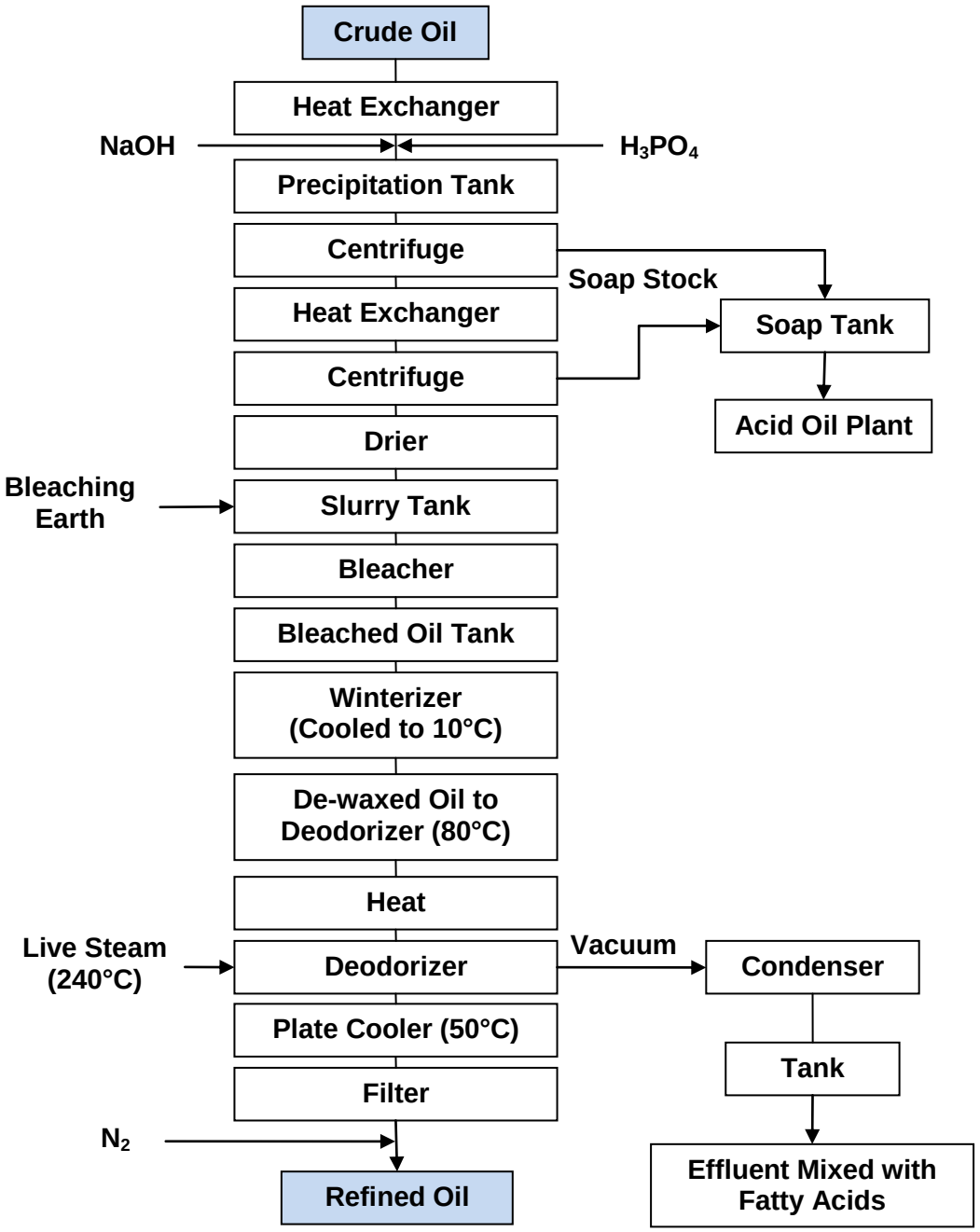


Figure 1.1 Schematic diagram of the refinery process employed at Company X

The factory is subdivided into four main plants located on the same premises. Three plants are mainly for production and the forth plant is an effluent

treatment plant. The three main production plants are: the refinery plant, which produces the refined vegetable oil; the acid oil plant, which produces soap stock and acid from fatty acids and sulphuric acid; and the soap plant which produces soap and candles from soap stock. All three production plants are responsible for the production of different kinds of effluents at variable quantities and strength as shown in Figure 1.2. The manufacture of VOE was the main focus of this research and hence the manufacturing process will only be mentioned.

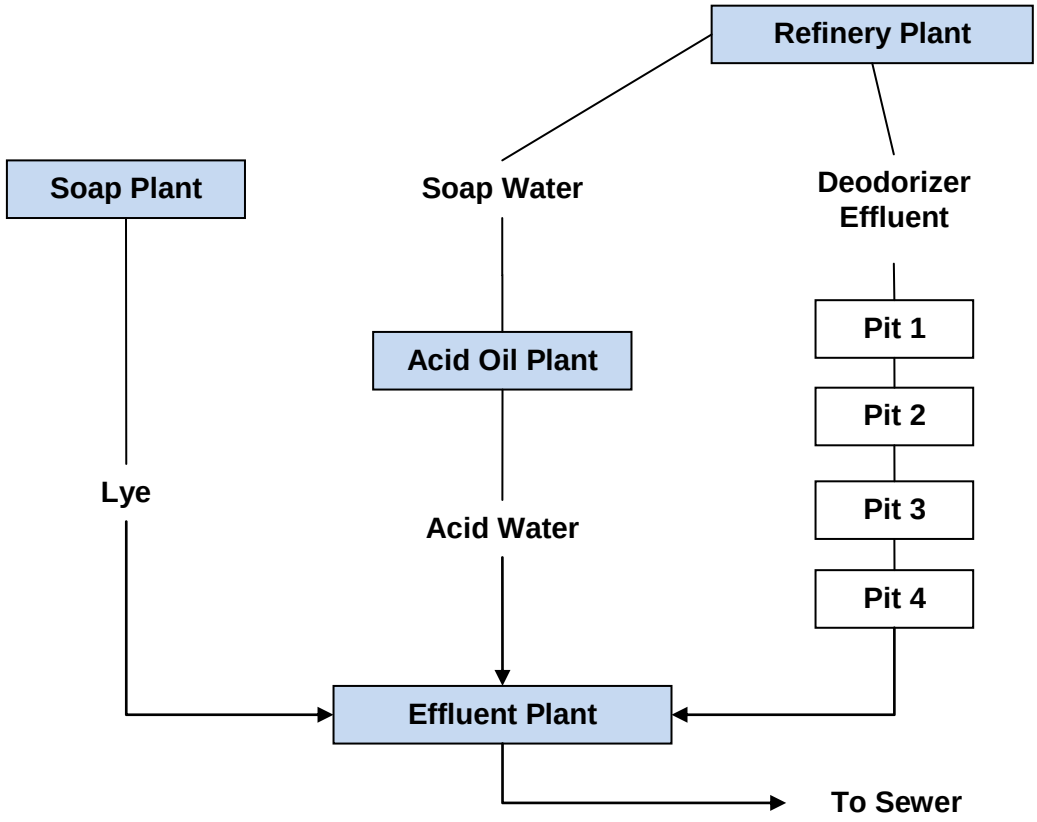


Figure 1.2 Schematic representation of the four plants at Company X and the effluents produced.

The volumes of effluent produced by each plant vary on a weekly basis depending on the refinery process employed as shown in Table 1.1. The

refinery produces 96 tons of effluent per day and the Soap plant produces 7 - 20 tons of lye per day. The Acid Oil plant produces 15 tons of acid water a day. To understand the overall quality and quantity of effluent produced by the factory, it is better to consider individually the unit operations of each plant, its main product and effluents. These will be described in more detail in the literature review.

Table 1.1 Typical effluent volumes produced by each plant per week at Company X

Day (24 hr)	Refinery Effluent (tons/day)	Lye (tons/day)	Acid Water (tons/day)
Monday	96	15	15
Tuesday	96	10	15
Wednesday	96	20	15
Thursday	96	7	15
Friday	Nil	Nil	15
Total	384	52	75

The processing of vegetable oil, both milling and refining depends on water availability. During vegetable oil processing, the industry consumes approximately 2 million cubic metres of water annually. A typical vegetable oil refining plant discharges about 40% of the incoming water to the sewer system and the remaining 60% is either vaporized in many cooling circuits, or else leaves the site in one of the secondary products or by-products. Hence, the Specific Water Intake (SWI) for the vegetable oil industry is very high compared to other industries in South Africa and must therefore be reduced. In a study conducted by Steffen *et al*. (1989), the SWI was found to range between 2.1 and 3.1 m^3/ton for milling and between 3.2 and 4.6 m^3/ton for refining. Based on these results, a target SWI of 2.0 m^3/ton for milling and 3.0 m^3/ton for refining was proposed. In addition to the proposed figure for each process, a further 5.0 m^3/ton SWI for a plant milling refining all products on site was proposed. It was then concluded that improved SWI could be achieved only by improving water management by the vegetable oil industry

(Steffen *et al.,* 1989). Another way of reducing the SWI is through incorporating the recycled water in the refining process

1.3 Chemical characterization of vegetable oil effluent

Before a wastewater treatment plant can be designed for biological nutrient removal (BNR), it is necessary to characterize the effluent quality and quantity. For this purpose, extensive monitoring of the effluent production is required, which includes the use of proper sampling techniques. Flow rates and water quality parameters often change sporadically, and these changes may affect the ability of a wastewater treatment plant to achieve consistent BNR.

Given the variable nature of the VOE, which results from the varying nature of the vegetable oil refining process and the necessity of attaining consistent BNR, it is necessary to collect samples that will represent "average" characteristics and approximate characteristics under more extreme conditions (Steffen *et al.,* 1989). According to Novotny (1998), a desirable sampling method is to collect 3 - 4 hour composite samples of wastewater. This would provide data that may be considered representative of average effluent characteristics throughout the day. Usually a careful review of flow monitoring records and reports generated by the effluent plant over the last couple of years, if present, tend to be helpful in assessing the periodic and seasonal characteristics of the wastewater throughout the year (Novotny, 1998).

Company X produced approximately 195 m^3 of effluent per day from the refinery process, during a 24 hour operational period. The effluent from the deodorization process was flocculated and used for the laboratory-scale biological treatment process. The characteristics of the (1) raw effluent, (2) pretreated effluent (3) prepared influent are shown in Table 1.2. The raw effluent was collected after the deodorization process at Company X. The pretreated effluent is the raw effluent that has been pretreated with

compound C40. The prepared effluent is the pretreated effluent that has been corrected after flocculation with the pH adjusted to ± 7.4, the COD diluted to ± 1000 mg/L and the C:N:P ratio maintained at 100:5:1 through nutrient supplementation. The prepared effluent will be used in the laboratory-scale biological treatment process. Due to the large number of analyses conducted per parameter, the results presented indicate the range i.e. highest and lowest values and the mean of all the analyses.

Table 1.2 Chemical characteristics of the raw effluent after deodorization at Company X and prepared effluent used in the biological treatment process

Parameter	(1) Raw Effluent		(2) Pretreated Effluent		(3) Prepared Influent	
	Range	Mean	Range	Mean	Range	Mean
pH	3.22 - 4.03	3.63	9.31 - 10.63	9.97	7.42 - 7.48	7.45
COD (mg/L)	5006 - 6790	5898	1117 - 1556	1337	918 - 1012	965
TKN (mg/L)	4.8 - 7.4	6.1	3.6 - 4.3	4.0	5.6 - 6.0	5.8

1.4 The need for pretreatment of vegetable oil effluent

Pretreatment methods are commonly used to treat wastewater in order to improve various conditions prior to biological treatment. The effluent produced from the deodorization process contains high levels of organics in the form of COD and FOGs. A trial run of the biological treatment process using raw untreated VOE conducted at the Centre for Water and Wastewater Technology showed that these concentrations caused shock loading of the mixed liquor in the laboratory-scale biological process, which prohibited it from functioning optimally with regard to COD and FOG removal. Hence the need to flocculate the raw VOE prior to biological treatment. This resulted in improved COD and TKN removal

The significance of this study was to elucidate the microbial populations present in a laboratory-scale process and understand the population

dynamics so that this information can assist in troubleshooting problems that may arrive in full-scale systems using biological treatment processes.

1.5 Aims and Objectives

The aim of this research was to evaluate biological treatment and to optimize and apply fluorescent *in situ* hybridization (FISH) and dot-blot hybridization in order to analyze the microbial community present in a laboratory-scale biological process treating VOE. The objectives were as follows:-

1. To setup and operate a laboratory-scale modified Ludzack-Ettinger process to treat VOE.
2. To determine steady-state conditions of the laboratory-scale process and achieve maximum process efficiency.
3. To analyze the microbial community present using FISH.
4. To analyze the microbial community using dot-blot hybridization and compare results obtained with FISH.

CHAPTER 2
Literature Review

2.1 The South African vegetable oil sector

The amount of vegetable oil that is produced by the South African vegetable oil sector depends very much on climatic conditions. Good rainfall patterns lead to large maize, groundnuts and sunflower crops, resulting in good seed production. However, drought has a negative impact on the industry, resulting in a decline in raw materials. To make up for the shortfall in local production, the balance of vegetable oil is imported, in crude form, to be refined in local refineries (Steffen *et al.,* 1989).

Vegetable oil production can be divided into two distinct stages: crude vegetable oil production in an oil mill; and crude vegetable oil processing conducted in a refinery. Thus the vegetable oil industry can be divided into two main groups based on the main production process being used. The milling industry produces crude vegetable oil from raw materials such as seed, and the refining industry purchases and refines crude vegetable oil into final products. In South Africa, two stages of processing are usually conducted on the same site, although marine oils and animal fats, where used are also purchased as such (Steffen *et al.,* 1989).

The principal product of vegetable oil refineries is liquid oil, which may be sold as cooking or salad oil, or may be further processed to increase the market value of the final product, for example, margarine, peanut butter and mayonnaise. Vegetable oil may be obtained from a large variety of monocotyledonous and dicotyledonous seeds. The most commonly grown oil bearing crops in South Africa are the sunflower, groundnuts and maize, although other seeds such as cotton and soya are also processed (Steffen *et al.,* 1989).

2.2 The vegetable oil refinery process and its effluents

The refining process is associated with the removal of phospholipids, colour bodies and other soluble impurities from the crude vegetable oil. The production of refined vegetable oil can be divided into two variable processes or stages viz., chemical refining and physical refining. Although the two stages are different from each other and produce different effluents, the products in both stages are similar in quality i.e. refined vegetable oil ready for commercialization. Chemical refining, also known as caustic refining, generally refers to the process designed to neutralize free fatty acids present in the crude vegetable oil by introduction of an alkali, followed by centrifugal separation of the heavy phase insoluble material. Physical refining refers to the process whereby the free fatty acids (FFAs) in the crude vegetable oil or degummed oils are removed by evaporation rather than by being neutralized and, subsequently removed as soapstock similar to the caustic refining process.

Degumming may be considered the first step in the refining process and is designed to remove the phosphatides that interfere with subsequent processing. Vegetable oils high in phosphorous such as soybean, corn and sunflower, may require degumming prior to the refining process. The primary reason for degumming is to either provide crude-degummed oil suitable for storage or long transit, to prepare crude vegetable oil for physical refining, or to produce lecithins. There are three main problems associated with the presence of gums in crude vegetable oil, which are:

- ✓ Phospholipids have excellent emulsifying abilities. Thus when discharged as soap stock, they introduce problems for oil-water separation in the acidulation process, thus resulting in increased product losses during chemical refining.
- ✓ Gums have the tendency to impart deep brown colouring to finished vegetable oils during the deodorizing stage as a result of the high temperature employed.

- ✓ They tend to form complex compounds with certain trace metals that adversely affect product stability.

Water degumming is effective only for water-hydratable phosphatides i.e. those having a greater affinity for the water phase rather than remaining in the oil phase. The addition of hot water and subsequent separation of the swollen globules by centrifugation, easily removes them. For non-hydratable gum, pretreatment of the vegetable oil with phosphoric acid or citric acid is required to render them hydratable. The hydratable gums are subsequently removed through the addition of small amounts of water followed by centrifugation. Many variations on this two stage process exist (Anderson, 1996a). The effluent produced during the degumming stage tends to contain quantities of phospholipids, inorganic phosphates (from phosphoric acid) and FOG.

2.2.1 Chemical refining

Caustic neutralization is the traditional first step for crude vegetable oil processing if degumming is not included as a pretreatment step. Caustic refining consists of five inter-related processes and each process produces its own unique effluent. The five processes are: (1) neutralization, (2) bleaching, (3) hydrogenation, (4) winterizing and (5) deodorizing. The advantage of caustic refining over the alternative method is its reduced sensitivity to the type of feedstock used (Anderson, 1996a).

2.2.1.1 Neutralization

Crude vegetable oil contains a percentage of FFAs. The FFAs or carboxylic acids are a product of natural degradation of triglycerides. Dilute caustic soda solution of up to 4 N strength is usually used for neutralization. The oil-alkali solution is thoroughly agitated to ensure intimate contact, normally using an inline high-shear mixer. Both the crude vegetable oil and caustic should be cooled to less than 38°C. Careful control of the operating conditions is required at this stage as the strong caustic soda used tends to saponify the

neutral triglyceride with the consequent loss of neutral oil (Anderson, 1996a). The neutralization process also helps with the removal of metals, particularly magnesium and calcium (Anderson, 1996a). Apart from saponification of free fatty acids in the crude vegetable oil, caustic addition tends to be more effective in hydration of gums than simple addition of hot water only. Thus, it is possible for crude vegetable oil containing low gum contents to be processed chemically without the need for an additional degumming pretreatment step (Anderson, 1996a).

The immiscible soap or soapstock, produced during neutralization, is separated from the neutralized vegetable oil using centrifuges or gravity settling, depending on whether the operation is continuous, semi-continuous or a batch process. Phosphoric acid may be added in the wash water to reduce the residual soap in the refined vegetable oil, and to provide a better split between the vegetable oil and aqueous phase. The soapstock is further treated in the acid plant (on-site) to produce acid oil (Steffen *et al.,* 1989; Anderson, 1996a).

Chemical refining with caustic soda gives rise to the most potent effluent generated at a vegetable oil processing plant. The resultant effluent stream is known as soapy water and contains quantities of FFAs, free oils, gums or phospholipids, sodium and phosphates (Eroglu *et al.*, 1990).

2.2.1.2 Bleaching

Bleaching is adsorptive process that is associated with the vegetable oil refining process. While the degumming operation is designed to remove phosphatides and caustic refining converts water soluble FFAs into soluble soaps, adsorptive bleaching provides the last practical opportunity to remove the remaining impurities, especially colour and phospholipids, to acceptable levels. The major colour pigments found in vegetable oil are chlorophyll (green) and carotenoids (orange) (Anderson, 1996a).

2.2.1.3 Dewaxing

Dewaxing which is sometimes called winterizing, refers to the removal of high melting point waxes extracted from certain seeds such as corn, sunflower and canola. The refined vegetable oil is cooled to approximately 5°C thus causing the high melting point esters and waxes to crystallize. These fat crystals are subsequently removed through filtering usually with assistance of diatomaceous earth as a filter media (Steffen *et al.,* 1989). The winterizing process is only necessary for vegetable oil products that are going to be marketed as such without any further processing. It is usually not necessary to winterize the vegetable oil that is to be, further hydrogenated. This stage of vegetable oil refining contributes little if any to the final effluent stream both qualitatively and quantitatively (Steffen *et al.,* 1989).

2.2.1.4 Deodorizing

This is typically the last step in the vegetable oil refining process. This step is included in almost all refining operations regardless of other unit operations used. The deodorization process is intended to remove the relatively volatile odoriferous compounds from the refined vegetable oil. This process involves steam distillation, under vacuum, which results in the removal of residual FFAs, aldehydes and ketones that are responsible for the unacceptable odours and flavours in the final refined vegetable oil. Removal of pigments is through thermal decomposition. These decomposition products, from the pigments, are subsequently distilled off from the final vegetable oil product. After the deodorization process has been completed, the refined vegetable oil is cooled in the lower tanks before being pumped for storage. Small quantities of citric acid may be added during the cooling stage as an anti-oxidant to prevent oxidation of the cooled vegetable oil (Steffen *et al.,* 1989).

Deodorization is the second greatest effluent generating stage following the neutralization process. Most of the effluent is produced during this stage consists of the distillate from the vegetable oil and contains volatile compounds responsible for the vegetable oil's characteristic odour, as well as

any remaining FFA's. A large quantity of water is also used during this stage for cooling purpose, which increases the SWI of the plant. Some of the stripping steam and the remaining FFA water vapours are mixed with the cooling water. After this has been re-circulated over cooling towers, it is then discharged down the drain to join the main effluent stream (Steffen *et al.,* 1989).

2.2.2 Physical Refining

Physical refining refers to the process whereby the FFAs in the crude vegetable oil or degummed vegetable oils are removed by evaporation rather than by being neutralized and, subsequently, removed as soapstock similar to the alkali refining process. The physical refining technique has two main advantages compared with the conventional caustic refining route; reduction in vegetable oil losses and the elimination of soapstock and its associated treatment problems (Anderson, 1996b).

The physical refining process, however, requires that the feedstock or crude vegetable oil be rigorously pretreated to ensure it is free from phosphatides, impurities, trace metals and earth-removable pigments. If these impurities were allowed to remain in the vegetable oil, the high temperatures used during the processes would darken the vegetable oil and result in a poor quality product. The extent of pretreatment necessary depends on the particular vegetable oil type and its quality. Pretreatment of high fatty acid containing vegetable oils such as maize and sunflower, prior to physical refining, may comprise the addition of phosphoric acid or citric acid at temperatures of approximately 95°C followed by high-speed centrifugation to remove the hydrated gums. The centrifuged vegetable oil is then dried, bleached and winterized before being sent for physical refining. Both continuous and semi-continuous units may be used in the physical refining process. Semi-continuous units involve pumping the vegetable oil into a vacuum de-aerator before entering the top heating section of the stripper. While in the stripper the oil heated to 270°C using thermal fluid from a

vaporizer (Steffen *et al.,* 1989). In a continuous unit physical refining is achieved by allowing the vegetable oil to flow over the top of a series of trays counter current to the up flow of stripping steam which is injected below the bottom of the tray. The refined vegetable oil is piped to a holding tank which provides the retention capacity for heat blanching before a final series of trays enables steam stripping of all remaining odoriferous material (Steffen *et al.,* 1989). The effluent streams resulting from physical refining are similar in quality to those produced during the degumming and deodorizing stages of caustic refining (Anderson, 1996b).

2.3 The acid oil plant and its effluents

The main product of the acid oil plant is the acid oil, which is produced from feedstock commonly known as soapstock. Soapstock is the by-product of crude vegetable oil neutralization with caustic soda during the chemical refining process. The resulting oil and water phase have very high concentrations of FOGs, total suspended solids (TSS), biological oxygen demand (BOD), COD loads, as well as, glycerine and FFAs. Due to these high concentrations, most processors include acidulation as part of the integrated facility.

Acidulation is one of the least desirable processes in the integrated facility. The process is rather difficult to perform effectively and it is generally the most cost ineffective process since it has no significant financial returns. The acidulation system is based on gravity separation that can be performed in either a continuous or a batch operation. The process involves collecting soapstock into an equalization or holding tank. The mixture is heated and then treated with sulphuric acid at a controlled pH of about 2 to 2.2 units. After the reaction with the acid, the mixture enters a series of holding and settling tanks where the oil and the aqueous phases separate. Acid oil is skimmed off the top surface, and then dried. The acid oil may be sold, as is, or may be passed through an evaporative heat exchanger to remove excess

water. The product is then sold as a feed supplement or may be used as a feedstock for soap and other industrial applications (Anderson, 1996a).

The acid water effluent, produced during acidulation, may be neutralized with lime or other alkaline materials prior to its discharge to the main effluent stream. The effluent in this stream is heavily polluted with high concentrations of COD, BOD, TSS and sulphates. Most of the contaminants in this stream by far exceed the municipal discharge standards. Table 2.1 provides the wastewater discharge limit values applicable in terms of the National Water Act of 1998 (Section 39).

Table 2.1 Wastewater general limit values applicable to discharge of wastewater into a water resource (The National Water Act, 1998).

Substance/Parameter	General Limit	Special Limit
Faecal coliforms (cfu's per 100 mL)	1000	0
Chemical Oxygen Demand (mg/L)	75	30
pH	5.5 - 9.5	5.5 - 7.5
Ammonia (ionized and un-ionized) 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)	70 - 150	50 - 100
Ortho-phosphate as phosphorous (mg/L)	10	1 (median) and 2.5 (maximum)
Fluoride (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)	0.001	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

The National Water Act is administered by the Department of Water Affairs and Forestry (DWAF) stipulates that discharging of domestic and industrial wastewater up to 2000 m^3 into water resources on any given day into a water resource is allowed provided that:

- ✓ 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°C.
- ✓ The discharge is not a complex industrial wastewater arising from industrial activities containing a complex mixture of substances that are difficult to chemically characterize and quantify, or one or more, 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 (The National Water Act, 1998).

2.4 The soap plant and its effluents

The main product of the soap plant is bar soap. No powdered soap is produced or manufactured on-site. The soap manufacturing process may be performed as either a batch or continuous operation. The acid oil, which contains fatty acids from the acidulation phase, is neutralized using strong caustic hydroxide solution. The FFAs, which result from the acid oil, react with excess sodium hydroxide to form sodium salts which precipitate out of solution. After centrifugation the soap is moulded into desired shapes before being sold (Anderson, 1996a).

The effluent stream produced in the plant is generally known as lye water which usually contains quantities of vegetable oils, FFAs, sulphates, and some sodium salts of FFAs. This stream is usually combined with acid oil water prior to its discharge to the final effluent stream.

2.5 Wash-down miscellaneous effluents

Wash-down effluents are those effluents that originate from regular cleaning of the vegetable oil factory. This cleaning is generally performed after a week's production or between changes of feedstock. Batch equipment may be cleaned after each batch has been processed. The cleaning of vessels is usually performed using live steam and hot water, to ensure maximum use of steam and the reduction in effluent volumes. Floor cleaning is conducted using hot water as necessary, which improves hygiene and reduces manual labour. The fats and oil bearing effluent stream resulting from cleaning is discharged to the main effluent stream via fat traps. The oil rich scum from the fat traps is recovered and recycled for reworking through the effluent treatment plant (Anderson, 1996a).

2.6 On-site effluent treatment employed at Company X

The main effluent streams at Company X that are generated from the four main plants (Figure 1.2) are channelled to the on-site effluent treatment plant. This plant is dedicated for treatment or pretreatment of incoming effluent prior to its discharge to the municipal sewer system. The three main effluents are the refinery effluent from the refinery and packaging plants; the acidic effluent from the acid oil plant; and lye from the soap plant. The effluent plant comprises two large holding tanks, pH correction tank, dissolved air floatation (DAF) unit and two settling tanks that are operated in series (Figure 2.1). Acidic effluents i.e. lye and acid water, are pumped and mixed together in the first holding tank. This effluent has a low pH of between 1 and 2 units and is highly corrosive to concrete because of its sulphate content. On the other hand the refinery effluent has a high pH ranging between 12 and 13 units. This effluent is pumped into a secondary holding tank. There are two methods of effluent treatment that are currently being used at the plant. The two effluent treatment methods are pH correction and physical separation of oil and grease using DAF. After DAF treatment, lime and/or ferric chloride are added to the effluent, which is followed by settling in the two settling tanks in

order to remove precipitated particles. The resultant effluent is discharged to the sewer system to be treated by Darvill Wastewater Works.

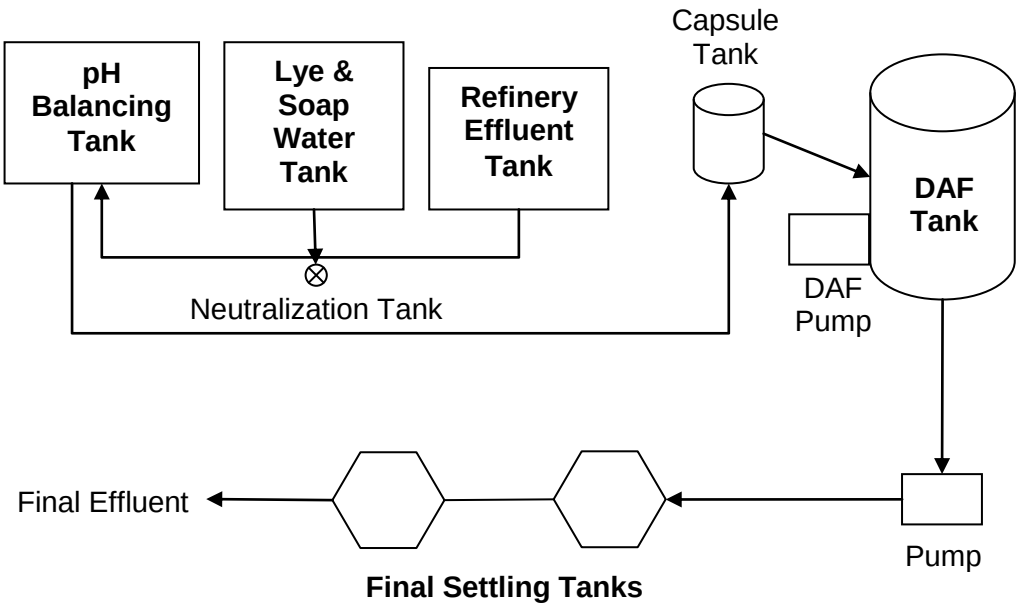


Figure 2.1 Schematic diagram of the effluent treatment plant at Company X in Pietermaritzburg.

2.6.1 pH correction

The effluents from the two holding tanks are mixed together into a single effluent stream. Due to pH differences between the two effluents, the two streams are mixed at different volume ratios by controlling the flow rates from the two tanks such that the desired final pH of between 5.5 and 8.5 units is achieved in the final combined effluent stream. This effluent is finally directed to the acid equalization tank for final pH adjustment. The effluent at this stage may be dosed with a polyelectrolyte such as lime or ferric chloride to coagulate fats and to precipitate phosphates. This effluent is now ready for DAF treatment.

2.6.2 Dissolved air floatation (DAF)

The DAF process is a separation technique that employs the production of micro size (10 - 100 µm diameter) air bubbles to enhance the floatation of oils and suspended materials not removed by gravity separation. The DAF system at Company X consists of three main unit processes, which are the pressurization system in the capsule, the floatation tank and the recycle system. In the capsule the effluent bubbles are formed by pressurizing all or part of the waste flow and introducing air into the pressurized cell. Subsequent depressurization in the floatation tank allows bubbles to form which adhere to any oil, fat or suspended material in the effluent bringing it to the surface (Boyer, 1996). The layer of frothy solids containing fats and oils formed on the surface is swept off into an inclined exit chute by a rotating arm or similar device (Boyer, 1996).

2.7 The effluent problem and its legislation

Only a single factory out of the estimated 16 vegetable oil factories in South Africa is treating its effluent completely using biochemical means. The remainder of the vegetable oil plants generally use DAF for physical removal of FOG followed by pH correction (Steffen *et al.,* 1989). Even after application of these treatment methods, the remaining emulsified grease tends to clog sewer pipes and pumps. The high organic (BOD and COD) and phosphorous loads create shock-loading problems for the receiving wastewater installations (Eroglu *et al.*, 1990).

At present the Department of Water Affairs and Forestry monitors water resources pollution from point sources by legislating that the effluents from industries must comply with the uniform discharge standards that are set at technologically attainable levels. These controls or regulations limit the rate of deterioration of receiving water bodies. The National Water Act, 1998 (Act 36 of 1998) is the controlling legislation for the control, development, utilization and protection of water resources in South Africa. The National Water Act, 1998 replaces the old water Act, 1956 (Act 54 of 1956), which

was motivated by the need for new legislation that would reflect democratic principles and equitable access to water resources by all symbolized by the slogan “Some for all, Forever” (Department of Water Affairs and Forestry, 1997). The Act provides for the compulsory purification of effluents by the user to specified standards and its subsequent disposal in a manner that will make it available for re-use.

The local authorities (the Pietermaritzburg-Msunduzi Transitional Local Council) and the municipal sewage plants (such as Darvill Wastewater Works) have increased pressure on industries to increase the efficacy of in-house effluent handling and treatment methods. Therefore industries are becoming more pro-active with regard to viable biological treatment methods which may be implemented on-site to supplement the existing physico-chemical effluent treatment methods.

2.7.1 The eutrophication problem

Eutrophication results from excessive nutrients in a lake or other body of water, usually caused by runoff of nutrients (animal waste, fertilizers, sewage) from the land, which causes a dense growth of plant life; the decomposition of the plants depletes the supply of oxygen, leading to the death of aquatic life. However, the natural eutrophication process is greatly accelerated by human activities increasing inputs of nutrients (Lilley *et al.*, 1997). The forms include nitrate, nitrite, ammonia, organic nitrogen (in the form of plant material or organic compounds), and phosphates (orthophosphate and others) (Cloern, 2001; Meuller & Helsel, 1996). Gross eutrophication takes place when inorganic soluble nitrogen and phosphorous levels in water approach concentrations of 0.3 mg/L and 0.015 mg/L respectively (Lilley *et al.*, 1997). When these conditions are met the presence of these nutrients stimulates the excessive growth of algae. This enhanced growth is often referred to an algal bloom. The consequences of this enhanced growth are reduced sunlight penetration of the water, reduced dissolved oxygen in the water, and decomposition of the algae which can

cause other aquatic organisms to die. Hypoxia is another consequence of eutrophication. Water with a low concentration of dissolved oxygen is termed hypoxic. In estuaries, lakes and dams, low oxygen usually means a concentration of less than 2 mg/L. Hypoxic waters do not have enough oxygen to support fish or other aquatic animals and is also caused by the presence of nutrients (Cloern, 2001; Meuller & Helsel, 1996)

Eutrophication in South Africa is further enhanced by prolonged storage times of dams and reservoirs, with the resultant accumulation of phosphate and nitrate together with higher summer temperatures algal growth is promoted. This eutrophication potential is further enhanced by a rapidly increasing population, industrial demands, and irrigation demands by the agricultural industry, irregular rainfall often leading to drought conditions and loss of source supply due to degradation of natural water resources (Joska & Bolton, 1994).

2.7.2 Sources of nutrients

Nutrients can come from many sources by human activities from both diffuse and point sources. Diffuse sources such as; fertilizers applied to agricultural fields; livestock; deposition of nitrogen from the atmosphere; erosion of soil containing nutrients are difficult to control as they are introduced in relatively small concentrations over wide areas (Lilley *et al.*, 1997). Nutrients introduced by point sources are easier to control since nutrients originate from residential or industrial areas are concentrated at a point by means of sewers, and treatment methods are readily available (Wentzel, 1992). In South Africa 80% to 90% of nutrients in water bodies originate from point sources with the vegetable oil industry being a major consumer and polluter of water (Steffen *et al.,* 1989). In anticipation of water shortages in South Africa the National Water Act (Act 36 of 1998) has made provisions for the compulsory treatments of effluents by industries (including municipal wastewater treatment works) to receiving water quality standards.

2.7.3 Nutrient elimination in wastewater treatment

Nitrogen compounds like ammonia and nitrate are the main components of fertilizers and wastewaters. Ammonia and nitrate are highly toxic to aquatic life and therefore their release into the environment has to be minimized. Ammonia concentrations of up to 0.2 mg/L ammonia has reported to have toxic effects on fish and invertebrates (Arthur *et al.*, 1987) while nitrate and nitrite has been found to be harmful to humans, especially young children (Scheider & Selenka, 1974). Nitrogenous compounds and phosphates resulting from sewage or industrial wastewaters contribute to the eutrophication of natural water bodies resulting in immeasurable ecological damage. Phosphates in natural water resources cause excessive growth of algae which is detrimental to the aquatic ecosystem. When algal blooms die in large numbers and decay a large pool of nutrients is released into the water body, which results in accelerated growth of other organisms. Consequently, the oxygen content of the water body is depleted and the lower water (hypolimnion) becomes anaerobic and eventually results in eutrophication significantly inhibiting other vertebrate animals in the water column (Lilley *et al.*, 1997).

The efficient elimination of nutrients is therefore one of the most important goals in modern wastewater treatment. Nitrogen removal takes place during a two phase process within biological wastewater treatment plants (Henze *et al.*, 1997). The first stage is known as nitrification and involves the transformation of ammonia to nitrate by ammonia- and nitrite-oxidizing bacteria under aerobic conditions. During the second stage nitrate is reduced to gaseous nitrogen, nitric and nitrous oxide under anaerobic conditions as is referred to as denitrification.

2.8 Conventional treatment of vegetable oil effluent

Food processing effluent including effluent from the vegetable oil processing industry is a complex mixture of settleable, suspended and dissolved materials. Complete treatment of these effluents requires a combination of

physical, chemical and biological treatment processes (Dalzel, 1994). The physical nature of fatty material is of great concern when considering any treatment method. The fatty contaminants in the VOE can be characterized in three ways viz. by polarity, biodegradability and physical characteristics. Polar contaminants are usually derived from animal and vegetable sources such as food industry operations including vegetable oil processing industry, whereas the non-polar contaminants are derived from petroleum and mineral sources and are generally non-readily biodegradable (Sutton *et al.*, 1994).

It used to be common practice to group effluents from the vegetable oil processing industry, which has polar and readily biodegradable fatty components, with effluents arising from the petrochemical industry, which is non-polar and non-biodegradable. As a result of this joint grouping of petrochemical effluents and the effluents from the food industry, the vegetable oil industry has been widely targeted as problematic (Grant, 1980).

2.8.1 Physical treatment

The effluent from vegetable oil processing plants carries an appreciable amount of fatty material or FOG. Prior to any form of treatment, it is desirable to install an oil/water separation system as the first phase. This will reduce the pollution load being discharged and also facilitate the recovery of re-usable fat. The separation of the water and oily phases from the VOE can be achieved using two simple methods, which are gravity settling and the DAF process.

2.8.1.1 Gravity or fat traps

The removal of fatty matter of FOG from effluent generated by the vegetable oil refining process has for many years been achieved through the use of fat traps. The gravity trap is usually installed as standard equipment on all process effluent streams and is the simplest form of physical treatment (Eroglu *et al.*, 1990). Fat traps are designed to produce a slow and gentle uniform flow through a tank, which allows density differences to bring the

fatty material to the surface without disturbing any accumulated scum and sludge. Fat removal is achieved by the use of surface scraping mechanisms (Grant, 1980; Dalzel 1994).

Fat traps are designed according the general settlement principle. According to Dalzel (1994) a typical fat trap has a length width ratio of 2:1, a retention time of 10 - 40 minutes and loading of 0.4 $m^3/m^2/h$ to 3 $m^3/m^2/h$ (volume of effluent to be treated per surface area of the fat trap per hour) at maximum flow rate. The problem with fat traps is that they are expensive and occupy a large surface area, which makes them unsuitable for many industrial applications, especially for small industrial applications (Grant, 1980; Dalzel 1994). The limitation of using gravity fat traps is that they are unable to reduce the emulsified fatty material content of wastewater to under 500 mg/L, the maximum concentration permitted to be discharged to municipal sewer systems (Eroglu *et al.*, 1990).

2.8.1.2 Dissolved air floatation (DAF)

A common problem with fat containing effluents is precipitation and emulsification due to pH, temperature, pumping and detergents. Under these conditions, gravity separation with a simple fat trap rarely gives satisfactory results and an alternative that is often used is assisted floatation, in this case DAF.

Sedimentation is the separation of solids from liquids under gravity. Floatation is the separation of solids from liquids by means of buoyancy. DAF systems are, therefore, suitable for the removal of substances such as oils that do not settle by gravity due to their low settling velocity (Kiely, 1997). There are three types of DAF systems: vacuum-floatation, micro-floatation and pressure-floatation. Pressure floatation is the most widely used (Letterman *et al.*, 1999).

In pressure floatation, air is dissolved in water under pressure of several atmospheres. The pressurized water then enters the floatation tank, containing the effluent or wastewater sample, by means of a pressure-release device. Different pressure-release devices may be used, such as: nozzles, needle valves or gate valves. In the pressure-release device, the pressure of the water is reduced to atmospheric pressure (Letterman *et al.*, 1999). The sudden reduction in pressure results in the dissolved air leaving the water as fine air bubbles (Quasim, 1994). The bubble size is approximately 10 - 100 µm in diameter (Letterman *et al.*, 1999). A pressure between 400 and 600 kPA (4 and 6 Bar) is recommended for the production of small bubbles (Edzwald, 1995).

Following pressurization and release of the water, fine air bubbles enter the floatation tank containing the wastewater. The floatation tank is divided into two zones: the contact zone and the separation zone. In the contact zone, there is opportunity for the contact between the fine air bubbles and the solid particles. This leads to the formation of “bubble-solid agglomerates” which are less dense than water. In the separation zone, the bubble-solid agglomerates, due to their lower density, rise to the surface of the floatation tank allowing for the separation of the solid particles from the wastewater (Letterman *et al.*, 1999).

The agglomerates that collect on the surface of the floatation tank are called “float” and may be removed either by flooding or by mechanical scraping. With flooding, the water level in the floatation tank is raised in order that the float and water overflow into a float collection trough. Mechanical scraping involves the movement of a scraper, with rubber blades, over the surface of the floatation tank, which pushes the float into a collection channel. The clarified wastewater can be removed from the bottom of the floatation tank (Letterman *et al.*, 1999).

2.8.1.3 Enhancement of DAF using coagulation and flocculation

The objective of coagulation is to promote the settling of solids that are suspended in the wastewater. Coagulation achieves this settling by utilizing chemical coagulants that promote agglomeration of the solids (Kiely, 1997).

Coagulation may be described as a destabilization process. This is because the chemical coagulant used alters the electrostatic charge of the particles so that they cease to repel each other, but rather, agglomerate or attach to one another. This increases their size and density resulting in an increase in their settling velocity. The particle suspension is no longer said to be stable since the particles agglomerate and destabilization is said to have taken place (Kiely, 1997).

After having pretreated the wastewater by the addition of a coagulant, the wastewater requires further treatment before being subjected to DAF. Flocculation is required, to facilitate the formation of large agglomerates of flocs (Letterman *et al.*, 1999). Flocculation is the agitation, or mixing, of the wastewater either mechanically or by diffused aeration. The purpose of flocculation is to promote, by slow mixing of the wastewater, the interaction and agglomeration of particles destabilized by the addition of a coagulant, resulting in the formation of large flocs (McGhee, 1991).

Following the pretreatment of the wastewater in the floatation tank by means of coagulation and flocculation, fine air bubbles are introduced into the floatation tank. The bubbles make contact with the coagulation-agglomerates which have settled to the bottom of the tank. This results in the formation of bubble-solid agglomerates which rise to the surface of the tank due to their lower density compared to water. Coagulation and flocculation may therefore be used to increase the amount of suspended particle removal by the DAF system.

2.8.2 Chemical treatment

Chemical treatment and/or pretreatment can, in many instances, improve the performance of physical and biological processes used in effluent treatment. The most commonly used chemical methods for effluent treatment are pH correction and coagulation to improve settlement rates by increasing particle size density (Eroglu *et al*., 1990; Dalzel, 1994; Lilley *et al*., 1997).

In South Africa, all but one vegetable oil manufacturer uses chemical treatment as the sole effluent treatment method to reduce the pollution load to discharge to the municipal sewer system or receiving river body (Steffen *et al.,* 1989). Chemical treatment is used mainly for carbonaceous (COD) and phosphorous removal from wastewater. Lime is the most widely used chemical coagulant for both COD and phosphorous reduction (Lilley *et al.*, 1997). Chemical phosphorous removal through phosphate precipitation can also be achieved using iron (Fe^{3+}) and aluminium (Al^{3+}) salts such as ferrous sulphate; ferric sulphate; ferric chloride; aluminium chloride; and aluminium sulphate (Grant, 1980; Dalzel, 1994; Loots *et al*., 1994; Lilley *et al*., 1997).

In the vegetable oil industry, lime and ferric chlorides are added to the neutralized effluent upstream of the DAF unit. Eroglu *et al*. (1990) reported that ferric chloride was the most effective coagulant during physicochemical treatability studies, and that it resulted in a BOD_5 reduction of 36%.

2.8.2.1 Advantages of chemical treatment

Chemical phosphorous removal in wastewater is reliable, and with strict control, a consistently low effluent phosphorous concentration can be achieved (Lilley *et al.*, 1997). Chemically bound phosphorous is not easily dissociated in water, which prevents the release of the bound phosphorous back into the water body (Loots *et al.*, 1994). When alum is used as a coagulant, it is possible to recover both aluminium and fatty material from fat/alum flocs through acid splitting (Grant, 1980).

2.8.2.2 Disadvantages of chemical treatment

The chemicals that are used during chemical treatment are corrosive in nature (strong oxidizing agents) and hence great care is required when handling, which in turn necessitates the use of expensive equipment that is resistant to corrosion (Lilley *et al.*, 1997). Chemical treatment of effluent is expensive due to the high price of chemicals. Pitman (1991) estimated that chemicals would cost the Johannesburg City council approximately 10 million Rand per year if they were to continue using chemical treatment alone. An additional problem associated with chemical usage, is the increased mineralization of water through the release of ions. Chemical coagulants usually contain chlorides or sulphates, which remain in solution, thus increasing the conductivity or salinity of the receiving water body (Lilley *et al.*, 1997).

2.9 Biological treatment of vegetable oil effluent

Biological treatment offers an efficient and cost effective means of treating VOE. Biological treatment of VOE may be carried out either aerobically, anaerobically or using a combination of both (Grant, 1980; Seng, 1980; Eroglu *et al*., 1990; Anderson, 1996b).

2.9.1 Anaerobic treatment process

Anaerobic digestion has long been practiced as a stabilization process for waste sewage treatment sludges, but the process has not been widely adopted for effluent treatment, with only very high strength industrial effluent being seriously considered (Grant, 1980). The effluent from the vegetable oil refining industry is loaded with sulphates, fats, and organic matter, which makes anaerobic treatment an attractive option prior to any aerobic treatment process. Studies by Eroglu *et al*. (1990) have shown that lime addition and activated sludge treatment does not bring an appreciable decrease in the sulphate concentration in the acidic effluent.

In the anaerobic digestion of high strength industrial waste containing high levels of sulphate, the two processes of concern are sulphate reduction and methane production, the latter being inhibited by the former (Eroglu *et al.*, 1990). In their study, Eroglu *et al*. (1990) reported a 60% sulphate reduction in an anaerobic filter reactor with a concurrent reduction of 60% of COD due to the oxygen demand exerted by dissolved sulphides in the treated effluent.

2.9.2 The activated sludge treatment process

The activated sludge treatment process is generally considered to have its origin in the aeration experiments which were carried out by Ardern and Lockett in Manchester, England in 1914 (Droste, 1997). Activated sludge is a suspended growth system which could be defined as a suspension of microorganisms, both living and dead in wastewater.

Since its inception, the activated sludge process has become one of the main methods used worldwide for the purification of wastewaters that contain biodegradable organic compounds, for example, domestic sewage (Horan, 1990; Droste, 1997). The conventional activated sludge treatment process involves two distinct operations usually performed in two separate basins; aeration in the aeration chamber and settling in a secondary settling tank (Figure 2.2).

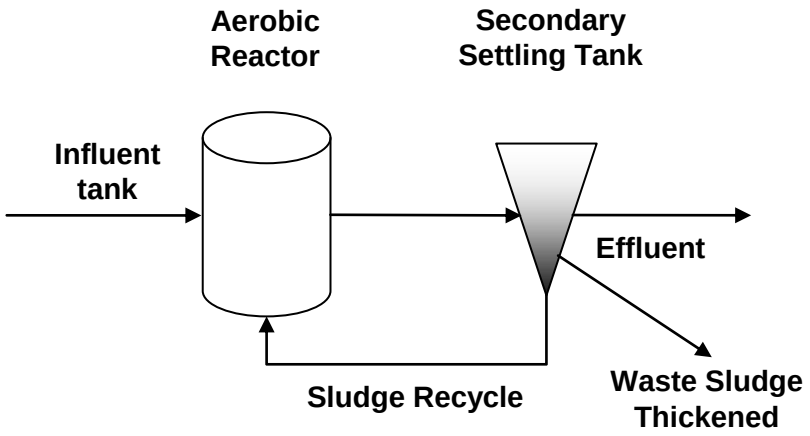


Figure 2.2 Schematic representation of a conventional completely mixed activated sludge system with hydraulic control of sludge age and recycle.

The principal unit in the activated sludge process is the aeration tank or the biochemical reaction vessel. The content of the aeration tank comprises an aerated mass of microorganism conglomerates, termed flocs, surrounded by the influent wastewater; this combined mixture of flocs and raw wastewater is termed the mixed liquor. The activated sludge flocs are agglomerates of microorganisms, inorganic and organic colloidal material and larger particulate matter, all held together in a compact organic matrix. A large number of protozoa, free-swimming ciliates and flagellates are found both in the mixed liquor and in the floc matrix (Horan, 1990). The flocs are mixed with incoming sewage in the presence of oxygen, supplied by aeration. In the aeration basin, some of the substrate is completely oxidized into harmless end products of carbon dioxide, water and other inorganic substances that are required to provide energy for the growth of microorganisms (Bitton, 1990; Horan, 1990; Droste, 1997).

The second phase in the activated sludge treatment process is the separation of the biomass and other suspended solids from the treated wastewater. This is accomplished in a secondary settling tank. Under the quiescent conditions of the secondary settling tank the activated sludge flocs settle rapidly to yield sludge with high concentrations of solids. A certain amount of the settled sludge is recycled back to the aeration tank while the remainder is removed from the system through wastage on a continuous or intermittent basis. Consistent wastage of sludge ensures that the concentration of biomass in the aeration chamber remains constant within the desired range (Quasim, 1994; Droste 1997). The clarified effluent at this stage is relatively devoid of any suspended particles and may be discharged into a watercourse after tertiary treatment, which includes chlorination or irradiation.

Optimal aeration of a conventional activated sludge process depends on the manipulation of the three basic design parameters, which are: organic loading rate (B_X) or the sludge age (R_S); maintaining the correct mixed liquor suspended solids value (MLVSS) and dissolved oxygen (DO) concentration in the mixed liquor (Horan, 1990). The B_X is frequently used as a key design parameter, since it use allows the determination of the required biomass without the need to make reference to the process kinetics coefficients. The choice of limiting values that may be assigned to the main design parameter to achieve a particular level of performance in the purification of a particular wastewater is most reliably made through pilot-plant operation. The final quality of the treated effluent is always a key consideration in the design and operation of any activated sludge process although other operation parameters also influence the choice of R_S and B_X. The more important factors are the degree of stabilization of the sludge biomass to be achieved and the requirement to produce a sludge biomass with good settling properties (Horan, 1990; Quasim, 1994).

2.10 Biological nutrient removal using the activated sludge process

Biological nutrient removal (BNR) refers to the removal of primary nutrients (carbon, nitrogen and phosphorous) from wastewater that could, subsequently cause eutrophication (Ekama & Wentzel, 1997). Nutrient removal is accomplished by manipulating activated sludge process configuration to create environmental conditions that are conducive to the optimal growth and activity of the microorganisms responsible for the removal of nutrients from wastewater (Ekama & Wentzel, 1997). BNR is mediated by a highly diverse mixed culture that develops in the modified activated sludge process. These mixed cultures work in sequence to remove different components at different stages of the process, that is, some lie dormant while others are actively metabolizing (Ekama & Wentzel, 1997).

2.10.1 Carbonaceous (COD) removal

Carbon in wastewater streams occurs in organic and inorganic forms. Heterotrophic organisms use organic compounds for their metabolism while inorganic compounds are metabolized by a group collectively termed autotrophs. Both forms of carbon are removed from wastewater through a series of oxidation and reduction (redox) reactions, oxidizing the carbon source to carbon dioxide and water. The carbon dioxide escapes into the atmosphere, thus removing carbon from wastewater.

The energy content of wastewater can be expressed using common substrate parameters, which are 5-day biological oxygen demand (BOD_5) and/or COD (Lilley *et al.*, 1997; Orhon & Artan, 1994a). The COD test is mostly used as a monitoring parameter at treatment works since it is quicker than the BOD_5 test and gives a more accurate reflection of the energy content of the system (Orhon & Artan, 1994a; Ekama & Wentzel, 1997; Lilley *et al*., 1997). In a BNR process, the carbonaceous material or the COD content of the system is divided into three main forms viz. non-

biodegradable; biodegradable and heterotrophic active biomass (Dold *et al.*, 1991; Orhon & Artan, 1994a; Wentzel *et al.*, 1995). The non-biodegradable COD has two sub-fractions, the non-biodegradable particulate and the non-biodegradable soluble. The biodegradable COD, also contains two sub-fractions, the slowly biodegradable (SBCOD), and the readily biodegradable (RBCOD) fractions. Both the RBCOD and SBCOD fractions are based wholly on the dynamic response observed in an activated sludge system (Wentzel *et al.*, 1995).

2.10.1.2 The COD test

In both the COD and BOD tests, the organic material concentration is calculated from the oxidant consumption necessary for the oxidation of the organic material. The main differences are the oxidant that is used and the operational conditions during the tests. In the case of COD, a sample of waste water containing organic material is placed in contact with a very strong inorganic oxidant, a mixture of dichromate and sulphuric acid with silver sulphate as a catalyst. The temperature is increased to the point of ebullition of the mixture, resulting in an increase of the oxidation rate. After two hours (the standard duration of the test) oxidation of the organic compounds is virtually complete. The resulting COD value can be determined by means of titration or with the aid of a spectrophotometer by reading the concentration of chromium (Cr^{3+}) formed (van Haandel & Lubbe, 2007).

2.10.1.3 The BOD test

The BOD test uses oxygen for the oxidation of organic material in the presence of microorganisms which are added at the beginning of the test, together with mineral nutrients and a buffer to maintain neutral pH. While in the COD test the complete oxidation of organic materials is complete within two hours, in the BOD test the oxidation rate can take several weeks to complete. Hence a standard period of 5 days has been introduced, even though it is well known that this period is not long enough to achieve complete oxidation. Since temperature has been found to affect the oxidation

rate, a standard temperature of 20°C is used. The organic material metabolized during the test is determined by the oxygen consumption and is called biodegradable material, while organic compounds that cause no measurable oxygen consumption are called non-biodegradable and are therefore not detected in the test (van Haandel & Lubbe, 2007).

2.10.2 Biological nitrogen removal

The key biological nitrogen removal reactions are nitrification and denitrification (Figure. 2.3). Other related reactions include ammonification (conversion of organic nitrogen to ammonia nitrogen) and nitrogen uptake for cell growth.

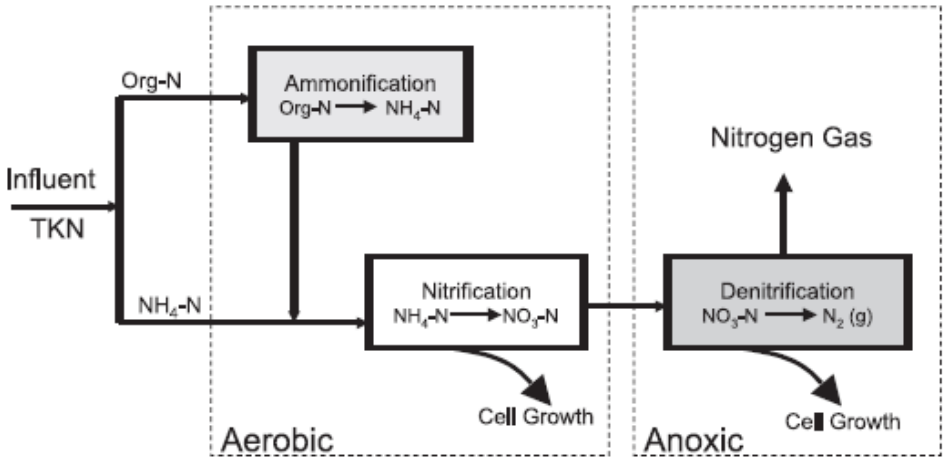


Figure 2.3 Biological nitrogen removal (Jeyanayagam, 2005).

2.10.2.1 Nitrification

Nitrification is the oxidation of ammonia to nitrite and nitrate. The key organisms involved are thought to be *Nitrosomonas* and *Nitrobacter* (Jeyanayagam, 2005). These are autotrophs that oxidize inorganic nitrogen compounds for energy:

Nitrosomonas:

$$2NH_4+ + 3O_2 \rightarrow 2NO_2^- + 2H_2O + 4H^+ + \text{New cells}$$

Nitrobacter:

$$2NO_2^- + O_2 \rightarrow 2NO_3^- + \text{New cells}$$

Consequently, organic substrate is not a prerequisite for the growth of nitrifiers. Nitrite accumulation is typically not encountered in a fully nitrifying system because *Nitrosomonas* is slower growing; however, there is some indication that at wastewater temperatures of above 25°C to 30°C, nitrite-to-nitrate conversion may become rate limiting, resulting in increased chlorine demand for disinfection (Jeyanayagam, 2005). Organisms other than *Nitrosomonas* and *Nitrobacter* can also mediate the nitrification process; therefore, the term ammonia oxidizing bacteria (AOB) is used to refer to them collectively. In BNR systems, nitrification is the controlling process for two reasons: (1) AOBs lack functional diversity. They represent about 2% of the microbial mass. (2) AOBs have stringent growth requirements and are sensitive to environmental conditions (Jeyanayagam, 2005).

2.10.2.2 Denitrification

Denitrification must follow nitrification to achieve significant total nitrogen removal. Denitrification is the reduction of nitrate to nitrogen gas by certain heterotrophic bacteria (Jeyanayagam, 2005). The process requirements are anoxic conditions and a source of readily biodegradable organic matter (RBOM). Anoxic refers to the presence of combined oxygen (nitrate and nitrite) and the absence of free or dissolved oxygen (Lilley *et al*., 1997). The simplified reaction is:

$$NO_3^- + \text{RBOM} \rightarrow N^2\ (\text{gas}) + CO_2 + H_2O + OH^- + \text{New cells}$$

Denitrification results in the recovery of 3.6 mg of alkalinity as $CaCO_3$ and 2.9 mg of oxygen per mg of NO_3-N reduced; therefore, by combining nitrification (aerobic) and denitrification (anoxic), partial alkalinity recovery and oxygen credit can be attained (Jeyanayagam, 2005). An additional benefit of incorporating an anoxic reactor is improved sludge settleability. The denitrification rate (g NO_3 reduced/g MLVSS.d), which determines the amount of nitrate denitrified, is primarily controlled by the availability of RBOM, and temperature (Jeyanayagam, 2005).

2.10.3 The modified Ludzack-Ettinger configuration

The first single sludge nitrificatrion-denitrification process was proposed by Ludzack and Ettinger in 1962. It was termed “single sludge” because the same sludge mass generates the nitrate and denitrifies it while utilizing the biodegradable substrate (influent sewage) as an energy source for denitrification (van Haandel *et al*., 1981). A schematic representation of this process is shown in Figure 2.4. It consists of two reactors in series, partially separated from each other. The influent enters the first reactor maintained in an anoxic state by stirring or mixing without aeration. The second reactor is aerated and nitrification takes place.

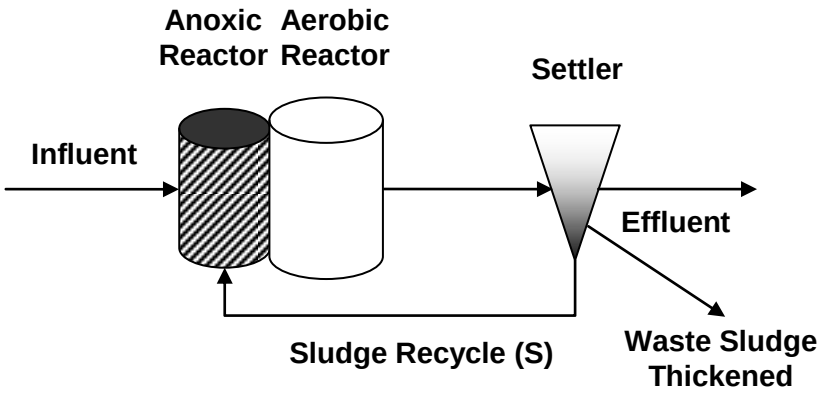


Figure 2.4 The Ludzack-Ettinger process for nitrogen removal (WRC, 1984).

Ludzack and Ettinger reported that the process gave variable dentrification results, possibly due to the lack of control over the interchange of the contents of the two reactors (WRC, 1984).

An improvement made by Barnard in 1973 allowed a significant improvement in control over process performance (Lilley *et al*., 1997). This was achieved by completely separating the anoxic and aerobic reactors, recycling the underflow from the settler to the anoxic reactor, and providing an additional recycle from the aerobic to the anoxic reactor. This process is known as the Modified Ludzack-Ettinger (MLE) process (Figure 2.5).

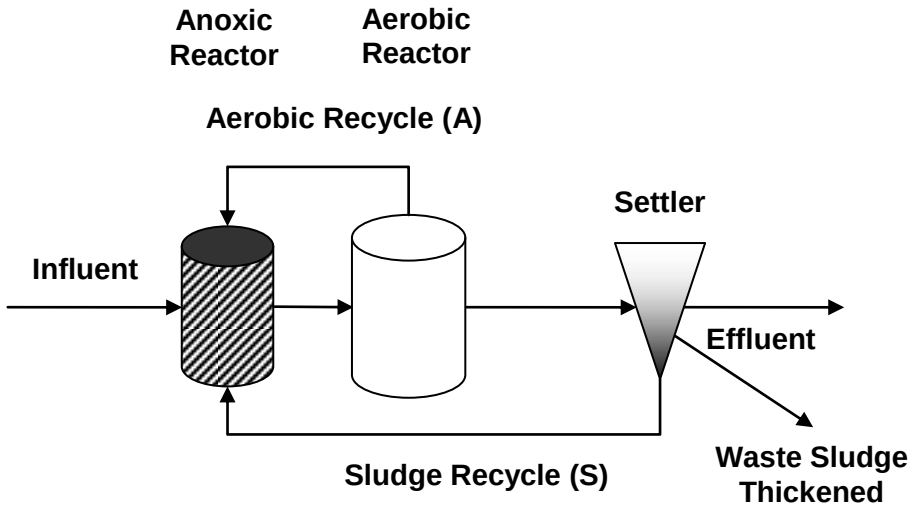


Figure 2.5 The modified Ludzack-Ettinger process for nitrogen removal (WRC, 1984).

In the MLE process influent enters the anoxic zone also called the “pre-denitrification reactor” or “primary anoxic reactor” directly and is placed ahead of the aerobic zone this gives rise to an improved rate of denitrification and a substantially higher reduction of nitrate, even when the pre-denitrification reactor of this process is substantially smaller than the aerobic

reactor. A recycle (A-recycle) from the aerobic zone recycles nitrite and nitrate back to the anoxic zone. The underflow recycle (S-recycle) from the settler or clarifier also recycles nitrite, nitrate and mixed liquor to the anoxic reactor (WRC, 1984). The only drawback of this configuration is that complete denitrification is cannot be achieved because part of the total flow from the aerobic reactor is not recycled to the anoxic reactor but is discharged directly with the effluent, resulting in nitrate being released into the effluent (WRC, 1984; Lilley *et al*., 1997).

Research conducted using an MLE process is significant in determining which group of microorganisms are predominant and significant in degrading organic wastes while the process is in steady-state (Muyima *et al.*, 1997). Steady-state behaviour of the MLE process is studied by conducting COD, TKN, OUR, MLSS and MLVSS analytical methods on a daily basis.

2.10.4 Monitoring of process performance

The oxygen utilization rate (OUR) is the rate at which microorganisms consume oxygen and is dependent on the amount DO available to microorganisms. When measured it is a combination of the oxygen required to oxidize both COD and nitrogen in the influent as well as the oxygen used by the organisms in endogenous respiration. In laboratory-scale processes it is measured by elevating the DO concentration to about 6 mgO/L and then stopping the supply. The DO concentration is then monitored and plotted against time. A linear plot should be obtained. The slope of the plot gives the OUR (Lilley *et al.*, 1997).

Chemical characterization of the wastewater entering (influent) and exiting (effluent) the process includes the determination of carbonaceous and nitrogenous materials. Carbonaceous material is characterized via the COD test (Clesceri *et al.*, 1998). Characterization of the nitrogenous material is done via the total Kjeldahl nitrogen test (Clesceri *et al.*, 1998). These

components of the influent and effluent wastewaters are routinely analyzed and the information gained is used to assess the performance of the process.

2.11 Conventional methods of microbial community analysis

Modern wastewater treatment processes rely on the action of complex microbial communities and have been accepted as “black boxes” for a long time. In recent decades great progress has been achieved in process engineering, with knowledge about community structure still lacking (Wagner & Amann, 1997). An understanding of the structure and function of these complex communities will aid in improving the design and operation of treatment plants.

Microbiological methods are inadequate in the identification of the microbial ecology in wastewater treatment works (Wagner & Amann, 1997). Microbial communities have mostly been analyzed using culture dependant methods, such as viable plate count and most probable number (MPN) technique. Culturing of bacterial from natural samples results in biases both in bacterial diversity (qualitative discrepancies) and in total bacterial numbers recovered (quantitative discrepancies), thus the number and diversity of bacterial populations are underestimated using these methods (Belser, 1979). Culture-based techniques do not provide reliable results on the microbial ecology of bacteria due to media selectivity. Studies performed on activated sludge showed that nutrient rich media favoured growth of heterotrophic organisms present in relatively low numbers and out-competed other bacteria, which were far more abundant in sludge samples (Wagner *et al.*, 1993). Viable but non-culturable organisms cannot be characterized by growth-based methods. Quantitative discrepancies are also significant. It has been reported that plate count results can range from 1% to 15% of the total number of cells determined by direct microscopic count (Manz *et al.*, 1994).

The use of *In situ* techniques can avoid quantitative and qualitative biases. The fluorescent antibody method has been used to characterize bacteria *in*

situ. However, its application has been limited in several ways (Wagner *et al.*, 1998). Extracellular polymeric substances (EPS) have been reported to hinder antibody penetration (Szwerinski *et al.*, 1985). Non-specific binding of detritus particles and fungal spores to antibodies may cause high background fluorescence (Wagner & Amann, 1997). In addition, uncultured bacteria cannot be studied since antibody production requires a pure culture of the target organisms. Molecular identification techniques have become increasingly popular for the detection and characterization of bacteria since they do not require the isolation and enrichment of bacterial strains. Fluorescent *in situ* hybridization (FISH) is one of the most commonly employed molecular techniques for the investigation of microorganisms in wastewater treatment plants (Wagner & Amann, 1997).

2.12 rRNA-based monitoring techniques

The ribosomal RNA (rRNA) approach is widely used for studying the microbial community structure of natural and man-made environments in a cultivation independent way. Ribosomal RNA genes provide particular advantages as a molecular marker in molecular methods, since all living cells contain ribosomes as a part of the cells apparatus for decoding RNA into protein. Firstly, rRNA is naturally amplified with bacteria containing between 10^3 and 10^5 ribosomes per cell. This natural amplification renders excellent sensitivity for hybridization assays (Amann, 1995a). Secondly, the cellular RNA content varies depending on the general metabolic activity or growth rate of a given species. Thirdly, rRNA are excellent molecules for discerning evolutionary relationships among bacteria because RNA molecules contain conserved and variable regions which make it possible to find general as well as specific target sites for probes. These regions are used for identification purposes. A practical reason for using rRNA is the public availability of large databases. They have enough sequence information to be used as a phylogenetic marker (Madiak *et al.*, 1999).

2.12.1 Nucleic acid hybridization

Ribosomal RNA-based studies in wastewater treatment plants have been performed in recent decades. Group and genus specific fluorescently labelled rRNA-targeted oligonucleotide probes were used to analyse directly the community structure of organisms in biological wastewater treatment plants, particularly in activated sludge systems by *in situ* hybridization (Daims *et al.*, 2001; Wagner & Amann, 1997). Activated sludge systems are one of the most commonly used processes in wastewater treatment plants (Lilley *et al.*, 1997). Oligonucleotide probes are short sequences of nucleic acids which are complimentary to a specific sequence of RNA. Hybridization is the artificial annealing of a double-stranded nucleic acid by complementary base pairing of two single stranded nucleic acids (Madigan *et al*., 2000).

Two different hybridization assays are commonly used in microbial ecology studies: FISH and dot-blot hybridization. Dot-blot hybridization requires the extraction of nucleic acids from fresh samples to be tested. Subsequently, nucleic acids are denatured, immobilized on membranes and hybridized with radioactive or nonradioactive probes (Robinson *et al.*, 2004). With FISH, the target nucleic acids are detected directly in the cells. To achieve *in situ* detection, cells are permeabilized to allow the probe access to the inside of cells. At the same time, the morphological integrity of the examined cells is maintained. This is usually achieved by fixing the cells with alcohols (ethanol) or aldehydes (paraformaldehyde) (Amann, 1995a). During the hybridization process, probes labelled with a fluorescent dye (e.g. rhodamine or fluorescein) bind to a signature sequence in the ribosomal RNA of the target organism(s) of interest (Stahl & Amann, 1991)(Figure 2.6).

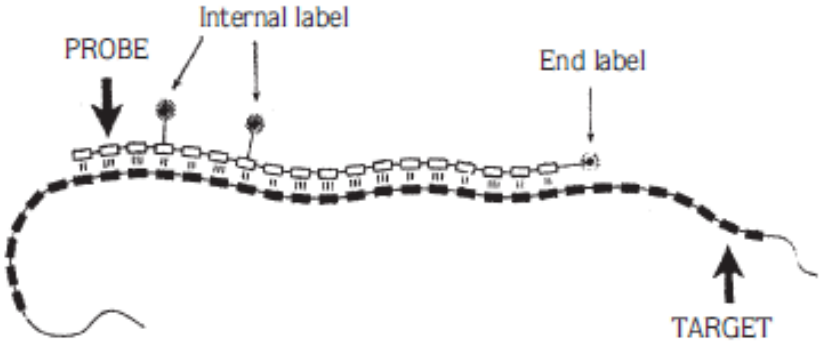


Figure 2.6 Base pairing between a fluorescently labelled oligonucleotide probe and target RNA (Manz *et al*., 1992).

The optimal temperature for the hybridization is a function of the base composition of the probe and complementary target sequence. This is determined empirically to avoid binding of the probe to the rRNA sequences with some sequence mismatches with the probe. Optimum hybridization conditions can be found by varying the formamide (FA) concentration in increments of 5% in the hybridization buffer with hybridization performed at a single temperature (Manz *et al.*, 1992). Addition of FA is one of the simple ways to discourage hydrogen bonding. It facilitates denaturation of the probe and the target DNA. During hybridization, a relatively high FA concentration should favour probe-target annealing but not excessive probe nontarget hybridization. In other words, relatively high formamide conditions increase the hybridization stringency (Gerhardt *et al.*, 1994). Binding of the fluorescently labelled probe to the target rRNA sequences allows visualization and enumeration of individual cells with the aid of an epifluorescent microscope (Manz *et al.*, 1992).

2.12.2 Potential advantages of FISH

2.12.2.1 Characterization of microbial diversity

FISH permits the rapid, simple and accurate detection of related groups of bacteria. FISH allows the *in situ* detection of bacterial species without requiring culturing. As a result, it is a potential tool for the identification of microbial ecology in wastewater treatment plants. This technique also allows the characterization of bacterial species which have not been cultured yet. Therefore, undiscovered diversity may be characterized by FISH.

2.12.2.2 Phylogenetic studies

The 16S rRNA sequences used in FISH provide phylogenetic information (the relationship between organisms) and can therefore distinguish between different populations independently of activity. The probes can also be designed for different levels of specificity. The more conserved regions allow differentiation between large phylogenetic entities like the domains Archea, Bacteria and Eucarya, and they also serve as targets for universal probes that react with all living organisms. Variable region sites can be used to identify certain genera, species, and infrequently also for subspecies or even a certain strain (Stahl & Amann, 1991).

2.12.2.3 Confocal scanning laser microscopy

Attempts have been made to combine fluorescent oligonucleotide probing with advanced microscopic techniques, such as confocal scanning laser microscopy (CSLM), for analysing the spatial distribution of specifically labelled target cells within activated sludge flocs or trickling biofilms (Wagner *et al.*, 1994a).

CSLM is an improved version of the optical microscope. In the confocal microscope, out-of-focus fluorescence is virtually eliminated and this increases contrast, clarity, and detection sensitivity (Wilkinson & Schut, 1998). CSLM significantly minimizes the effects of background and out-of-focus fluorescence (Wagner *et al.*, 1994a; Wagner *et al.*, 1994b). CSLM has

another advantage over epifluorescent microscopy. Conventional epifluorescent microscopy is adequate for thin samples, where all information within the field of view is found in a single focus plane. However, CSLM offers the third dimension, by a technique known as optical sectioning, for thick samples without the need for mechanical sample sectioning. Stacks of optical sections taken at successive focal planes (known as z series) can be reconstructed to produce a three-dimensional view of the specimen (Matsumoto, 1993).

2.12.2.4 Kinetic growth studies

In theory, *in situ* growth rates and physiological activities may be estimated by measuring the fluorescence conferred by the rRNA-targeted oligonucleotides in combination with digital image analysis as the cellular quantity of rRNA is closely related to the growth rate of cellular micro-organisms. Quantification of the probe conferred signal intensity of single cells seems to be an appropriate tool for estimating their physiological state *in situ* (DeLong *et al.,* 1989; Poulsen *et al.,* 1993). It has been demonstrated that there is a linear relationship between the average fluorescence intensity per cell volume and the growth rate of the cell culture (DeLong *et al.,* 1989; Poulsen *et al.,* 1993). Therefore, growth kinetic parameters may be determined with this technique.

2.12.2.5 Enumeration of microorganisms

Until recently, the enumeration of micro-organisms was limited to cultivation-based methods. These methods underestimate the number of bacterial cells. FISH allows the detection of one to three orders of magnitude more cells than plate counts in environmental samples; 60 - 90% of all cells present in activated sludge can be detected with DNA-intercalating dye (DAPI). FISH can also visualize a similar quantity of cells. Enumeration of bacterial species in activated sludge systems using FISH has been investigated successfully in previous research (Coskuner, 2000; Davenport *et al.*, 2000).

2.12.3 Problems associated with FISH

2.12.3.1 Problems with permeabilization

Successful entry of the probe into the cell is the first and main step of the FISH. Most micro-organisms have been permeable to short oligonucleotide probes following fixation (DeLong *et al.,* 1989; Giovannoni *et al.*, 1988). Although a variety of fixatives have been evaluated, autofluorescence is generally minimized by fixation in formaldehyde.

2.12.3.2 Non-uniform cell penetration

Successful cell permeabilization does not guarantee that hybridization of the targeted rRNA sequence with a probe will occur (O'Donnell & Head, 1997). It is uncertain whether oligonucleotide probes will be able to permeate all cell types and find 16S rRNA target sequences (Muyzer & Ramsing, 1995). High stringency is required to manage even cell penetration.

2.12.3.3 No signals

Sometimes no fluorescence signal is obtained because of a very low concentration of cells or low RNA content. It has been found to be difficult to detect less than 10^3 - 10^4 cells per mL (Amann *et al.*, 1995). This indicates a high detection limit. Hence, the sensitivity of FISH increases with an increased number of cells metabolizing actively.

2.12.3.4 Target site accessibility

The target sequence in the rRNA is believed to be inaccessible due to strong interactions with ribosomal proteins or highly stable secondary structure elements of the rRNA itself. If the pure culture cells give a strong hybridization signal with a universal probe while they are not giving signal with specific probe, this generally indicates poor accessibility of the target site. *In situ* accessibility can sometimes be improved by the addition of formamide to the hybridization buffer (Manz *et al.*, 1992).

2.12.3.5 Background autofluorescence

The auto-fluorescence of some bacteria, such as phototrophs (Muyzer & Ramsing, 1995), and the background fluorescence of inorganic particles is often much stronger than the fluorescence of the specific probe binding. Bleaching of fixed cells before hybridization, and the use of fluorescent dyes with emission wavelengths that do not coincide with the auto-fluorescence, are potential solutions to this problem (DeLong *et al.*, 1989).

2.13 Current applications of FISH in wastewater treatment plants

FISH has been applied in studies conducted to identify filamentous and non-filamentous bacteria in WWTP, particularly in activated sludge processes. Microorganisms related to phosphate removal, nitrification processes and bulking and foaming problems have been investigated (Wagner *et al.*, 1994c; Juretschko *et al.*, 1998).

2.13.1 Polyphosphate accumulating organisms

Enhanced biological phosphate removal (EBPR) in anaerobic-aerobic activated sludge systems has generally been linked to *Acinetobacter* spp. This was supported by the detection of *Acinetobacter* spp. in the microbial consortia in the anaerobic and aerobic compartments of two sewage treatment plants with EBPR in Germany (Wagner *et al.*, 1994c). Furthermore, in this study, filamentous bacterium of the Eikelboom type 1863 showed positive hybridization reactions with the probe GAM42a, which is targeted to gamma-subclasses of *Proteobacteria* and with the *Acinetobacte*r specific probe. Therefore, it was understood that there is a close affiliation of the filamentous bacterium of the Eikelboom type 1863 with the genus *Acinetobacter*.

2.13.2 Foam causing bacteria

The formation of stable, often chocolate-coloured, and viscous foams or scums on the surfaces of activated sludge aeration tanks causes a number of problems. It reduces oxygen transfer to aeration basins when surfaces of

basins were aerated mechanically. It also causes poorer effluent quality and carriage and dispersal of microbial pathogens by the wind. Finally, the drying out of foam results in cleaning and odour problems (Sodell & Seviour, 1990). No definite ways have been identified to prevent or control the accumulation of foam. In addition, it has been observed that strategies that work in one WWTP, may not work in another. Therefore, it is necessary to understand both the taxonomic diversity and ecology of the organisms which cause the foaming problem. The true extent of the taxonomic diversity and identity of *Actinomycetes* were studied using FISH (Goodfellow *et al.*, 1996). It was supported that the term Nocardia foams does not cover the taxonomic range of *Actinomycete* associated with foams (Goodfellow *et al.*, 1998).

2.13.3 Nitrite and ammonia oxidizing bacteria

There are many studies investigating the microbial ecology of nitrifying plants with *in situ* hybridization techniques. Early molecular studies sought to find *Nitrobacter* species in activated sludge systems since these species were thought to be responsible for nitrite oxidation due to their growth during standard enrichment and isolation procedures (Henze *et al*., 1997). However, *Nitrobacter* species were not observed with *in situ* hybridization in recent studies (Wagner *et al*., 1996; Juretschko *et al*., 1998; Schramm *et al*., 1998). *In situ* hybridization studies using 16S rRNA-targeted probes specific for the nitrite oxidizing bacteria (NOB) of the genus *Nitrobacter* did not detect any targeted cells in a variety of nitrifying environments, which highlighted the importance of non-*Nitrobacter* NOB for the nitrification process (Juretschko *et al.*, 1998).

It has been suggested that unknown NOB were important in activated sludge (Burrell *et al.*, 1998) and *Nitrospira*-like bacteria were found to be a dominant population in an industrial WWTP receiving sewage with a high ammonia concentration (Juretschko *et al.*, 1998) and in a nitrite oxidizing bioreactor (Burrell *et al.*, 1998). Schramm *et al*. (1996) have studied the *in situ* localization of *Nitrosomonas* spp. and *Nitrobacter* spp. with *in situ*

measurements of concentrations of O_2, and NO_2^-/NO_3^- using micro-electrodes. They found a good correlation between the structure and function of nitrifiers using FISH. Where the nitrifiers were located, nitrification was also observed in the same zones. These studies revealed that the common belief that *Nitrobacter* spp. were the important nitrite oxidizers in wastewater treatment as a mere artefact of cultivation (Schramm *et al*., 1998).

CHAPTER 3

Evaluation of a Laboratory-Scale Biological Process for the Treatment of Vegetable Oil Effluent

3.1 Introduction

The vegetable oil refining industry produces effluents containing large quantities of fats, oils, sodium, phosphates as well as a host of other pollutants. Common methods of treatment of effluents generated from vegetable oil refineries are restricted to either physical separation of oils and grease through the use of gravity fat traps or dissolved DAF followed by pH correction, which provide a considerable reduction in organic loading. However, the remaining emulsified grease tends to block sewer pipes and clog pumps with discharge standards not being met (Eroglu *et al.*, 1990; Mkhize & Bux, 2001). Therefore, biological treatment methods are being sought after. Aerobic treatment has been attempted however, organic shock loads cause problems while running such a process. Currently, global trends are focusing on the application of biological treatment processes as a final polishing step of effluent treatment. A comparative evaluation of treatment alternatives for wastewaters from an vegetable oil refinery conducted by Eroglu *et al.* (1990) demonstrated that activated sludge treatment proved successful in degrading the majority of the FOG.

In activated sludge processes it is necessary to create zones that are either aerobic (dissolved oxygen present), anaerobic (absence of dissolved oxygen and nitrate) or anoxic (nitrite and nitrate present, but deficient in oxygen) to effect biological nutrient removal. The anaerobic zone is required for the effective removal of phosphorus while the anoxic zone is necessary for the removal of nitrate. Various configurations have been developed for the removal of these nutrients. Selection is dependent on the TKN/COD ratios of the wastewater being treated (Lilley *et al.*, 1997). The mixed population of microorganisms present in the sludge grows, as it utilizes the organic material from the influent wastewater entering the system. Biological

processes that occur within the system and through wasting a part of the sludge from the aerobic reactor ensure that the effluent produced is low in carbon and suspended solids. The amount of sludge wasted depends on the characteristics of the influent and the biological processes occurring within the system (Schroeder, 1977). Microscopic evaluation of the sludge reveals that it is formed mainly by a heterogeneous population of microorganisms which are usually present as flocculating cells (Lilley *et al.*, 1997).

Carbonaceous materials are broken down under aerobic conditions by flocculating heterotrophic microorganisms. A small percentage of the energy derived from the breakdown is used for synthesis of new cells, while the remaining fraction is eventually lost as heat. Dead microorganisms separate from the sludge flocs and are eventually discharged in the effluent lowering the COD of the sludge before discharging the sludge as effluent (Lilley *et al.*, 1997).

In this aspect of the study a laboratory-scale MLE process was evaluated for the biological treatment of VOE focussing on COD and TKN removal. However, nitrogen was found to be limiting in the VOE and therefore needed to be supplemented in order to maintain the integrity of the biological process.

3.2 Methods and Materials

3.2.1 Sampling of vegetable oil effluent

Raw effluent was obtained after the deodorization process from Company X. Composite samples were collected in 22 x 25 L plastic containers and transported to the laboratory and stored in a cold room at 4°C to prevent further biological activity. The effluent was allowed to reach room temperature prior to pretreatment. COD (Appendix 1) and TKN (Appendix 2) concentrations were determined according to *Standard Methods* (Clesceri *et al.*, 1998). The COD concentration was determined by firstly oxidizing all the organic material in the sample by refluxing the sample in a known amount of

potassium dichromate. The organic content of the sample was then measured indirectly by determining the amount of Cr^{3+} reduced by titration with ferrous ammonium sulphate. The TKN determination consisted of digesting the sample with sulphuric acid to produce ammonium sulphate, the solution was then distilled with sodium hydroxide to convert the ammonium salt to ammonia. The amount of ammonia (hence the amount of nitrogen in the sample) was determined by back titration. The end of the condenser was dipped into a solution of boric acid. The ammonia reacts with the acid and the remainder of the acid is then titrated with a sodium carbonate.

3.2.2 Pretreatment of vegetable oil effluent

The COD concentration of the raw effluent ranged from 5006 mg/L to 6790 mg/L. Therefore, in order to prevent organic shock-loading of the biological treatment process the effluent was pretreated using a commercial flocculent compound C40 (Chemserve Trio, South Africa). A 300 L plastic vessel was filled with 300 L of raw effluent and allowed to reach room temperature (20°C). Compound C40 was added to the effluent with slow stirring (3000 rpm) using a Heidolf overhead stirrer (Heidolf, UK) until a final C40 concentration of 6 g/L was obtained. However, the amount of C40 required for complete flocculation varied amongst effluent batches due to the inconsistent nature of the refinery process. Clarification was reached after 10 to 15 min. The supernatant (flocculated effluent) remained in the vessel for 24 - 48 h to facilitate efficient gravity settling of the flocculent. The clear supernatant was decanted and transferred to a clean vessel in a cold room at 4°C. The initial pH of the effluent was acidic (pH 3.2 - 4.0) but on addition of the flocculent, the pH became basic (pH 9.0 - 10.0). The COD concentration of the pretreated effluent ranged from 1100 mg/L to 1400 mg/L and was diluted to ±1000 mg/L with tap water. The final pH was adjusted to pH 7.4 by the addition of concentrated sulphuric acid (±100 mL).

3.2.3 Nutrient supplementation of vegetable oil effluent

TKN and TP determinations revealed that nitrogen and phosphorus were limiting in the pretreated effluent (both < 0.5 mg/L), i.e. their concentrations were lower than the carbon to nutrient ratio required to maintain a biological treatment process. Therefore, nitrogen and phosphorus were added to the pretreated effluent in the form of ammonium chloride and potassium dihydrogen orthophosphate salts. Since the COD concentration was ±1000 mg/L, 50 mg/L of ammonium chloride and 10 mg/L of potassium dihydrogen orthophosphate were added with mixing (3000 rpm) to the pretreated effluent such that a COD:N:P ratio of 100:5:1 was obtained. This ratio is required to maintain a biological treatment process. However, the ratio ultimately depends on the characteristics of the raw wastewater to be treated (WRC, 1984). If the C/N or C/P ratios are too low, as in the case of domestic wastewaters, only very limited N or P removal occurs. For optimal system operation ratios of 100:5:1 and 150:5:1 were proposed to define the minimum nutrient requirements for activated sludge systems designed for carbon removal (Orhon & Artan, 1994b). After nutrient supplementation the pH of the flocculated effluent was adjusted to pH 7.4 with concentrated sulphuric acid. The supplemented VOE was stored in a cold room at 4°C in a 300 L influent tank and used as the influent for the biological process.

3.2.4 Design and operation of the laboratory-scale biological treatment process

The laboratory-scale biological treatment process was modelled upon the MLE process but focused on carbon removal since nitrogen was found to limiting (Lilley *et al.*, 1997). The unit was designed and manufactured by the Department of Civil Engineering, University of Cape Town, South Africa. The process was seeded with mixed liquor sampled from the aerobic zone of Darvill Wastewater Treatment Purification works (Pietermaritzburg, South Africa), located in the direct vicinity of the vegetable oil refinery. A schematic representation of the laboratory-scale MLE process is shown Figure 3.1.

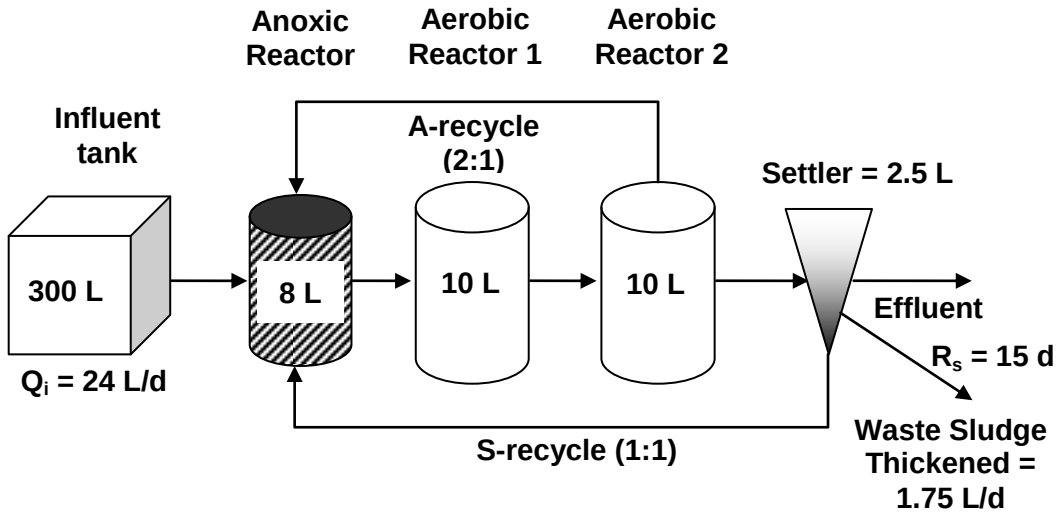


Figure 3.1 Schematic representation of the laboratory-scale MLE process used to treat vegetable oil effluent.

The laboratory-scale MLE process was fed supplemented VOE from a 300 L influent tank at an influent flow rate (Q_i) of 24 L/d. the flow bin was kept refrigerated in a cold room at 4°C. The process consisted of a primary anoxic reactor (8 L) followed by two secondary aerobic reactors (10 L) providing a total volume of 20 L. An aerobic-recycle (A-recycle) between the anoxic and aerobic zones was setup at a 2:1 ratio with respect to the influent flow rate. A settler (2.5 L) was set up at a 60° to the horizontal. The settled sludge was returned to the anoxic reactor via a sludge recycle (S-recycle) at a ratio of 1:1 with respect to Q_i. A sludge age (R_s) of 15 d was maintained by wasting 1750 mL of mixed liquor daily from aerobic reactor 2 and replacing the volume with tap water. The process was operated in an air conditioned room with the temperature set at 20°C. The process was fed 15 batches of supplemented effluent in total with each batch lasting a period of 10 d.

3.2.5 Monitoring of process performance

Daily analyses were conducted in order to determine steady-state conditions and to monitor the performance of the laboratory-scale MLE process. Daily results obtained for each batch was averaged over the 10 days of treatment and the standard deviation for each batch calculated. Chemical oxygen demand (Appendix 1) and TKN (Appendix 2) analyses were conducted on samples decanted from the influent tank and effluent buckets. MLSS and MLVSS (Appendix 3) determinations were performed on mixed liquor obtained from daily wasting. The MLSS was determined by evaporating an aliquot of mixed liquor at 105°C in a pre-weighed stainless steel crucible and determining the weight of the solids from the sample. The organic portion of the mixed liquor suspended solids is represented by MLVSS which comprises non-microbial organic matter as well as dead and alive microorganisms including their debris. MLVSS is determined after volatilization of the dried sample at 550°C (Bitton, 1999).

All analyses were performed according to Standard Methods (Clesceri *et al.*, 1998). The OUR profile of the mixed liquor in the aerobic reactor was monitored continually using an automated technique (Randall *et al.*, 1991) with the lower and upper dissolved oxygen limits set at 2.0 mgO/L and 5.0 mgO/L respectively. A DO probe (Yellow Springs Inc., U.S.A.) attached to a DO meter (Hi Tech Microsystems, South Africa) was immersed in the mixed liquor of aerobic reactor 1. An air pump connected to the DO meter sparged air through a porous air stone until the DO in the reactor reached 5 mgO/L at this point a relay automatically switched the pump off and the time taken for the DO to reach 2 mgO/L measured by the DO meter, the air switched on again and the cycle repeated. For each cycle, the slope of the DO-time data during the unaerated period was determined using linear regression and automatically calculated by the DO meter, thus giving the OUR. The data from the DO meter was downloaded to a PC on a daily basis and mean OUR data for the day recorded. The organic load into the laboratory-scale process

was expressed as the food to microorganism (F/M) ratio (Lilley *et al*., 1997). The F/M ratio was calculated as follows:

$$\text{F/M ratio} = \frac{Q_i \times COD}{MLSS \times V}$$

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

3.2.6 Statistical analysis

Statistical analyses were performed using Microsoft *Excel* spreadsheet software including the 'Analysis Toolpak addin'. Averages and standard deviations were performed on the results obtained during the treatment of each batch of VOE. The Pearson product-moment correlation coefficient (r) was used to investigate the association between the influent COD and the F/M ratio of the laboratory-scale biological treatment process.

3.3 Results

3.3.1 Pretreatment of vegetable oil effluent

The results of means COD concentrations determined for the fifteen batches of raw VOE collected from Company X and the corresponding mean COD concentrations of the batches pretreated with C40 are represented in Figure 3.2. Detailed results of daily monitoring of the laboratory-scale MLE process obtained during steady-state were averaged and standard deviations calculated according to each of the 15 batches of VOE treated are listed in Table A2 in Appendix 4**.**

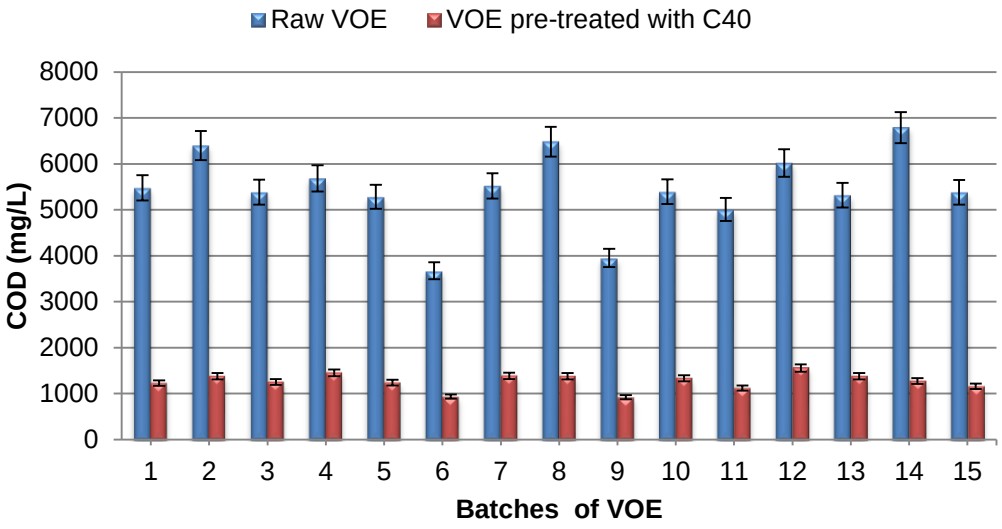


Figure 3.2 COD concentrations of the raw VOE and VOE pretreated with compound C40.

The COD of the raw VOE varied since the raw effluent quality and concentration appeared to vary on a monthly basis depending on the source crude vegetable oil available. The COD concentration of the raw VOE ranged from 3700±200 mg/L (Batch 6) to 6790±322 mg/L (Batch 14) represented in Figure 3.2. The efficacy of the pretreatment procedure was evaluated on the basis of COD removal. The assessment of the efficacy of the commercial flocculent C40 showed that the COD removal efficiency ranged from 72% (Batch 13) to 81% (Batch 14), with an average of 73% of the COD of raw VOE being removed (Figure 3.3). The pretreatment step using C40 produced VOE with a COD concentration ranging from 950±50 mg/L (Batch 6 or 9) to 1556±64 mg/L (Batch 12) (Figure 3.2), which was diluted to a COD of ± 1000 mg/L to be further treated biologically. The pH of the raw VOE on collection was acidic and ranged from pH 3.2 - 4.0 and basic after treatment i.e. pH 9.0 - 10.0 which required neutralization prior to biological treatment.

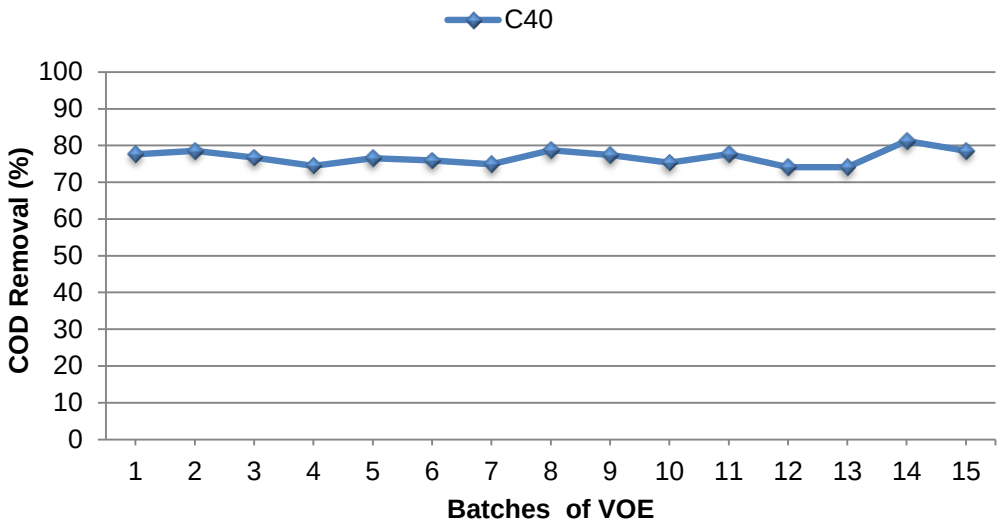


Figure 3.3 Pretreatment of VOE with compound C40 showing COD removal efficiency

3.3.2 The MLE process

The laboratory-scale MLE process was operated for a period of 8 months. During the first two months of operation the process experienced a period of acclimation as the Darvill seed sludge became acclimatized to the new substrate consisting of VOE. During the acclimation period the process exhibited erratic behaviour in terms unstable MLVSS concentrations and OUR's as well as poor COD and TKN removal efficiencies. Results of process monitoring during the acclimation period are presented in Table A1 appearing in Appendix 4. COD and removal was erratic and ranged from 60 - 81% and TKN removal efficiency ranged from 35 - 70%. Following the two month acclimation period the process was deemed to be in steady-state, hence all subsequent observations in the following Chapters are based on the hypothesis that the process was operating under optimal conditions with maximum COD and TKN removal efficiencies. The percentage COD and TKN removal efficiencies are presented graphically in Figure 3.4.

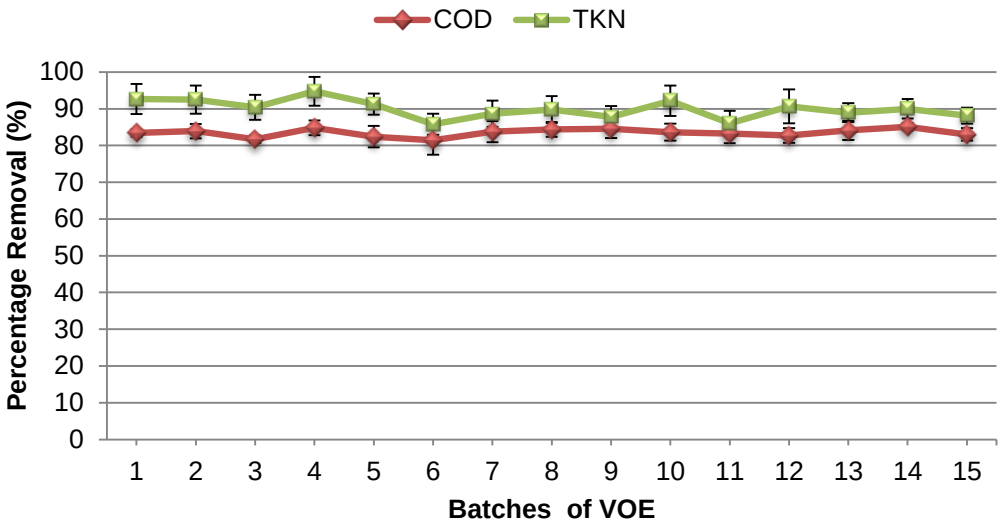


Figure 3.4 Percentage COD and TKN removal efficiencies of the laboratory-scale MLE process

The mean influent COD concentration of the 15 batches VOE ranged from 918±43.2 mg/L to 1012±104 mg/L producing an effluent COD concentration ranging from 137±23.6 mg/L to 206±26 mg/L (Table A2 in Appendix 4). Inconsistencies in the strength of the raw effluent COD amongst batches prevented the desired influent COD from being achieved in some batches of supplemented VOE. Mean supplemented influent TKN concentrations of the 15 batches of VOE ranged from 45.4±3.5 mg/L to 53.1±1.8 mg/L and effluent TKN ranged from 2.6±0.7 mg/L to 6.4±1.6 mg/L (Table A2 in Appendix 4). This demonstrated that the laboratory-scale MLE process was capable of removing on average 84% of the influent COD and 90% of the influent TKN concentrations from the supplemented VOE (Figure 3.4).

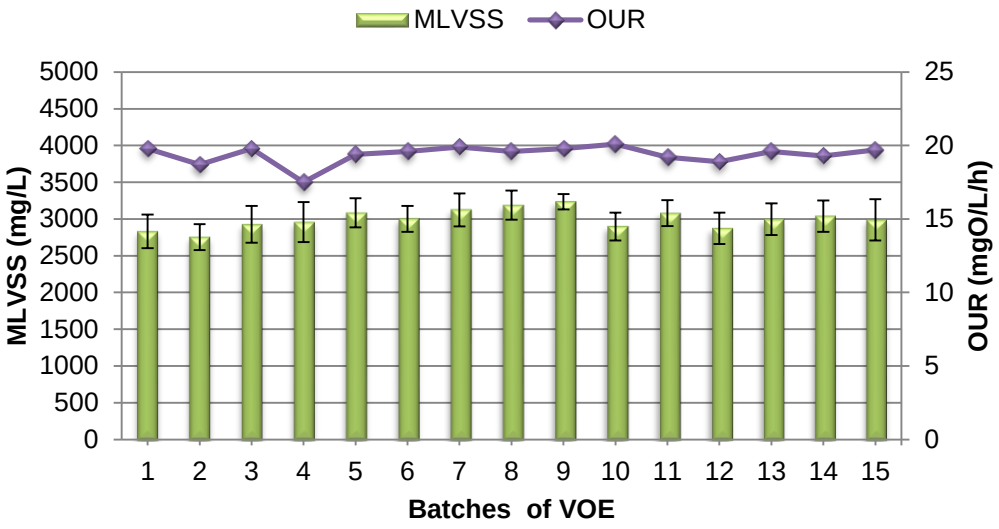


Figure 3.5 MLVSS concentrations and OUR profile of the aerobic mixed liquor of the laboratory-scale MLE process.

Average MLVSS concentrations per batch of VOE treated of the aerobic mixed liquor ranged from 2754±282 mg/L to 3234±474 mg/L and averaged 3000 mg/L during the treatment of 15 batches of VOE (Figure 3.5). The viability of the microorganisms in the activated sludge was monitored by OUR and ranged from 17.5±0.2 mgO/L/h to 20.1±0.1 mgO/L/h and averaged 19 mgO/L/h (Figure 3.5). The mean OUR was slightly erratic but reflected a similar trend to the COD removal capacity of the process (Figure 3.5). However, blockages in pipes due to settling of sludge and occasional mechanical problems with peristaltic pumps causing reactor overflows which possibly caused a loss of MLVSS resulting in the slightly diminished process efficiency in batches 10 through 15.

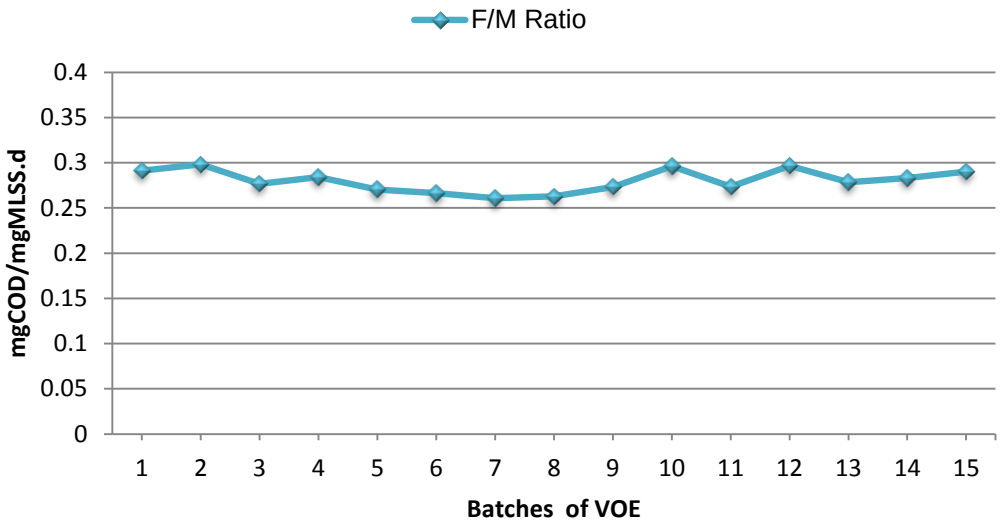


Figure 3.6 **Food/microorganism (F/M) ratios calculated for each of the 15 batches of VOE treated.**

The F/M ratio was kept between 0.26 – 0.30 by controlled wastage of 1.75 L/d of aerobic mixed liquor and maintaining the influent COD concentration at ±1000 mg/L. Variations in the influent COD concentrations resulted in slightly erratic F/M ratios. A Pearson product-moment correlation coefficient value of 0.58 was calculated indicating no correlation between influent COD and the F/M ratio of the laboratory-scale process.

3.4 Discussion

Prior to the large scale pretreatment of the VOE the effluent was characterized in terms of pH and COD concentration. Current findings substantiate previous research by Saw *et al*. (1987) that demonstrated raw effluent pH measurements of pH 2.0 and COD concentrations ranging from 1010 mg/L to 8200 mg/L, which compared favourably with the COD of the raw effluent collected (Figure 3.2). The wide range of results was ultimately due to inconsistencies in the strength of the effluent collected from Company X.

Industrial wastewaters are usually characterized as having remarkably high organic loads. Complete treatment of these wastewater pollutants cannot be achieved by the adoption of a single treatment method. Chemical and biological treatment methods are often combined to optimize the overall process and minimize the economic costs incurred (Bertanza *et al*., 2001). The COD of untreated wastewaters of vegetable oil manufacturing industries range from 8000 - 10 000 mg/L, depending on the seedstock (Eroglu *et al*., 1990). Hrudey (1982) reported the failure of activated sludge processes due to lipid overloading, which coincided with a substantial increase in effluent turbidity. These effluents must therefore be pretreated in order to remove the majority of the FOG using physical/chemical means so that the effluent is amenable to biological treatment.

After consultation with Chemserve Trio, a local supplier of flocculants and coagulants, it was decided that compound C40 would best suit this application and a dosage of 5-10 g/L was suggested. Exceeding the recommended dosage resulted in the treated effluent taking up the excess C40 which had an adverse effect of coagulating the mixed liquor in the laboratory-scale process, thus reducing process efficiency. No information was available from the supplier about the chemical structure of coagulant C40. However the physical structure resembled that of an organoclay. Bentonite is an organoclay commonly used to remove oil from water (Alther, 2000). It is made up of two or more mineral oxide layers which are stacked parallel units of silica and alumina sheets. These layers are expandable and are known for their absorption capacity. These clay particles bind or adsorb to the opposite electrical charges on an organic molecule causing it to destabilize and coagulate. Clays with a high cation exchange capacity and larger pore sizes will also impact on the binding of organic molecules (Alther, 2000). A study conducted by Beccari *et al*. (1999) made use of bentonite for the pretreatment of olive mill effluent (OME) prior to anaerobic digestion. Their findings showed that at a 4 g/L dosage a COD removal efficiency of 42% can be achieved. However, further trials to enhance the efficacy of

bentonite using higher concentrations i.e. 10 g/L and 15 g/L produced marginal increases in COD removal i.e 47% and 45% respectively indicating that increasing the coagulant concentration from 10 g/L to 15 g/L resulted in a decrease in COD removal efficiency rather than increasing COD removal as would be expected. When comparing the latter to our findings, the COD removal efficiencies of C40 (bentonite) was relatively good at 77%, considering a coagulant dosage of 6 g/L was used. Sengul (1989) using coagulants such as poly-electrolytes when treating sunflower oil effluents, showed COD removal rates of 76% and also demonstrated pretreatment as a necessary step prior to biological treatment. Betazzi *et al*. (2007) confirmed that pretreatment using physico-chemical processes improved the efficiency of biological treatment.

Following the period of acclimation, the biological process was operated for the duration of 15 VOE batches. During this period, process performance reflected steady-state conditions with consistent COD and TKN removal efficiencies (Figure 3.4), OUR's and MLVSS concentrations (Figure 3.5). As shown in Figure 3.4, the process achieved a mean COD removal capacity of 84% from an influent COD of ±1000 mg/L. Current findings support work of other researchers. Reddy *et al*. (2003) demonstrated an average COD removal capacity of 81% using a similar laboratory-scale MLE process to treat VOE. Boukchina *et al*. (2007) showed 84% COD removal using aerobic treatment to treat olive mill wastewater, although the initial COD was 250 mg/L. Öztürk *et al*. (1990) using a laboratory-scale activated sludge process to treat VOE, achieved a lower COD removal capacity of 72%. Mkhize *et al*. (2000) achieved 70% COD removal using an anaerobic/aerobic sequencing batch reactor to treating VOE. In addition, other biological treatment processes using biomass rich in fungi showed a COD removal capacity of 86% under aerobic conditions (Caffaz *et al.*, 2007).

The TKN removal efficiency of the biological process averaged 90% removal (Figure 3.4). However, nitrogen removal was not the main focus of this study

since nitrogen was found to be limiting in the VOE and was therefore supplemented in the influent feed at a C:N:P ratio of 100:5:1 in order to sustain biological activity. All microorganisms require certain basic nutrient elements. Carbon, nitrogen and phosphorus are required by microorganisms in balanced amounts, the ratios can differ but a reasonable estimate for the ratio is C:N:P of 100:5:1. The C:N:P ratio ultimately depends on the characteristics of the raw wastewater. In activated sludge processes however, this ratio can differ (WRC, 1984).

The OUR reflects the rate at which microorganisms use oxygen (Lilley *et al*., 1997). It is not common practice to characterize the transformation kinetics of lipids in activated sludge using OUR. However, in this study OUR measurement was used rather as an indication of the metabolic activity of the activated sludge biomass of the biological process. An average OUR of 19.4 mgO/L.h was measured in the aerobic reactor (Figure 3.5). This was considerably lower than OUR's measured in biological processes treating domestic wastewaters and thus indicated a lower biological activity of the biomass. Padayachee *et al.* (2006) demonstrated an average OUR of 31.0 mgO/L.h within the aerobic reactor of a laboratory-scale MLE process treating domestic wastewater. The comparatively lower OUR could be attributed to the oily nature of the VOE resulting in possible lipid overloading of the activated sludge biomass, although the VOE was pretreated (Hrudey, 1982). Banerji (1974) suggested that high FOG loadings may cause the activated sludge floc to become coated with hydrophobic material, thereby limiting oxygen transfer efficiency and reducing the OUR. However, the MLVSS followed the same pattern as the OUR profile. The biomass concentration of the aerobic mixed liquor of the process was determined using MLVSS analysis. By wasting of 1.75 L of sludge per day an average MLVSS concentration of ±3000 mg/L was maintained in the aerobic reactor (Figure 3.5). Results of MLVSS determinations (Figure 3.5) indicate a high concentration of volatile suspended solids in the aerobic reactor which consists mainly of organic matter and dead or living microorganisms (Bitton,

1999). This indicates a significantly high biomass concentration present in the mixed liquor of the laboratory-scale biological process.

The F/M ratio is an important parameter in the operation of activated sludge processes and is used to describe the organic loading and potential food availability to the microbial population (Droste, 1997). The F/M ratio and sludge age are all interrelated and impact on the process performance and the ratios reflected in this study were low and maintained between 0.26 and 0.30 (Figure 3.6). Since having a higher microorganism concentration (>0.1 - 0.4), less substrate is available for biomass concentration and therefore produces less biomass per unit of organic load. High quality effluent with a low COD concentration is produced with good floc formation, but the oxygen requirement is greater (Seviour *et al*., 1999). Increasing the F/M ratio of the system has been reported to have an overloading effect on the biomass, resulting in poor system performance overall (Casey *et al*., 1995).

3.5 Conclusion

Pretreatment of the raw VOE using commercial flocculent C40 or removed the majority of the emulsified fats and oils thus reducing the organic load by 77%, thereby rendering the VOE amenable to biological treatment.

After a two month acclimation period the laboratory-scale MLE process reached steady-state conditions demonstrated by consistent COD and TKN removal efficiencies.

The COD removal capacity of the laboratory-scale process averaged 84% indicating that the majority of the influent COD is biodegradable.

The VOE had the following effects on the activated sludge biomass process of a laboratory-scale MLE process: (i) a reduced OUR due to possible lipid overloading and (ii) an elevated MLVSS concentration.

- ✓ Biological treatment can be used to successfully treat VOE. However, it would be practical as a polishing step in the treatment of VOEs as a pretreatment step would be probably be necessary.

CHAPTER 4

Microbial Community Analysis of the Laboratory-scale Biological Treatment Process Using Fluorescent *in situ* Hybridization

4.1 Introduction

The use of molecular methods, specifically hybridization with rRNA targeted oligonucleotide probes, provides novel insights with respect to the structure and dynamics of microbial communities in activated sludge (Daims *et al.*, 2001). According to Activated Sludge Model no.2 (Henze *et al.*, 1995) heterotrophic organisms comprise several groups; the ordinary heterotrophs, which grow aerobically and are responsible for COD removal, denitrifying organisms growing anoxically, and the fermenters, which grow anaerobically. Previously, culture dependant techniques such as MPN method and heterotrophic plate counts, have been used to characterize and enumerate these communities in activated sludge (Banks & Walker 1977; Lotter and Murphy 1985). However, only 15% of the indigenous bacteria in activated sludge could be cultivated (Kämpfer *et al.*, 1996; Wagner *et al.*, 1993). These limitations have led to techniques using the 16S rRNA approach. In particular, fluorescent in situ hybridization (Amann *et al.*, 1995), polymerase chain reaction and denaturing gradient gel electrophoresis (Muyzer *et al.*, 1993) have been used extensively to conduct microbial community analysis.

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 labelled oligonucleotide probes, because they are highly conserved and naturally amplified, and can therefore be used in determinative studies in microbiology (Amann *et al.*, 1990). Using selected regions within larger rRNA molecules (16S and 23S rRNA) as hybridization targets for synthetic oligonucleotides, probe specificity to individual phyla or species, can be freely adjusted. In addition, DeLong *et al*. (1989) showed that probe binding varied with ribosomal content and reflected cell growth rate, viz., metabolically active cells will produce

intensified fluorescence, because of their increased rRNA content. The application of FISH for microbial community analysis of activated sludge processes could be considered a novel approach with a comparatively higher degree of success. Dual staining of samples with probe EUB338 and DAPI a DNA intercalating dye (Hicks *et al.*, 1992) gives not only an indication of the metabolic activity of bacteria, but also that cells had sufficient rRNA for detection, were permeabilized for probes by standard fixation procedures. Therefore, a high EUB:DAPI ratio in activated sludge would indicate a highly metabolically active bacterial population.

In probing COD removing activated sludges from various municipal plants with oligonucleotide probes specific for *Proteobacteria,* Wagner *et al*. (1993) demonstrated the dominance of *Proteobacteria*, which together comprised 60 - 75% microbial cells stained with DAPI. Wagner and Amann (1997) reported members of the beta-P*roteobacteria* as playing a major role in the microbial consortia of activated sludge plants and alpha- and gamma-*Proteobacteria* classes being less abundant. Padayachee *et al*. (2006) hybridized mixed liquor from a laboratory-scale MLE process treating domestic wastewater and confirmed that 75 - 80% of total DAPI stained were detected by probe EUB indicating that bacterial cells dominated the mixed liquor. Results from this study also confirm dominance of alpha- and gamma- subclasses of *Proteobacteria* in the mixed liquor of the aerobic and anoxic reactors.

In this Chapter, the microbial composition of the laboratory-scale biological process was determined using FISH to identify the bacterial communities that may be implicated in the biological treatment of VOE.

4.2 Methods and Materials

4.2.1 Sampling of activated sludge mixed liquor

A sample was taken from the seed sludge obtained from Darvill WWTP (Pietermaritzburg, South Africa) which was used as the inoculum for the laboratory-scale MLE process. Samples for FISH analysis were taken from

the aerobic reactor of the laboratory-scale biological process on the 7th day of each of the 15 batches of VOE treated. Grab samples of 15 mL of mixed liquor were collected and decanted into a 15 mL centrifuge tube and kept on ice. Activated sludge biomass was harvested from the mixed liquor by centrifugation (5000 x *g* for 10 minutes at 4°C), washed twice with ice-cold phosphate buffered saline [PBS; 130 mM sodium chloride, 10 mM sodium phosphate buffer (pH 7.2)] and resuspended to original sample volume in 50% (v/v) ethanol in PBS (Amann, 1995b).

4.2.2 Fixation of bacterial cells

Gram-negative cells were rendered permeable to probes by fixation in ice-cold 4% (w/v) paraformaldehyde/PBS solution for 1.5 h (Appendix 5). Gram positive cells were fixed in 15 mL polypropylene centrifuge tube by the addition of ice-cold 98% ethanol at a final concentration of 50% (v/v) (Roller *et al*., 1994). Fixed samples were stored in 50% (v/v) ethanol in PBS at -4°C until required for hybridization.

4.2.3 Floc disruption and cell dispersion

In order to disrupt activated sludge flocs and facilitate the release bacterial cells, fixed samples were sonicated at low frequency with sonication parameters optimized at 5 W for 5 min (Appendix 6). Improved visualization of individual cells was achieved by dispersion with Igepal CA-630 (Sigma, St Louis, MO, USA). Igepal CA-630 a nonionic, nondenaturing detergent was added to sonicated samples at a final concentration of 0.1% (v/v) and vortexed briefly prior to spotting.

4.2.4 Cell immobilization on microscope slides

A volume of 10 µL of sonicated sample was spotted onto each well of a Teflon coated microscope slide pretreated with poly-L-lysine solution (Appendix 7). Spots were allowed to air dry before dehydrating through an ethanol series of 60, 80 and 98% (v/v) for 3 min each (Amann, 1995b). Dehydrated slides were stored upright in a dessicator at room temperature.

4.2.5 FISH and DAPI staining

Oligonucleotide probes were purchased from MWG-BIOTECH AG (Ebersberg, Germany) at a 0.01 µM scale, HPSF (high-purity salt-free) purified and modified on the 5' end with either a tetramethylrhodamine-5-isothiocyanate (TRITC) or 5(6)-carboxyfluorescein-N-hydroxysuccinimide (FLUOS) ester. Table 4.1 illustrates the oligonucleotide probes, FA and sodium chloride concentrations used during hybridization. These probes were selected due to the fact that within the *Proteobacteria*, the alpha-, beta- and gamma-subclasses and the subclass *Actinobacteria* (Gram-positive bacteria with high G+C DNA content) within the class *Firmicute* (Gram positive) predominate in activated sludge (Bond *et al*., 1999).

Table 4.1 Details of probes and hybridization conditions for *in situ* and dot-blot hybridization

Probe	Sequence (5' - 3')	Specificity	% FA*	M NaCl†	Reference
EUB338	GCTGCCTCCCGTAGGAGT	*Bacteria*	20	0.19	(Daims *et al.*, 1999)
EUB338-II	GCAGCCACCCGTAGGTGT	Planctomycetales	20	0.19	(Daims *et al.*, 1999)
EUB338-III	GCTGCCACCCGTAGGTGT	Verrucomicrobiales	20	0.19	(Daims *et al.*, 1999)
ALF1b	CGTTCGYTCTGAGCCAG	Alpha-*Proteobacteria*	20	0.19	(Wagner *et al.*, 1993)
BET42a	GCCTTCCCACTTCGTTT	Beta-*Proteobacteria*	35	0.08	(Yeates *et al.*, 2003)
GAM42a	GCCTTCCCACATCGTTT	Gamma-*Proteobacteria*	35	0.08	(Yeates *et al.*, 2003)
HGC69a	TATAGTTACCACCGCCGT	*Actinobacteria*	25	0.15	(Roller *et al.*, 1994)

*Percentage of formamide (FA) (%v/v) in the hybridisation buffer

†Molarity of sodium chloride in the wash buffer.

Hybridizations were performed in a 50 ml polypropylene centrifuge tube, isotonically equilibrated with hybridization buffer as outlined by Amann (1995b). A volume of 10 µL of hybridization buffer/probe mixture containing; 50 ng probe (5 ng/µL), 0.9 M NaCl, 0.01% SDS, 20 Mm Tris/HCl, pH 7.2 and X% (v/v) FA was applied to each dehydrated spot (specific FA percentages

are given in Table 4.1 and hybridized at 46°C for 1.5 h. Probes EUB338, EUB338-II and EUB338-III were used in an equimolar mixture called EUBmix according to Yeates *et al*. (2003). Probes BET42a and GAM42a were hybridized simultaneously to increase specificity due to the single mismatch at position 1033 between the target sequences of these probes (Yeates *et al.*, 2003). Hybridization was stopped by rinsing unbound probe from slides with wash buffer containing; 20 mM Tris/HCl, 0.01% SDS, 5 mM EDTA and Y M NaCl (specific NaCl molarities are given in Table 1) prewarmed to 48°C. Slides were transferred to a 50 ml polypropylene centrifuge tube filled with prewarmed wash buffer and incubated for 20 min at 48°C. Buffer salts were removed by dipping the slides briefly in deionized water, excess water shaken off and slides were air dried. Spots were stained after hybridization with 10 µL of 0.25 µg/mL DAPI solution for 10 min in the dark, rinsed with deionized water and allowed to air dry. Slides were mounted in VECTASHEILD® anti-fading mounting medium (Vector Laboratories, Burlingame, CA) and laminated with clear nail polish.

4.2.6 Microscopy and image analysis

Hybridizations were viewed under a Zeiss Axioplan microscope (Carl Zeiss, Göttingen, Germany) fitted for epifluorescence with a 50 W high pressure mercury lamp and filter sets 02 to visualize DAPI stained cells (blue), 09 to visualize fluorescein stained cells (yellow/green) and 15 to visualize rhodamine stained cells (red). Images were captured using a CCD camera (Hamamatsu, Japan). From each hybridization thirty random fields using 400X magnification were selected for enumeration. Cells bearing a detectable fluorescent signal were enumerated using Zeiss KS300 image analysis software (Carl Zeiss, Göttingen, Germany). Relative probe percentages for each bacterial group were calculated by dividing the number of specific probe conferred cells by the number of cells bound to probe EUBmix in each field and expressed as a percentage of EUBmix.

4.2.7 Statistical analysis

Statistical analyses were performed using Microsoft *Excel* spreadsheet software including the 'Analysis Toolpak addin'. Averages and standard deviations were performed on all relative probe percentages. The paired *t*-test was used to determine the effect of VOE on the bacterial populations in the activated sludge mixed liquor. The Pearson product-moment correlation coefficient (r) was used to investigate the association between the EUB:DAPI ratio and MLVSS concentration of the laboratory-scale biological treatment process. Analysis of variance (ANOVA) single factor, with Alpha set at 0.05 was used to determine differences amongst the four bacterial populations investigated.

4.3 Results

A detailed microbial community analysis of the bacterial population as per phylogenetic classification is presented in Figure 4.2. These results were expressed as average percentages calculated by dividing the number of probe specific counts by the number of cells that have bound to probe EUBmix. A sample of 30 random microscopic fields under 400x magnification was used. The total probe percentage for each sample was calculated by adding together the average probe percentages. The microbial community profile of the seed sludge obtained from the aerobic zone of Darvill Wastewater Treatment Purification Works is identified as "Darvill". This sample served as the control to determine microbial community shifts when exposed to VOE during the biological treatment process. Samples 1 to 15 represent grab samples of mixed liquor collected during the treatment of each of the batches of VOE respectively. The probe EUBmix (Table 4.1) was used to assess the overall physiological state and activity of the bacterial communities present in the activated sludge biomass since substrate degradation is coupled to biomass growth i.e. cells obtain their energy from substrate degradation (Kalmbach *et al*., 1997).

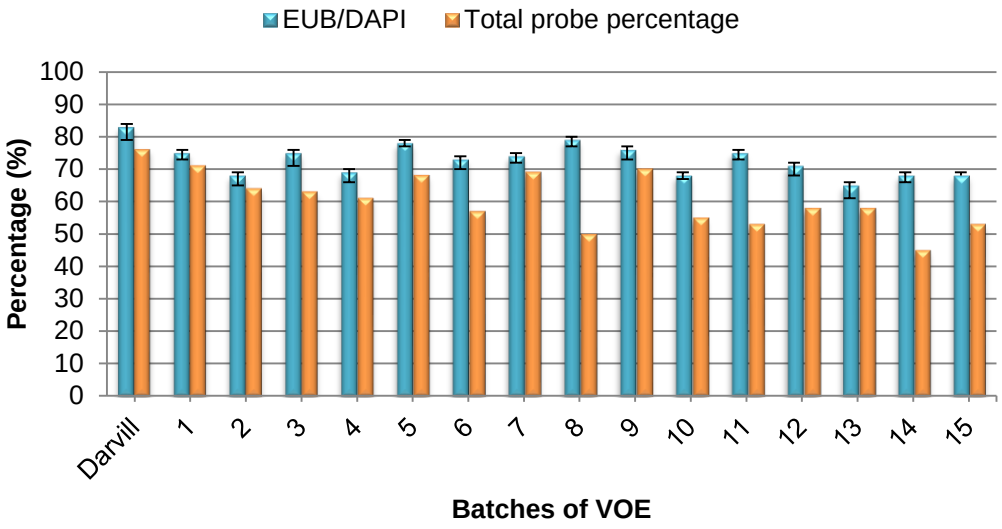


Figure 4.1 Percentages of EUBmix-hybridized cells relative to DAPI and total probe percentages calculated for the laboratory-scale biological process.

Hybridization of samples with EUBmix showed that the Darvill seed sludge had an EUB/DAPI ratio of 83±3% (Figure 4.1). Samples from the biological treatment process demonstrated a slight decrease in EUB:DAPI ratio which ranged from 65±3% to 78±5% (Figure 4.1) during steady-state conditions as demonstrated by consistent COD and TKN removal efficiencies of 84% and 90% respectively. The results of MLVSS concentration and EUB:DAPI ratios of samples 1 to 15 from the laboratory-scale biological process were analyzed using the Pearson correlation coefficient in order to determine whether a correlation between the two exists. A Pearson correlation coefficient value of 0.68 was calculated for the laboratory-scale process.

These results confirm that the majority of the cells belonged to the domain *Bacteria*. The community profile of the Darvill seed sludge demonstrated the beta-sublasss of *Proteobacteria* to predominate in the sludge i.e. cells hybridizing with probe BET42a accounted for 34±2% of cells detected by

EUBmix followed by the alpha-subclass with probe ALF1b detecting 21±2% of EUBmix cells and the gamma-subclass with probe GAM42a accounting for 16±1% of EUBmix confirmed cells (Figure 4.2). The *Actinobacteria* represented by probe HGC69a constituted only 5±2% of EUBmix cells (Figure 4.2).

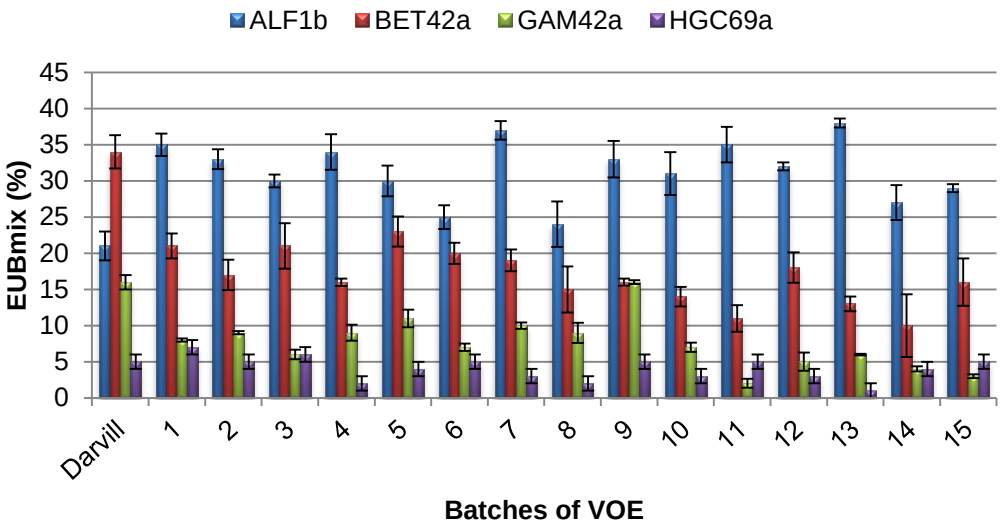
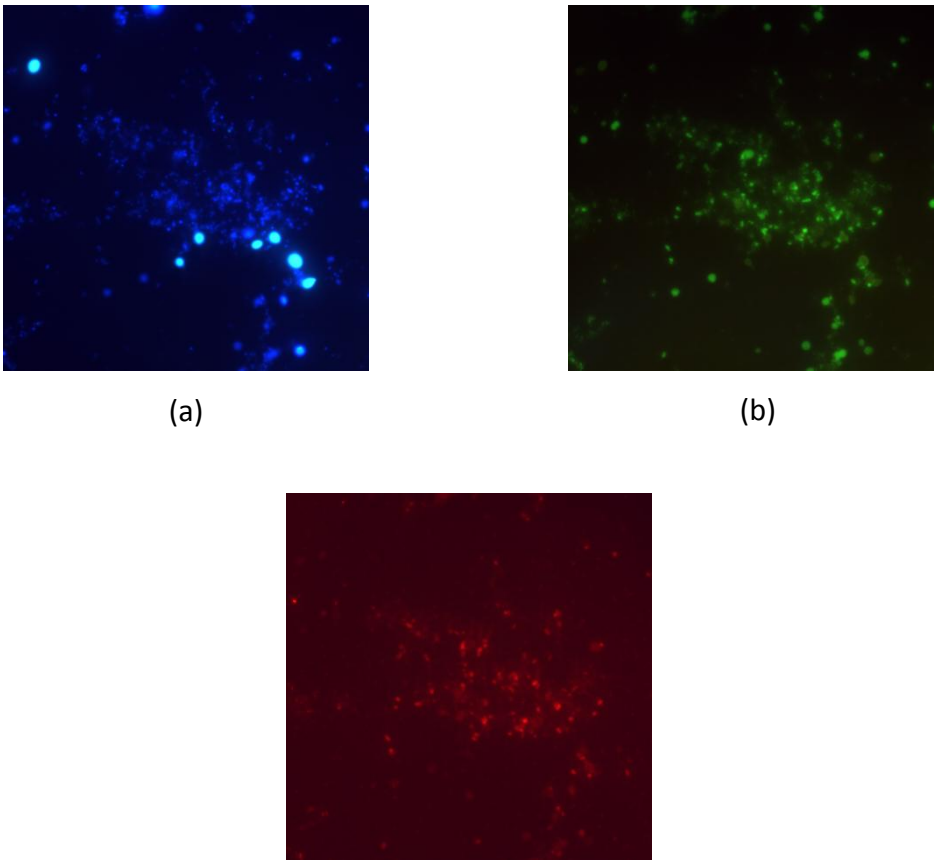


Figure 4.2 Percentages of group-specific probes relative to EUBmix counts of the aerobic mixed liquor of the laboratory-scale biological process.

On comparison of the profiles of the seed sludge sample with samples 1 to 15 from the biological process, a population shift in the microbial communities was observed (Figure 4.2). Results of the paired t-test indicated there was a significant difference in the alpha-*Proteobacteria* (P = 1.3%), beta-*Proteobacteria* (P < 0.001%), gamma-*Proteobacteria* (P < 0.001%) and *Actinobacteria* (P = 1.1%) populations in the seed sludge and lab-scale process samples. The alpha-*Proteobacteria* population increased by 10% on average in samples 1 to 15 and hence became the predominant population constituting on average 31% of EUBmix cells with relative percentages

ranging from 21±1% in batch 3 to 38±1% in batch 13 (Figure 4.2). During this period the process was at steady state and performing optimally. The mean influent COD was 969 mg/L and removal efficiency was averaging 84%. This population shift was possibly as result of the activated sludge adapting to the selective pressure of the pretreated VOE resulting in the dominance of the alpha-*Proteobacteria* over the other populations detected by the set of probes used. The next abundant population in samples 1 to 15 was the beta-*Proteobacteria* which diminished by 50% and averaged 17% of EUBmix hybridized cells ranging from 10±4% in batch 14 to 30±3% in batch 3 (Figure 4.2). The gamma-subclass of *Proteobacteria* also showed a 50% reduction in relative numbers and accounted for 8% of cells detected by EUBmix and ranged from 2±0.6% in batch 11 to 16±0.3% in batch 9. The *Actinobacteria* population represented by probe HGC69a showed the lowest presence with a 1% decrease overall and represented 4% of EUBmix cells with relative percentages ranging from 1±0.3% in batch 13 to 7±5.4% in batch 1 (Figure 4.2). Photomicrographs of activated sludge from the aerobic reactor of the biological treatment process stained with DAPI and hybridized using group specific probes are presented in Figures 4.3 (a - c). Figure 4.3a shows blue cells stained with DAPI representing the total number of cells in the field. Figure 4.3b is a photomicrograph of the same microscopic field using the fluorescein filter set to visualize yellow cells hybridized by the probe EUBmix representing bacterial cells. While Figure 4.3.c is a photomicrograph of the same microscopic field using the rhodamine filter set to view red cells stained with the ALF1b probe indicating that they are from the alpha-subclass of *Proteobacteria*.



(a) (b)

(c)

Figure 4.3 **Photomicrograph (400x) of activated sludge mixed liquor of the aerobic reactor stained with DAPI (a), and hybridised with fluorescein labelled probe EUBmix (b) and rhodamine labelled probe ALF1b (c).**

4.4 Discussion

The Darvill seed sludge sample was profiled and used as a control to determine any shifts in the microbial population when the mixed liquor was subjected to the pretreated VOE. The EUB:DAPI ratio was used to determine the effect of VOE on the activated sludge biomass of the laboratory-scale MLE process as a whole, while changes in individual bacterial populations

were monitored using FISH with probes specific for the *Actinobacteria* and members of the alpha-, beta- and gamma-subclasses of *Proteobacteria*.

Results of hybridization with EUBmix revealed that 83±3% of DAPI stained cells in the Darvill seed sludge and 72% of cells stained with DAPI in the aerobic mixed liquor of the biological treatment process (samples 1 to 15) bound to probe EUBmix and can therefore be assumed to be metabolically active, belonging to the domain *Bacteria* (Figure 4.2). Since the mixed liquor of laboratory-scale biological process exhibited lower EUB:DAPI ratios than the seed sludge during steady-state conditions, this would possibly indicate a slightly diminished contribution of the bacteria in the biological process when compared with a full scale wastewater treatment process such as Darvill WWTP.

The lack of correlation between MLVSS concentrations and EUB:DAPI ratios (Pearson correlation coefficient of 0.68) demonstrates that MLVSS analysis is an indirect method which is not sensitive to changes in metabolic activity and therefore does not give a true reflection of the metabolic activity of the activated sludge biomass. These findings validate the importance of molecular techniques such as FISH in accurately quantifying the active biomass concentration in activated sludge mixed liquors. Washing of mixed liquor samples twice with PBS prior to fixation proved successful in removing excess oils from the flocs which minimized background noise, thereby improving visualization during epifluorescent microscopy. Figure 4.3 displays the *in situ* distribution of bacterial simultaneously stained with DAPI and hybridized with probes EUBmix and ALF1b. Sonication parameters for adequate floc disruption were optimized at 5 W for 5 min. The composition and distribution of the bacterial populations as per phylogenetic classification system in the aerobic mixed liquor of the laboratory-scale biological process are presented in Figure 4.2. The results are expressed as percentages of probe specific counts divided by EUB338mix counts. As mentioned above the microbial community profile of the Darvill seed sludge served as a control

to determine possible population shifts that may have occurred resulting from exposure of the activated sludge to VOE during biological treatment. The community structure profile of the seed sludge demonstrated the beta-*Proteobacteria* to be the numerically dominant group of *Bacteria*, i.e. 34±2% of bacterial cells represented beta-*Proteobacteria* (BET42a/EUBmix), followed by alpha-*Proteobacteria* at 21±2% (ALF1b/EUBmix), the gamma-*Proteobacteria* at 16±1% (GAM42a/EUBmix) and *Actinobacteria* at 5±2% (HGC69a/EUBmix) (Figure 4.2). These results reflect a similar scenario in other full scale wastewater treatment processes. Wagner *et al*. (1993) identified members of the beta-*Proteobacteria* predominating in aerated activated sludge systems with 42% of cells binding to BET42a with respect to probe EUB. Wong *et al*. (2005) surveyed wastewater treatment plants in Japan and found the beta-*Proteobacteria* to be the most abundant group in two treatment plants and accounted for 20% and 30% of EUBmix-stained cells respectively. Current findings also support previous studies on activated sludge from enhanced biological phosphorous removal (EBPR) processes indicating the dominance of the beta-subclass of *Proteobacteria* (Bond et al., 1999; Mudaly *et al.*, 2000).

Distinct differences between the microbial communities present within the seed sludge sample and laboratory-scale biological process (samples 1 to 15) were observed using the paired t-test. In the laboratory-scale biological process, it was evident that the alpha-*Proteobacteria* became numerically predominant binding to 31% of EUBmix detected cells, followed by beta-*Proteobacteria* and gamma-*Proteobacteria* constituting 17% and 8% of EUBmix stained cells, respectively (Figure 4.2). The *Actinobacteria* represented only 4% of EUBmix stained cells (Figure 4.2). A similar observation was made by Stoffels *et al*. (1998) using a trickle bed bioreactor to treat aromatic compounds whereby the seed inoculum comprising of gamma-*Proteobacteria* when inoculated into the fermenter, resulted in predominance of members of the alpha-*Proteobacteria* and beta-*Proteobacteria*. In the current research, the response to pollutant adaptation

resulted in the process selecting the appropriate bacteria to degrade the substrate, thereby enforcing dominance of the alpha-*Proteobacteria.* The decrease in the beta-*Proteobacteria* could be attributed to the toxicity of the substrate (VOE) to the bacteria within the subclass. Substrate uptake is a very important factor 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 other Proteobacterial populations in relation to the alpha-*Proteobacteria*. The overall comparative predominance of alpha-*Proteobacteria* throughout the process treating VOE further supported the theory of acclimation, i.e. members of the alpha-*Proteobacteria* had adapted to the substrate.

Comparative analysis of the seed sludge and the laboratory-scale biological process using group-specific probes showed dominance of the beta-*Proteobacteria* in the seed sludge and the alpha-*Proteobacteria* in the laboratory-scale biological process. It is common knowledge that bacterial populations in full scale wastewater treatment processes are dominated by the beta-*Proteobacteria* and therefore the change in the population dynamics in the process treating VOE could be attributed to the nature of the substrate, which comprised primarily VOE. A significant finding by Kaewpipat and Grady (2002) showed that 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 community composition of the processes, the TKN removal efficiencies were similar, demonstrating that different microbial communities can be functionally similar.

Current findings were also in agreement with research by Layton *et al*. (2000), who conducted a detailed microbial community profile of an activated sludge system treating wastewater from a chemical manufacturing process. On acclimation, the percentage of 16S rDNA cloned sequences showed

representatives of the alpha-*Proteobacteria* predominating, followed by the beta-*Proteobacteria* and *gamma-Proteobacteria*. The microbial community of a dairy wastewater plant was shown to be numerically dominated by spindle shaped bacteria belonging to the “Flavobacter-Bacteriodes” phylum (Manz *et al.*, 1994). These findings reiterate that the microbial community profile of municipal WW plants treating domestic WW and comprising predominantly beta-*Proteobacteria* (Wagner *et al.*, 1993) certainly differ from those treating industrial effluents. Population shifts become evident once the seed sludges are exposed to the industrial wastewaters (influent) and can be attributed to substantial differences in the chemical composition of waste streams. Industrial wastewaters generally have a much higher total organic carbon load when compared to municipal (Layton *et al.*, 2000). With reference to the current research, the VOE was a complex substrate comprising a range of lipids, which necessitated initial hydrolysis of the substrate by the bacteria before the substrate could be metabolized, resulting in a limiting step in the degradation process. FISH analysis confirmed that the gamma-*Proteobacteria* and *Actinobacteria* were numerically low in both processes. Previous research has shown that the gamma-*Proteobacteria* were less abundant in municipal activated sludge (Wagner *et al*., 1993; Manz *et al*., 1994). Low percentages of *Actinobacteria* were also verified by Wagner and Amann (1997) who found only 9% of all bacteria present in activated sludge samples originating from a municipal sewage treatment plant were *Actinobacteria*.

The sum of the average group-specific probes percentages (ALF1b, BET42a, GAM42a and HGC69a) of the seed sludge was 76% (Figure 4.1) and when compared to the EUB/DAPI ratio (83±3%) was found to be 7% slightly lower. A similar fashion was observed when comparing total probe percentages to EUB:DAPI ratios of the laboratory-scale process batch samples. The sum of the average group-specific probes percentages for the laboratory-scale biological process was 60% which was 11% lower than the average EUB:DAPI ratio of 72% (Figure 4.1). These differences suggest that some of

the bacterial populations have not been accounted for. These populations could possibly belong to other divisions such as the Cytophaga-Flavobacterium-Bacteroides phylum (Kämpfer *et al.*, 1996) or low G + C Gram-positive bacteria (Snaidir *et al.*, 1997) or even possibly hitherto undiscovered taxonomic ranks. This trend was seen throughout all samples with the total probe percentages being lower than the EUB/DAPI ratios. Sample 8 and sample 11 showed the largest differences between total probe percentages and EUB/DAPI ratios. However, this phenomenon did not impact on the COD removal efficiency or the OUR of the process at that time. This could be due to bacterial subclasses that were not accounted for, possibly contributing towards overall COD removal. It is also possible that upon acclimation, the laboratory-scale process could have selected for a highly specialized population that could not be detected by the group-specific probes used. Theoretical limitations of rRNA probes involve the target RNA molecule. The 16S RNA may be too highly conserved to discriminate between closely related populations. Different species may have near identical 16S RNA sequences (Fox *et al*., 1992). In such cases, the 23S rRNA is used. Another limitation is derived from the fact that the RNA diversity has only been partially described (Amann *et al*., 1995). This means that even if a probe was designed to be specific on the most complete current data set and shown to be specific for a range of test organisms, it may still hybridize with as yet unknown organisms (Amann and Ludwig, 2000).

The applied value of the research is in the accurate elucidation of the microbial communities involved in the biological treatment of VOE, using novel molecular techniques. This information will aid plant operators and researchers to understand the microbial population dynamics involved, with the ultimate objective of trouble-shooting and optimizing biological treatment of VOE.

4.5 Conclusion

A comprehensive knowledge of the bacterial communities mediating activated sludge processes within wastewater works can only originate from a combination of various approaches. In this Chapter the applicability of FISH was demonstrated to be an accurate measure of the metabolic activity of the activated sludge biomass as well as describing changes within the various bacterial communities present in a steady-state biological treatment process. FISH is more sensitive than conventional methods and could be regarded as a more accurate technique than MLVSS analysis in determining the active biomass fraction of activated sludge mixed liquor. FISH gives a more reliable result as it measures the active biomass fraction directly or *in situ* and is sensitive to changes in cellular RNA content as only metabolically active cells are counted. Comparative evaluation of the microbial profiles of the Darvill seed sludge and samples from the laboratory-scale process showed that although there was a shift in population dominance from beta in the seed sludge to alpha-*Proteobacteria* when treating VOE, no major population shifts were observed during the 15 batches treated where alpha-*Proteobacteria* was largely dominant**.** The results obtained conclusively demonstrate that in a laboratory-scale MLE process treating VOE, dominance of the alpha-*Proteobacteria* over members of the beta-*Proteobacteria*, gamma-*Proteobacteria* and *Actinobacteria* occurred. This could be attributed to pollutant adaptation of the alpha-*Proteobacteria* to the VOE, resulting in the dominance of the alpha-*Proteobacteria*.

CHAPTER 5

Microbial Community Analysis of the Laboratory-scale Biological Treatment Process Using Dot-Blot Hybridization

5.1 Introduction

Molecular techniques based on rRNA sequence analysis are used in the identification and quantification of microorganisms in wastewater treatment plants (Daims *et al*., 2001). These techniques are used to avoid the limitations of microscopic or culture based methods. Ribosomal RNA targeted hybridization probes are commonly used (Wagner & Amann, 1997; Daims *et al*., 2001). Two hybridization formats are generally used: fluorescent *in situ* hybridization (FISH) and dot-blot hybridization using extracted rRNA immobilized on membranes. Dot-blot hybridizations are suited for multiple samples and are amendable to quantitative analysis (Keith *et al.*, 2005). The ribosome content of a specific subclass is reflected by dot-blot hybridization and gives a measure of the contribution of a group to the total metabolic activity (Hoppert, 2003). The quantitative dot-blot hybridization method using 16S rRNA targeted oligonucleotide probes can be used to determine the relative rRNA abundance of a specific bacterial group relative to the total rRNA, as long as it is above the detection limit (Robinson *et al.*, 2004). Since the rRNA of a microbial cell varies according to its activity and physiological history (Flärdh *et al.*, 1992), data obtained cannot be translated to cell numbers. In addition, varying RNA extraction efficiencies from different microbial populations and degradation of rRNA during the procedure might bias the method by affecting probe target sites in different ways (Robinson *et al.*, 2004).

In the dot-blot format total RNA is isolated from environmental samples and immobilized on a membrane together with a dilution series of known reference RNA standards. Subsequently, the membrane is hybridized with probes labelled either radioactively or with digoxigenin (DIG). After a stringent washing step the amount of target rRNA hybridized is quantified

with a Phosphor Imager in the case of radioactively labeled probes or a gel documentation system when DIG-labelled probes are used. Results are usually expressed as nanograms (ng) of target rRNA per weight or volume of the environmental sample e.g. mgRNA/gVSS in wastewater treatment. Alternatively the amount of population specific rRNA can be detected with the specific probe and expressed as a fraction of the total (bacterial) RNA using the bacterial to EUB338 (Wagner *et al.*, 1993) or total 16S rRNA using a universal probe specific for all known organisms (Raskin *et al.*, 1994). Quantitative blot analyses have been applied to wastewater microbiology for the detection of problematic filamentous bacteria responsible for activated sludge foaming (De Los Reyes *et al.*, 1997; 1998; Keith *et al.*, 2005) and for spatial distributions of autotrophic ammonia- and nitrite-oxidizing bacteria (Mobarry *et al.*, 1996). In this Chapter the microbial community profile of the laboratory-scale biological treatment process was determined by dot-blot hybridization with DIG-labelled oligonucleotides in order to verify the bacterial communities identified by FISH (Chapter 4) which have been implicated in the biological treatment of VOE.

5.2 Methods and Materials

5.2.1 Sampling of activated sludge mixed liquor

For comparative analysis of dot-blot hybridization with FISH analysis, the same frequency of sampling was used i.e mixed liquor samples were taken after the 7th day during each of VOE batch treated. A sample of the Darvill seed sludge was also taken. Therefore, a total of 16 samples were taken and analyzed. Mixed liquor samples were kept on ice and subsequently stored at -20°C in a 50 mL polypropylene centrifuge tube.

5.2.2 Extraction of total RNA

A modification of the method of Lane (1996) was used for the extraction of total RNA. Activated sludge was harvested by centrifugation (5000 x *g* for 10 minutes at 4°C) in a microfuge (Biofuge fresco, Heraeus GmbH, Germany), washed twice with PBS and supernatant discarded. Approximately 200 µL of

cell pellet was added to a 2 mL bead beating vial filled one-third (±700 µL) with acid washed, baked glass beads (0.106 µm Sigma, U.S.A) (Appendix 8). A volume of 750 µL of bead beating buffer (50 mM NaOH, 10 mM disodium EDTA. Filter sterilized) and 50 µL of 20% SDS were added to the bead beating vial. The remaining portion of the vial was filled with buffer-saturated phenol (Appendix 9) and beat for 4 minutes at 4600 rpm using a Mini-Beadbeater (Biospec Products, U.S.A). The vial was heated to 60°C in a hot block for 10 - 15 minutes, beat for 2 more minutes and spun at 1000 x *g* for 10 minutes at 4°C to separate the phases. The top (aqueous) layer was transferred to a clean Eppendorf microfuge tube and 400 µL of buffer-saturated phenol was added and vortexed. A volume of 100 µL chloroform-isoamyl alcohol was added and vortexed. The vial was spun again at 1000 x *g* for 10 minutes at 4°C. The top layer was transferred and the phenol-chloroform extraction was repeated. The top layer was transferred and 1/10 volume of 3 M NaOH, pH 5.1 and two volumes ethanol at -20°C was added and precipitated overnight at -20°C. The RNA was pelleted at 10 000 x *g* for 30 minutes at 4^{o}C and the supernatant discarded. The pellet was air-dried for 15-20 minutes in a laminar flow cabinet before gently re-suspending in 500 µL of sterile deionized water. The concentration and purity of RNA was determined spectrophotometrically by measuring the absorption at 260 nm (A_{260}) and 280 nm (A_{280}) using a UV-visible spectrophotometer (Ultrospec 2000, Pharmacia Biotech) and determining the A_{260}/A_{280} ratio (Appendix 10).

5.2.3 Dot-blot hybridization

Dot-blot hybridization was performed according to (Manz *et al*., 1992). RNA was denatured by adding 3 volumes of formamide/formaldehyde (37%)/3-(N-morpholino)-propanesulfonic acid (MOPS buffer) [5:1.62:1]. After incubation at 60°C on a heating block, samples were diluted to 1 µg, 0.5 µg, 0.25 µg, 0.125 µg, 0.0625 µg and 0.0312 µg in sterile deionised water to a final volume of 100 µL and applied to positively-charged nylon membrane (Roche Diagnostics GmbH, Germany) using a dot-blot manifold (Appendix 11). RNA was immobilized by baking the membranes in an oven at 120°C for 30 min.

The membranes were prehybridized for 1.5 h at 46°C with 100 mL solution containing 5 x SSC, 2% blocking reagent, 0.1% N-lauroylsarcosine and 0.02% SDS . Hybridizations were performed at 46°C for at least 12 h using a solution of 0.1% N-lauroylsarcosine, 4% blocking reagent (Used to decrease the background in non-radioactive hybridization and detection of nucleic acid hybrids), 0.01% SDS, 0.9 M NaCl, 20% formamide (for probes ALF1b, EUBmix), 45% formamide (for probes BET42a, GAM42a) or 25% formamide (probe HGC69a), and 5 pmol/mL of DIG-labeled oligonucleotide probe. Information regarding probe sequences is given in Table 4.1 (Chapter 4).

Following hybridization the membranes were washed twice at 48°C for 15 min in 100 mL washing buffer (probes ALF1b, EUBmix: 20 mM Tris, 0.01% SDS, 190 mM NaCl; probes BET42a, GAM42a: 20 mM Tris, 0.01% SDS, 44 mM NaCl). Hybridization solutions and wash buffers used in hybridizations were prepared from stock solutions (Appendix 12). Probe-conferred DIG molecules were detected using anti-DIG-antibodies coupled with alkaline phosphatase and colorimetric substrate reagents nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Roche Diagnostics GmbH, 2000) (Appendix 13). Images of the dots on the membranes were captured using the G-Box gel documentation system using a CCD camera with white Epi overhead lighting (Syngene, USA). Images were analyzed using Gene Tools software package (Syngene, USA). The abundances of specific groups of organisms (alpha, beta, gamma *Proteobacteria* and *Actinobacteria*) are expressed as percentages of the total bacterial 16S rRNA in the sample. The total bacterial rRNA was quantified with the bacterial probe EUBmix. Ribosomal RNA was isolated from the following pure cultures of bacteria and used as positive controls for the purpose of confirming probe specificity and to eliminate any possible systematic errors;

- *Escherichia coli* was used to confirm probe EUBmix, specific for all hitherto sequenced species affiliated to the kingdom *Bacteria*.

- *Acetobacter acetii* was used to confirm probe ALF1b, specific for the alpha-subclass of *Proteobacteria*.
- *Alcaligenenes faecalis* was used to confirm probe BET42, specific for the beta-subclass of *Proteobacteria*.
- *Acinetobacter calcoaceticus* was used to confirm probe GAM42a, specific for the gamma-subclass of *Proteobacteria.*
- *Corynebacterium glutamicum* was used to confirm probe HGC69a, specific for the *Actinobacteria*.

5.2.4 Statistical analysis

Statistical analyses were performed using Microsoft *Excel* spreadsheet software including the 'Analysis Toolpak addin'. Averages and standard deviations were performed on all relative probe percentages. ANOVA single factor, with Alpha set at 0.05 was used to determine differences amongst the group-specific probe hybridizations of the seed sludge and the laboratory-scale biological process. The Pearson product-moment correlation coefficient (r) was used to correlate values obtained from dot-blot hybridization and *in situ* hybridization.

5.3 Results

An RNA yield of 102.4 mgRNA/VSS was isolated from the Darvill seed sludge sample with purity ratio of 1.82 (Table 5.1). Purity ratios of the RNA isolated from the activated sludge biomass of the laboratory-scale biological process ranged from 1.59 to 1.73 (Table 5.1). These ratios are lower than those determined for pure RNA (1.95 – 2.10) suggesting the contamination of protein (Lane, 1996). These proteins may originate from bacterial cells and biofilm made up of extracellular polymeric substances (EPS) (Denecke, 2006). However, a second ethanol precipitation step resulted only in a moderate improvement in RNA purity. The RNA yield from the mixed liquor ranged from 77.1 mgRNA/gVSS to 90.6 mgRNA/gVSS (Table 5.1).

Table 5.1. RNA purity and yield from mixed liquor of the laboratory-scale biological process

Sample/VOE Batch	Purity (A_{260}/A_{280})	Yield (mgRNA/gVSS)
Darvill	1.82	102.4
1	1.68	87.6
2	1.59	78.4
3	1.69	73.8
4	1.59	77.1
5	1.64	83.0
6	1.62	90.6
7	1.71	76.8
8	1.69	76.5
9	1.71	73.6
10	1.73	83.8
11	1.68	86.3
12	1.66	84.2
13	1.70	75.2
14	1.65	81.0
15	1.63	79.2

Statistical analysis of the results of dot-blot hybridizations of the seed sludge using ANOVA single factor showed $P < 0.001\%$. This indicated a significant difference between the alpha-, beta- and gamma-*Proteobacteria* subclasses detected in the seed sludge. The community structure of the seed sludge using dot-blot hybridization showed the highest abundance of beta-*Proteobacteria* RNA molecules, which represented 47±5% of the total RNA community detected by probe EUBmix (Table 5.2). The alpha-*Proteobacteria* represented 28±4% of EUBmix hybridized RNA, while the gamma-*Proteobacteria* comprised 14±3% of EUBmix RNA (Table 5.2). Ribosomal RNA from the *Actinobacteria* subclass was not detected in the seed sludge.

Table 5.2 **Community structure analyses of the Darvill seed sludge and mixed liquor samples from the laboratory-scale biological process as determined by dot-blot hybridization**

Sample/VOE Batch	EUBmix (%)		
	ALF1b	BET42a	GAM42a
Darvill	28±4	47±5	14±3
1	16±1	n/d*	n/d*
2	26±6	n/d*	n/d*
3	55±3	15±4	6±1
4	45±5	17±3	7±3
5	48±8	23±5	4±1
6	49±2	26±3	8±6
7	54±7	19±6	6±3
8	42±6	23±7	4±2
9	51±5	18±5	7±5
10	44±7	14±3	3±1
11	41±4	21±4	4±1
12	51±3	19±7	7±2
13	44±5	27±3	5±2
14	46±2	22±8	4±2
15	50±3	19±4	8±3

* Below the detection limit of the method and therefore not detected.

Statistical analysis was also performed on the dot-blot hybridization results of samples 1 to 15 of the laboratory-scale biological process which showed a statistically significant difference between the alpha-, beta- and gamma-*Proteobacteria* populations ($P < 0.001\%$, ANOVA single factor). Dot-blot hybridization revealed that on average 43% of the total community rRNA, hybridized by probe EUBmix, belonged to the alpha-*Proteobacteria* which predominated in the process and ranged from 16±1% in batch 1 to 54±7% in batch 3 (Table 2). The beta-*Proteobacteria* probe BET42a bound to 16% of the EUBmix hybridized rRNA molecules, ranging from 14±3% in batch 10 to 26±3% in batch 6 and was not detected in batches 1 & 2. While the GAM42a

probe hybridized to 5% of the EUBmix confirmed rRNA molecules and ranged from 4±1% in batch 5 to 8±6% in batch 6 and was not detected in batches 1 & 2 (Table 5.2). Ribosomal RNA from the *Actinobacteria* subclass was not detected in any of the batches of VOE treated.

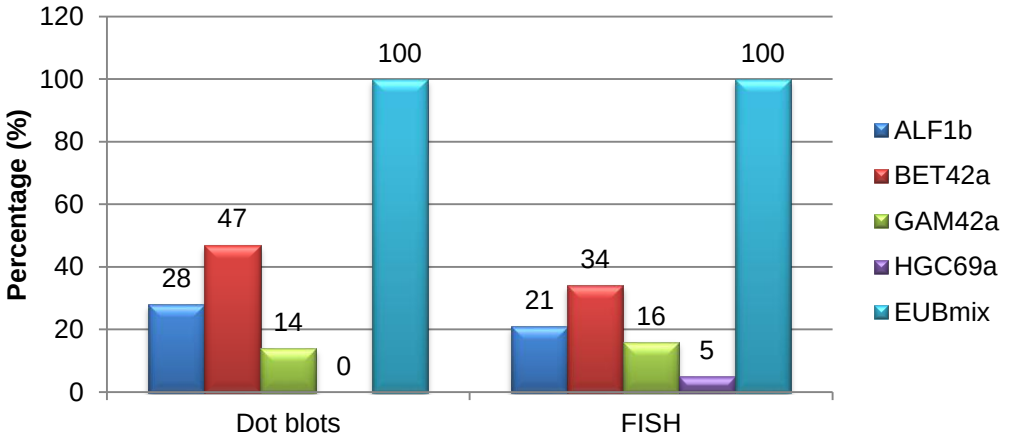


Figure 5.1 **Comparison of the community structure analyses of Darvill seed sludge as determined by dot-blot hybridization of extracted RNA and *in situ* hybridization.**

Figure 5.1 combines the results of microbial community analysis of the seed sludge using dot-blot and *in situ* hybridization. Figure 5.2 combines the results of microbial community analysis of the laboratory-scale biological process using dot-blot and *in situ* hybridization. The FISH results of the laboratory-scale biological process are expressed as averages of all 15 batches of VOE treated. There was perfect correlation (r = 1.0, Pearson correlation coefficient) between the results obtained from dot-blot and *in situ* hybridization in the seed sludge and samples 1 to 15 from the laboratory-scale biological process.

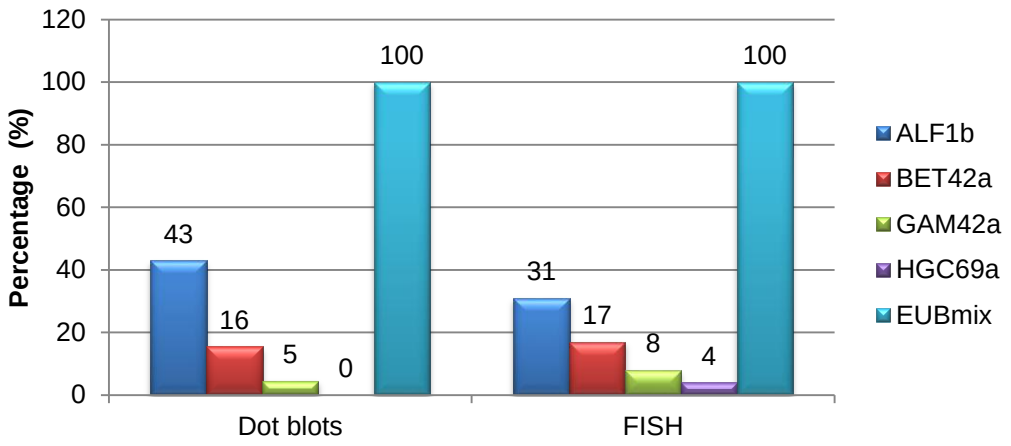


Figure 5.2 Comparison of the community structure analyses of aerobic mixed liquor samples as determined by dot-blot hybridization of extracted RNA and *in situ* hybridization.

5.4 Discussion

Organism abundance was estimated from the fractional contribution of its specific rRNA molecules to the total ribosome population. The results presented using dot-blot hybridization indicate that the alpha-, beta- and gamma-*Proteobacteria* are indeed metabolically active in the Darvill seed sludge and activated sludge in the laboratory-scale biological process. Hybridizations with the four group-specific probes were quantified relative to EUBmix, thus determining the contributions of the alpha-, beta- and gamma-*Proteobacteria* populations to the total bacterial rRNA. In the mixed liquor samples from the laboratory-scale biological process, rRNA molecules hybridizing with probes ALF1b (43%) and BET42a (16%) were more frequent than rRNA molecules hybridizing with probe GAM42a (5%) (Table 5.2). The amount of rRNA from the *Actinobacteria* group could not be detected by dot-blot hybridization in any of the samples analysed as it was below the detection limit of the method and hence gave no hybridization signal. This was confirmed by hybridization of RNA isolated from the positive control, a culture of *Corynebacterium glutamicum*.

Quantification of targeted groups by FISH and by dot-blot hybridization gave comparable results, indicating that the incidence of nonspecific labelling to fluorescence signal was negligible and did not affect sensitivity. The relative distribution of the alpha-, beta- and gamma-subclasses of *Proteobacteria* determined by dot-blot and *in situ* hybridization in samples 1 to 15 from the laboratory-scale biological process (Figure 5.1) were in agreement (r = 1.0, Pearson correlation coefficient) and both demonstrated the dominance of the alpha subclass of *Proteobacteria* over the beta-*Proteobacteria*, gamma-*Proteobacteria* and *Actinobacteria* subclasses, respectively. Results of dot-blot hybridizations and in situ hybridization of the seed sludge were also in agreement (r = 1.0, Pearson correlation coefficient) confirming dominance of the beta-*Proteobacteria* over the alpha- and gamma-*Proteoabacteria*, respectively. However, slight discrepancies between the two techniques were observed.

On comparison of results of the two techniques, dot-blot hybridization revealed an overestimation of alpha-*Proteobacteria* by 12% when compared with *in situ* counts. The subclass beta-*Proteobacteria* was found to be underestimated by 1% and the gamma-*Proteobacteria* by 3% using dot blot hybridization. FISH seems to be a more suitable method for the detection of *Actinobacteria* as compared with dot-blot hybridization. Underestimations using *in situ* hybridization could be attributed to the contribution of auto fluorescence of unbound probe not washed away during stringent washing steps or non specific binding of the probe to debris inherent in the mixed liquor such as extracellular polymeric substances (EPS). A key step for culture-independent nucleic acid approaches is the direct extraction of nucleic acids from environmental matrices. The parameters critical to effective recovery of nucleic acids include the efficiency of cell lysis, efficiency of nucleic acid recovery after lysis, and purification from contaminating protein like EPS. Although many methods have been successfully used, the effective recovery of nucleic acids from environmental samples is still a challenge. Differences in nucleic acid extraction efficiencies

amongst samples can lead to misrepresentation of the abundance of rRNA molecules present in a sample even before membrane hybridization. The mechanical method of cell lysis using bead-beating was originally chosen to rule out such artefacts by ensuring uniform extraction of RNA from the sample (Lane, 1996).

Due to varying sizes and ribosomal content of bacterial cells, i.e. larger cells consequently might have a higher cellular ribosome content, relative cell numbers cannot reliably be extrapolated from the rRNA abundance values determined by dot-blot hybridization. However, as already suggested by Stahl *et al*. (1988) these values could be valid measures for the contribution of a defined group to the total metabolic activity of the activated sludge biomass.

Activated sludge systems are usually dominated by beta-Proteobacteria (Mudaly *et al*., 2000; Wong *et al*., 2005; Padayachee *et al*., 2006). Dot-blot analysis confirms the pronounced shift in the microbial population structure from beta-*Proteobacteria* dominance in a full scale wastewater treatment process (Darvill WWTP) to that of the alpha-*Proteobacteria* in the treatment of VOE initially detected by FISH. These findings reinforce the theory that the reduction in beta-*Proteobacteria* abundance in the aerobic reactors of the biological treatment process was caused by the sensitivity of this group to the VOE. Alpha-*Proteobacteria* constitutes the predominant group of bacteria within the VOE-amended sludge community. As mentioned previously, this finding most likely reflects the involvement of this group of bacteria in VOE degradation.

Variations of the dot-blot technique for example the slot-blot, whereby a manifold with a slot configuration instead of a circular blot, is used in many applications. Mobarry *et* al. (1996) demonstrated An Environmental application of quantitative slot blot hybridization of nitrifying activated sludge and biofilm samples. Results obtained suggested the occurrence of novel

populations of ammonia oxidizers. Slot blot hybridization using domain- or group-specific oligunucleotides have been applied to better describe the predominance of microbial populations in an upflow anaerobic sludge bed (UASB) reactor treating brewery wastewater with archeal cells representing about 40.8% and 62.9% of the total RNA isolated from the granular sludge (Lui *et al*., 2002).

Dot-blot hybridization has been used to investigate the occurrence of the bacterium responsible for the formation of activated sludge flocs *Zooglea ramigera*. Hybridization with probes designed for characteristic regions of the 16S rRNAs of *Z. ramigera* ATCC 19544T (T5 type strain) and two misclassified strains, *Z. ramigera* ATCC 25935 and ATCC 19623 found that relatively high levels of *Z. ramigera* cells (up to approximately 10% of the total number of cells) and typical morphology tended to be linked to overloading of sewage plants. The probe directed to rejected type strain *Z. ramigera* ATCC 19623 bound to only a few cells (Rossello-Mora *et al*., 1995*)*. Dot-blot hybridization has also been used to screen for organisms producing polyphosphate kinase (PPK) in activated sludge carrying out EBPR**.** Dot-blot analysis of total RNA extracted from sludge demonstrated that the Type I *ppk* mRNA was present, indicating that this gene is expressed during EBPR (MacMahon *et al.,* 2002).

5.5 Conclusion

The application of dot-blot hybridization confirmed the dominance of beta-*Proteobacteria* in the Darvill seed sludge and dominance of the alpha-*Proteobacteria* in the laboratory-scale process. The *Actinobacteria* were not detected in any of the samples using dot-blot hybridization as the RNA concentration was below the detection limit of the method.

The results of community profiling using dot-blot hybridization and FISH analysis showed perfect correlation, hence dot-blot hybridization can be successfully used to verify cell counts obtained using FISH. However, relative

cell numbers should not be extrapolated from RNA abundance values due to varying ribosomal content as a consequence of varying cell size. While FISH gives a good indication of cell numbers, dot-blot hybridization can provide an insight into the contribution of individual microbial populations to the total metabolic function of the activated sludge mixed liquor. Using both methods will give a more accurate qualitative and quantitative description of the microbial community active in wastewater treatment processes.

CHAPTER 6

General Conclusion and Recommendations

6.1 Conclusion

The first and second objectives of this research were achieved with the operation of a laboratory-scale biological treatment process to steady-state achieving maximum process efficiency. During steady-state conditions an overall COD removal capacity of 84% and TKN removal capacity of 90% were achieved. These findings indicate that a substantial portion of the VOE is biodegradable organic matter. However, the appropriate remediation technology must be optimized on a case to case basis and cannot be generalized for the greater vegetable oil sector due to individual problems experienced by each industry.

The high organic load of the raw VOE necessitated a pretreatment step to remove the high FOG and prepare the effluent for biological treatment. Ferric chloride and alum combined with DAF are the most popular choices of effluent treatment in the vegetable oil sector; however the commercial flocculent C40 was selected since it was proven to be more efficient, less labour intensive and cheaper than DAF.

The application of rRNA-based monitoring techniques used in this study which included FISH and dot-blot hybridization were successfully used to identify and describe the complex structure of the Proteobacterial populations present within the aerobic mixed liquor of the laboratory-scale biological process. Fluorescent *in situ* hybridization of the aerobic mixed liquor of the biological process indicated that members of the domain *Bacteria* predominated (EUB:DAPI ratios) however, these ratios were slightly lower than those obtained from a similar laboratory-scale biological process treating domestic wastewater, possibly indicating a slightly diminished contribution of the bacteria in the biological process treating VOE when compared with the domestic process. A clear population “shift” was evident

when comparing FISH results obtained from the biological process treating VOE compared to the Darvill seed sludge. The laboratory-scale process was dominated by members of the alpha *Proteobacteria* when compared to the beta *Proteobacteria*, gamma *Proteobacteria* and *Actinobacteria*.

Dot-blot hybridization confirmed dominance of the alpha *Proteobacteria* but revealed an overestimation of alpha *Proteobacteria* when compared with *in situ* counts. This could be attributed to auto fluorescence of unbound probe not washed away during stringent washing steps or non-specific binding of the probe to extracellular polymeric substances (ECP). FISH and dot-blot results reiterate previous findings indentifying the *Proteobacteria* group as dominating wastewater treatment plants.

It can therefore be concluded that the community profiles of activated sludge processes treating industrial effluents such as VOE are different to domestic activated sludge processes and are dependent and determined by the substrate being treated.

6.2 Recommendations

Current findings recommend the application of a combination of chemical pretreatment using C40 and/or a generic equivalent with the aid of an activated sludge process optimized to suite the specific wastewater treatment needs of the vegetable oil company.

Current pretreatment methods employed by the vegetable oil sector in South Africa have been limited to the use of ferric chloride, alum and other poly-electrolytes and more commonly used is DAF. As a result of the improved efficiency of compound C40 as compared to these methods, future research should focus on identifying the active ingredient(s) in C40 to further reduce cost and improve its performance. C40 far surpasses conventional technology for large scale application onsite at industry since it has proven to

be comparatively more efficient, cheaper and requires less space than DAF proving a more practical option.

The information gained from monitoring the microbial community of the process using novel rRNA-based hybridization techniques can be used to further optimize the system and reduce perturbations. Future studies should focus on FISH and dot-blots using genus and species-specific probes to provide more information on the community comprising the “specialized” community of the activated sludge process treating VOE. In this way specialised consortia of bacteria can be used to improve the efficiency of biological treatment of VOE.

Future investigations focussing on other novel molecular approaches such as sequencing, denaturing gradient gel electrophoresis (DGGE) or confocal scanning laser microscopy (CSLM) could provide more detailed phylogenetic information with regard to affiliation of the community members implicated in the treatment of VOEs.

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APPENDIX 1

Chemical oxygen demand (COD) test (Clesceri *et al.*, 1998)

Principle:

COD is defined as the amount of specified oxidant that reacts with the sample under controlled conditions. The quantity of oxidant that consumed is expressed in terms of its oxygen equivalence. Due to its unique chemical properties, the dichromate ion ($Cr_2O_7^{2-}$) is the specified oxidant and is reduced to the chromic ion (Cr^{3+}). Both organic and inorganic components of a sample are subject to oxidation, but in most cases the organic component predominates and is of the greater interest. COD is a defined test; the extent of sample oxidation can be affected by digestion time, reagent strength and sample COD concentration. COD is often used as a measurement of pollutants in wastewater and natural water bodies.

Reflux apparatus:

The COD test apparatus consists of a 250 mL flat round-bottomed Erlenmeyer flask with ground-glass 24/40 neck and 300 mm jacket Liebig condenser with 24/40 ground-glass joint, and hotplate having sufficient power to ensure adequate boiling of the contents of the refluxing flask.

Reagents:

1.1 Standard potassium dichromate ($K_2Cr_2O_7$) solution, 0.25 N

Dissolve 12.259 g of $K_2Cr_2O_7$ (lab grade) previously dried at 103°C for 3 hours and then cooled in a desiccator, in dH_2O and make up to 1000 mL.

1.2 Sulphuric acid (H_2SO_4) reagent

Dissolve 15 g of silver sulphate (Ag_2SO_4) in 2500 mL of concentrated (<98%) H_2SO_4 using a magnetic stirrer. Dissolution takes place between 1 and 2 days.

1.3 Standard ferrous ammonium sulphate [$Fe(NH_4)_2(SO_4)_2$], 0.05 N

Dissolve 100 g of $Fe(NH_4)_2(SO_4)_2.6H_2O$ (analytical grade) in dH_2O using a 5 L volumetric flask. Add 100 mL of concentrated H_2SO_4 and make up to 5000 mL.

Standardization:

Pipette 5 mL of standard 0.25N $K_2Cr_2O_7$ solution into an Erlenmeyer flask, and dilute to 50 mL with dH_2O. Add 15 mL of the H_2SO_4 reagent and allow to cool before titrating with 0.05N $Fe(NH_4)_2(SO_4)_2$ titrant, using 2 drops of ferroin indicator.

$$\text{Normality } Fe(NH_4)_2(SO_4)_2 = \frac{\text{mL } K_2Cr_2O_7 \text{ x } 0.25}{\text{mL Fe } NH_4\ _2\ SO_4\ _2}$$

1.4 Ferroin indicator

Dissolve 1.485 g 1,10-phenanthroline monohydrate, together with 0.695 g of $FeSO_4.7H_2O$ in dH_2O and dilute to 100 mL.

1.5 Mercuric sulphate ($HgSO_4$) powder.

Procedure:

1. Collect the correct number of clean, dry 250 mL Erlenmeyer flasks with the ground glass 24/40 necks. Two flasks are used for each test. Include 2 extra flasks if influent COD is being tested and 2 extra flasks if reactor or effluent COD is being tested.
2. Place approximately 0.04 g of $HgSO_4$ powder in each flask. This is the equivalent to the volume of two match heads.
3. Place 8 - 10 glass beads glass beads (± 3 mm diameter) in each flask.
4. For each influent sample, pipette 10 mL of unfiltered influent into each of the two flasks. Pipette 10 mL of dH_2O into each of the two flasks (these are the blanks).

5. Add 5 mL of $K_2Cr_2O_7$ solution to each of the two flasks.
6. In turn, carefully add 15 mL of the H_2SO_4 reagent to each flask, ensuring that no vapour escapes from the flask. This is most easily done by pouring the acid down the wall of the flask while the flask is tilted. Immediately attach the flask to the jacket condenser.
7. Once acid has been added to each flask ensure that the flasks are level on the heating pad. Allow the contents of the flask to boil for two hours. Ensure that the water flow rate in the condensers is swift enough to condense all vapour rising up the condensers.
8. Allow the flasks to cool to room temperature with the condensers still in position.
9. Pour approximately 80 mL of dH_2O through the top opening of each of the condensers into the sample mixture, ensuring that the side walls of the condensers are well washed.
10. Remove the flasks from the condensers and the heating pad and add 2 drops of ferroin indicator to each flask.
11. Titrate with standardized 0.05N $Fe(SO_4)_2(NH_4)_2$ solution until one drop of titrant changes the solution colour to reddish brown.

Calculation:

$$mgCOD/L = \frac{(a - b) \times N \times 8000}{mL\ sample}$$

where:
- a = average mL $Fe(SO_4)_2(NH_4)$ used to titrate blank of same volume as sample.
- b = average mL $Fe(SO_4)_2(NH_4)$ used to titrate sample.
- N = normality of $Fe(SO_4)_2(NH_4)$.

APPENDIX 2

Total Kjeldahl nitrogen (TKN) test (Clesceri *et al.*, 1998)

Reagents:

2.1 Mercuric sulphate ($HgSO_4$) solution

Dissolve 40 g red mercuric oxide (HgO) in 250 mL of 1 H_2SO_4:5 dH_2O (50 mL:250 mL) and dilute to 800 mL with dH_2O.

2.2 Sulphuric acid (H_2SO_4)-mercuric sulphate ($HgSO_4$)-potassium sulphate (K_2SO_4) solution

Dissolve 333.75 g of K_2SO_4 in 1800 mL of dH_2O and add 500 mL of concentrated H_2SO_4. Add 62.5 mL $HgSO_4$ solution and dilute to 2500 mL. This reagent crystallizes at temperatures lower than 14°C.

2.3 Sulphuric acid (H_2SO_4) solution, 7N

Dilute 485 mL concentrated H_2SO_4 (>98%) in dH_2O and make up to 2500 mL with dH_2O.

2.4 Digestion mixture

Mix the two solutions made up in 2) and 3) above to give 5000 mL of digestion mixture.

2.5 Mixed indicator

Mix two volumes 0.2% methyl red in 95% alcohol with 1 volume of 0.2% methylene blue in 95% alcohol. This solution must be made up fresh every 30 days.

2.6 Boric acid (H_3BO_3)

Dissolve 100 g of H_3BO_3 in dH_2O. Add 100 mL of mixed indicator (or 40 mL blue and 80 mL red), and dilute to 5000 mL.

2.7 Sulphuric acid (H_2SO_4) solution, 0.02 N

Prepare a stock solution of 0.1 N H_2SO_4 by diluting 2.8 mL of concentrated H_2SO_4 to 1000 mL. Dilute 200 mL of the 0.1 N H_2SO_4 stock solution to 1000 mL with dH_2O to give a 0.02 N stock solution.

2.8 Standard Sulphuric acid (H_2SO_4) solution, 0.001 N

Dilute 100 mL of 0.02 N H_2SO_4 stock solution to 2000 mL to give approximately a 0.001N H_2SO_4 solution. Standardize this solution as outlined in Section 102.3 (c) “Alkalinity”, p. 54 (Clesceri *et al.*, 1998).

2.9 Sodium hydroxide (NaOH) - sodium thiosulphate solution ($Na_2S_2O_3.5H_2O$)

Dissolve 500 g NaOH and 25 g $Na_2SO_3.5H_2O$ in dH_2O and make up to 1000 mL.

NB. This procedure is done in a polypropylene container as the reaction is highly exothermic.

Procedure:

1. Pipette 5 mL influent sample or a 10 mL reactor or effluent sample into a 30 mL micro Kjeldahl flask.
2. Add 10 mL digestion mixture (4.) add three glass beads approximately 3 mm in diameter. (Prepare a blank by placing a volume of dH_2O equal to that of the sample with digestion mixture and beads, in a second Kjeldahl flask. Follow the same procedure with the sample and blank).
3. Digest the sample mixture on heating pad until sample is clear, and leave for another 20 min.
4. Transfer the Kjeldahl flask to the micro-steam distillation apparatus and allow to cool for approximately 30 min.
5. Pipette 25 mL of H_3BO_3 solution (6.) into a 100 mL Erlenmeyer flask and place in position on the steam distillation apparatus with the nozzles of the condenser immersed in the solution.

6. Add about 10 mL of dH_2O to the contents of the Kjeldahl flask to dissolve the sediment.
7. Add 7 mL of the NaOH solution (9.) to the contents of the Kjeldahl flask via the top opening of the steam distillation apparatus, and immediately seal the apparatus by placing the steam line in position.
8. Steam-distil until the volume of the H_3BO_3 solution in the Erlenmeyer flask is approximately 40 - 50 mL.
9. Titrate the contents of the Erlenmeyer flask for the sample and the blank with standardized 0.001 N H_2SO_4 (8).

Calculation:

$$\text{TKN mgN/L} = \frac{(a - b) \times N \times 14000}{\text{mL sample}}$$

where: a = mL 0.001 N H_2SO_4 for sample
b = mL 0.001 N H_2SO_4 for blank
N = actual normality of 0.001 N H_2SO_4

APPENDIX 3

Determination of mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) (Clesceri *et al.*, 1998)

A. Determination of MLSS:

1. Decant 100 mL of activated sludge mixed liquor into a polypropylene centrifuge tube and centrifuge at 5000 x g for 10 min.
2. Discard the supernatant. Using a spatula, quantitatively transfer the pelleted sludge to a clean pre-weighed crucible.
3. Place the crucible into an oven at 105°C for 24 h. Remove crucible from oven and allow to cool in a dessicator.
4. Reweigh the crucible.
5. Determine the MLSS according to the following calculation:

$$\text{MLSS (mg/L)} = \frac{\text{mass of (crucible + sludge) in grams} - \text{mass of crucible in grams x 10}}{\text{100 mL}}$$

B. Determination of MLVSS:

1. Place the pre-weighed crucible from MLSS determination in a muffle furnace at 550°C for 1 h.
2. Remove crucible from furnace and allow to cool in a dessicator.
3. Reweigh the crucible.
4. Determine the MLVSS according to the following calculation:

$$\text{MLVSS (mg/L)} = \frac{\text{mass of (crucible + sludge) in grams} - \text{mass of (crucible + ash) in grams x 10}}{\text{100 mL}}$$

APPENDIX 4

Table A1. Results of daily monitoring of the laboratory-scale biological process treating vegetable oil effluent during the acclimation period.

Date	VOE Batch	COD influent (mg/L)	COD effluent (mg/L)	COD removal (%)	TKN influent (mg/L)	TKN effluent (mg/L)	TKN removal (%)	MLVSS (mg/L)	OUR (mgO/L/h)
16-Apr		1118	443	60.4	51.8	9.4	81.9	2667	6.80
17-Apr		1024	406	60.4	53.3	11.1	79.2	2545	29.2
18-Apr		968	185	80.9	52.3	6.9	86.8	2374	23.3
19-Apr		969	297	69.3	56.6	13.5	76.1	2034	20.7
20-Apr		932	312	66.5	46.9	10.1	78.5	2122	41.7
21-Apr		958	228	76.2	45.8	6.9	84.9	2634	17.6
22-Apr		977	283	71.0	46.9	9.0	80.8	2430	24.3
23-Apr		911	316	65.3	48.0	10.1	79.0	2287	18.4
24-Apr		940	221	76.5	50.1	5.6	88.8	2512	19.3
25-Apr		890	231	74.0	53.3	6.9	87.1	2432	18.9
Mean	**A1**	**969**	**292**	**70.1**	**51**	**9.0**	**82**	**2404**	**22.0**
Std		**64.3**	**82.5**	**7.0**	**3.5**	**2.4**	**4.3**	**207**	**9.02**
26-Apr		1009	310	69.3	45.4	21.6	52.4	3253	19.3
27-Apr		951	303	68.1	41.5	14.4	65.3	3436	18.7
28-Apr		959	487	49.2	45.4	33	27.3	3621	19.6
29-Apr		902	349	61.3	38.7	9.6	75.2	3376	22.2
30-Apr		927	376	59.4	45.3	15	66.9	3840	6.80
01-May		910	384	57.8	40.5	25	38.3	3846	22.3

Table A1. Continued

02-May		929	343	63.1	49.0	25.8	47.3	3274	7.7
03-May		877	372	57.6	47.6	31.6	33.6	3638	18.7
04-May		862	371	57.0	46.2	27.8	39.8	3521	7.6
05-May		875	356	59.3	44.8	12.7	71.7	3114	8.9
Mean	**A2**	**920**	**365**	**60.2**	**44**	**22**	**52**	**3492**	**15**
Std		**45.0**	**50.7**	**5.8**	**3.2**	**8.3**	**17.1**	**247**	**6.5**
06-May		969	364	62.4	52.6	15.2	71.1	3725	9.7
07-May		960	298	69.0	53.6	18.4	65.7	3865	23.4
08-May		964	258	73.2	49.0	11.2	77.1	3567	8.6
09-May		947	352	62.8	52.0	22	57.7	3482	24.3
10-May		955	385	59.7	56.8	16.4	71.1	3835	21.2
11-May		901	380	57.8	50.5	11.3	77.6	3275	46.2
12-May		875	275	68.6	54.0	14.8	72.6	3602	24.3
13-May		896	259	71.1	52.6	13.4	74.5	3421	25.1
14-May		915	272	70.3	51.2	9.2	82.0	3624	12.7
15-May		932	285	69.4	49.8	11.2	77.5	3732	23.1
Mean	**A3**	**931**	**313**	**66**	**52**	**14**	**73**	**3613**	**22**
Std		**32.9**	**51.5**	**5.3**	**2.3**	**3.9**	**7.0**	**186**	**10.7**
16-May		1079	396	63.3	41.8	25.5	39.0	3263	19.7
17-May		1002	316	68.5	43.3	42	3.0	3338	23.4
18-May		933	335	64.1	42.3	41	3.1	3256	15.3
19-May		904	313	65.4	46.6	22.5	51.7	3178	17.6
20-May		926	323	65.1	36.9	25	32.2	3032	12.5
21-May		1054	284	73.1	38.0	23.6	37.9	3233	15.4
22-May		1028	246	76.1	40.1	20.8	48.1	3035	19.8

Table A1. Continued

23-May		965	242	74.9	43.3	23.2	46.4	2899	20.4
24-May		1050	244	76.8	47.5	29.2	38.5	2755	21.3
25-May		1038	262	74.8	49.0	22	55.1	2594	19.6
Mean	**A4**	**998**	**296**	**70**	**43**	**27**	**36**	**3058**	**19**
Std		**61.7**	**49.8**	**5.4**	**5.3**	**7.7**	**18.5**	**245**	**3.3**
26-May		1063	230	78.4	46.1	26	43.6	2794	27.2
27-May		943	213	77.4	47.5	23.6	50.3	3128	23.4
28-May		1037	223	78.5	43.6	24.4	44.0	3007	19.5
29-May		943	202	78.6	52.0	20.8	60.0	3128	21.2
30-May		988	241	75.6	49.0	23.6	51.8	3074	24.8
31-May		1022	174	83.0	46.1	10.2	77.9	2976	18.1
01-Jun		990	192	80.6	46.3	16.8	63.7	2865	20.2
02-Jun		962	189	80.4	49.0	16.4	66.5	2932	20.6
03-Jun		925	256	72.3	47.5	9	81.1	3026	23.3
04-Jun		903	214	76.3	47.5	16.6	65.1	2976	22.7
Mean	**A5**	**978**	**213**	**78**	**47**	**19**	**60**	**2991**	**22**
Std		**51.7**	**25.1**	**3.0**	**2.2**	**5.9**	**13.0**	**108**	**2.7**
05-Jun		977	197	79.8	54.7	17.2	68.6	3081	21.4
06-Jun		841	223	73.5	57.7	10.6	81.6	3164	20.9
07-Jun		989	183	81.5	55.4	16.4	70.4	2862	20.2
08-Jun		964	179	81.4	50.5	9.0	82.2	2986	22.3
09-Jun		932	195	79.1	55	10.0	81.8	3143	22.1
10-Jun		941	187	80.1	54.5	14.3	73.8	2854	23.7
11-Jun		941	164	82.6	54.5	16.4	69.9	2818	20.4
12-Jun		948	153	83.9	51.5	22.4	56.5	3067	21.7

13-Jun		997	145	85.5	48.5	21.3	56.1	3023	21.8
14-Jun		973	168	82.7	54.8	21.3	61.1	3064	20.2
Mean	**A6**	**950**	**179**	**81**	**54**	**16**	**70**	**3006**	**21**
Std		**44.2**	**23.0**	**3.3**	**2.7**	**4.9**	**10.0**	**123**	**1.1**

Table A2. Results of daily monitoring of the laboratory-scale biological process treating vegetable oil effluent during steady-state.

Date	VOE Batch	COD influent (mg/L)	COD effluent (mg/L)	COD removal (%)	TKN influent (mg/L)	TKN effluent (mg/L)	TKN removal (%)	MLVSS (mg/L)	OUR (mgO/L/h)
16-Jun		919	187	79.6	50.4	4.4	91.3	2799	20.0
17-Jun		784	146	81.4	46.5	4.2	91.0	2715	19.2
18-Jun		988	177	82.1	50.4	2.2	95.6	2533	19.0
19-Jun		1034	156	84.9	43.7	3.4	92.2	2972	19.9
20-Jun		984	156	84.2	50.4	5.6	88.9	2927	19.3
21-Jun		892	159	82.1	45.5	5.6	87.7	3145	20.6
22-Jun		1080	191	82.4	48.0	3.4	92.9	2518	18.6
23-Jun		971	173	82.2	46.5	2.2	95.3	3133	19.3
24-Jun		957	166	82.6	46.5	2.2	95.3	2551	20.9
25-Jun		1009	125	87.6	40.4	1.2	97.0	3020	21.2
Mean	**1**	**962**	**164**	**82.9**	**46.8**	**3.4**	**92.7**	**2831**	**19.8**
Std		**82.4**	**19.8**	**2.2**	**3.2**	**1.5**	**3.1**	**243**	**0.9**
26-Jun		941	131	86.0	51.0	3.8	92.5	2816	18.5
27-Jun		947	139	85.3	52.1	4.6	92.0	2743	19.1
28-Jun		1009	155	84.7	47.1	2.8	94.1	3016	18.7
29-Jun		998	162	83.7	47.1	5.5	88.4	2513	18.6
30-Jun		1013	166	83.6	54.0	4.1	92.5	3007	18.6
01-Jul		957	147	84.7	48.8	2.8	94.3	2601	18.6
02-Jul		976	131	86.5	42.0	3.7	91.3	2560	18.7
03-Jul		937	147	84.3	42.0	4.1	90.3	2889	18.7

Table A2. Continued

04-Jul		875	178	79.7	54.0	2.3	95.8	2230	18.6
05-Jul		927	174	81.3	45.3	2.8	93.9	3162	18.8
Mean	**2**	**958**	**153**	**84.0**	**48.3**	**3.6**	**92.5**	**2754**	**18.7**
Std		**42.6**	**16.7**	**2.1**	**4.4**	**1.0**	**2.2**	**282**	**0.2**
06-Jul		949	162	82.9	47.6	4.8	89.9	3537	19.1
07-Jul		1032	169	83.6	48.6	5.7	88.3	2845	22.1
08-Jul		936	127	86.4	44.0	3.5	92.1	3188	20.3
09-Jul		925	144	84.4	47.0	6.8	85.5	2793	19.6
10-Jul		961	162	83.1	51.8	5.1	90.2	2499	19.4
11-Jul		895	211	76.4	45.5	3.5	92.4	2926	20.0
12-Jul		943	180	81.0	49.0	4.6	90.6	2988	19.3
13-Jul		882	183	79.2	47.6	5.1	89.3	2534	19.0
14-Jul		963	158	83.5	46.2	2.9	93.8	2655	19.9
15-Jul		965	204	78.8	44.8	3.5	92.3	3294	19.3
Mean	**3**	**945**	**170**	**81.9**	**47.2**	**4.5**	**90.4**	**2926**	**19.8**
Std		**41.5**	**25.7**	**3.0**	**2.3**	**1.2**	**2.4**	**336**	**0.9**
16-Jul		986	135	86.3	54.4	2.7	95.0	3084	17.2
17-Jul		932	92	90.1	55.5	3.2	94.2	2996	17.3
18-Jul		982	110	88.8	50.2	2.0	96.1	3025	17.4
19-Jul		997	166	83.4	50.2	3.9	92.3	2979	17.4
20-Jul		1080	153	85.8	57.6	2.9	95.0	3122	17.5
21-Jul		1057	140	86.7	52.0	2.0	96.2	2794	17.6
22-Jul		1005	133	86.8	44.8	2.6	94.2	2653	17.6
23-Jul		927	151	83.7	44.8	2.9	93.5	2730	17.6
24-Jul		917	128	86.1	57.6	1.6	97.2	2824	17.7

Table A2. Continued

25-Jul		927	166	82.1	48.3	2.0	95.9	3377	17.7
Mean	**4**	**981**	**137**	**86.0**	**51.6**	**2.6**	**95.0**	**2958**	**17.5**
Std		**56.4**	**23.6**	**2.4**	**4.7**	**0.7**	**1.5**	**215**	**0.2**
26-Jul		977	211	78.4	35.8	3.1	91.3	3087	20.3
27-Jul		1065	218	79.5	37.1	2.1	94.2	3170	20.2
28-Jul		952	204	78.6	47.3	4.8	89.7	2868	20.0
29-Jul		971	204	79.0	37.2	1.4	96.1	2992	19.8
30-Jul		962	138	85.6	44.1	2.2	94.9	3154	19.6
01-Aug		900	149	83.4	35.4	6.6	81.1	2677	19.4
02-Aug		1028	131	87.3	56.4	3.8	93.3	2724	19.1
03-Aug		972	127	86.9	58.3	4.6	92.0	3573	18.8
04-Aug		962	178	81.5	58.4	7.5	87.1	3429	18.6
05-Aug		936	189	79.8	56.3	5.5	90.1	3170	18.3
Mean	**5**	**973**	**175**	**82.0**	**46.4**	**4.2**	**91.0**	**3085**	**19.4**
Std		**45.8**	**35.4**	**3.5**	**9.9**	**2.0**	**4.4**	**285**	**0.7**
06-Aug		981	182	81.5	46.8	3.8	91.8	2214	21.2
07-Aug		915	220	76.0	48.3	8.2	83.0	3390	20.3
08-Aug		944	129	86.4	47.3	6.4	86.4	3038	21.4
09-Aug		894	197	78.0	51.6	7.3	85.8	3077	17.8
10-Aug		963	163	83.1	41.9	8.2	80.3	2967	21.4
11-Aug		955	155	83.7	40.8	5.9	85.7	3154	19.6
12-Aug		933	148	84.2	41.9	8.1	80.8	3249	18.1
13-Aug		942	170	81.9	43.0	5.9	86.4	3035	17.4
14-Aug		913	212	76.8	45.1	5.9	87.0	3133	19.4
15-Aug		891	197	77.9	48.3	4.0	91.7	2764	19.4

Table A2. Continued

Mean	**6**	**933**	**177**	**80.9**	**45.5**	**6.4**	**85.9**	**3002**	**19.6**
Std		**29.7**	**29.4**	**3.6**	**3.5**	**1.6**	**3.9**	**323**	**1.5**
16-Aug		906	166	81.7	45.5	1.3	97.2	3363	19.6
17-Aug		931	131	85.9	45.2	6.4	85.8	2679	19.7
18-Aug		914	135	85.3	44.2	5.8	86.9	2466	19.6
19-Aug		1011	121	88.0	40.9	4.5	89.0	3110	19.6
20-Aug		881	111	87.5	49.5	5.8	88.3	3480	19.5
21-Aug		866	131	84.8	45.5	4.5	90.1	3119	19.4
22-Aug		1013	124	87.7	46.3	5.1	88.9	3312	19.3
23-Aug		991	166	83.3	53.8	5.1	90.5	2872	20.6
24-Aug		990	200	79.8	53.8	6.4	88.1	3357	20.8
25-Aug		1007	142	85.9	48.4	7.2	85.2	3492	20.9
Mean	**7**	**951**	**143**	**85.0**	**47.3**	**5.2**	**89.0**	**3125**	**19.9**
Std		**57.3**	**27.0**	**2.7**	**4.1**	**1.6**	**3.3**	**350**	**0.6**
26-Aug		959	120	87.5	53.1	4.5	91.5	3538	20.5
27-Aug		973	127	86.9	48.4	2.8	94.2	2496	20.4
28-Aug		905	127	85.9	64.6	7.3	88.6	2981	20.2
29-Aug		879	195	77.9	68.0	5.6	91.8	3552	20.0
30-Aug		964	146	84.9	45.6	6.2	86.2	3312	19.8
31-Aug		968	195	79.9	47.3	3.4	92.8	2647	19.6
01-Sep		1012	153	84.8	44.6	4.4	90.0	3801	19.3
02-Sep		986	176	82.2	50.3	8.9	82.2	3196	19.0
03-Sep		935	142	84.8	39.0	4.0	89.7	3120	18.8
04-Sep		888	135	84.8	45.0	3.4	92.4	3246	18.5
Mean	**8**	**947**	**152**	**84.0**	**50.3**	**5.1**	**89.9**	**3189**	**19.6**

Table A2. Continued

Std		**43.9**	**27.7**	**3.1**	**9.1**	**1.9**	**3.6**	**404**	**0.7**
05-Sep		948	110	88.4	48.5	5.9	87.8	3382	19.8
06-Sep		971	132	86.4	39.7	5.2	86.6	3043	19.7
07-Sep		948	157	83.4	52.4	5.8	88.7	3476	19.7
08-Sep		848	126	85.2	52.3	7.3	85.9	2907	19.6
09-Sep		956	119	87.5	42.4	5.5	86.9	3376	19.7
10-Sep		929	151	83.8	48.2	6.5	86.4	3403	19.5
11-Sep		919	163	82.2	54.5	5.9	89.1	2084	19.5
12-Sep		929	126	86.5	52.6	6.1	88.7	3298	19.5
13-Sep		861	132	84.7	54.2	5.2	90.3	3608	20.8
14-Sep		871	160	81.6	52.3	5.9	88.7	3767	20.2
Mean	**9**	**918**	**138**	**85.0**	**49.3**	**5.9**	**87.9**	**3234**	**19.8**
Std		**43.2**	**18.8**	**2.2**	**5.1**	**0.6**	**1.4**	**474**	**0.4**
15-Sep		1196	179	85.0	48.6	2.9	94.0	2952	20.1
16-Sep		906	175	80.7	47.8	3.8	92.0	2861	20.2
17-Sep		965	155	84.0	48.6	4.1	91.6	3066	20.1
18-Sep		997	203	79.6	52.0	4.0	92.3	2729	20.2
19-Sep		1010	138	86.3	53.7	5.1	90.5	2921	20.1
20-Sep		824	146	82.2	52.0	4.6	91.2	2786	20.1
21-Sep		997	130	86.9	55.4	3.8	93.1	2943	20.2
22-Sep		1087	138	87.3	55.4	5.1	90.8	2918	20.3
23-Sep		1101	179	83.7	57.9	4.1	92.9	2929	21.8
24-Sep		1037	175	83.1	48.6	4.1	91.6	2885	17.8
Mean	**10**	**1012**	**162**	**83.9**	**52.0**	**4.2**	**92.0**	**2899**	**20.1**
Std		**104**	**23.7**	**2.6**	**3.5**	**0.6**	**1.1**	**93**	**1.0**

Table A2. Continued

25-Sep		810	221	72.7	55.4	6.7	87.9	3147	20.4
26-Sep		1007	182	81.9	54.3	5.3	90.1	3248	19.7
27-Sep		967	178	81.6	55.5	6.0	89.1	3357	18.3
28-Sep		967	173	82.1	45.2	5.3	88.2	3245	17.9
29-Sep		1001	182	81.8	40.2	6.0	85.0	3124	18.6
30-Sep		1007	250	75.2	45.2	6.9	84.6	2938	18.5
01-Oct		987	230	76.7	46.4	3.3	92.8	3097	19.7
02-Oct		1041	211	79.7	37.2	9.7	73.7	2718	18.5
03-Oct		1033	221	78.6	38.4	6.7	82.4	2799	19.9
04-Oct		1011	216	78.6	39.2	5.5	85.9	3127	20.5
Mean	**11**	**983**	**206**	**78.9**	**45.4**	**6.1**	**86.0**	**3080**	**19.2**
Std		**65.6**	**26.0**	**3.2**	**7.1**	**1.6**	**5.3**	**203**	**1.0**
05-Oct		1075	216	79.9	42.2	3.6	91.5	2868	18.6
06-Oct		998	142	85.7	43.6	2.4	94.4	3120	18.7
07-Oct		929	165	82.2	55.4	5.5	90.1	3370	18.8
08-Oct		900	165	81.6	43.6	1.6	96.3	2362	18.9
09-Oct		922	161	82.6	51.8	2.5	95.1	2502	18.9
10-Oct		1050	133	87.3	41.2	7.5	81.7	3429	19.0
11-Oct		1024	147	85.7	49.5	4.3	91.4	3192	19.0
12-Oct		961	174	81.8	68.3	5.3	92.2	2942	19.0
13-Oct		1046	170	83.8	68.3	8.6	87.5	2261	19.0
14-Oct		1034	216	79.1	66.0	6.3	90.4	2683	19.1
Mean	**12**	**994**	**169**	**83.0**	**53.0**	**4.8**	**91.1**	**2873**	**18.9**
Std		**61.7**	**27.9**	**2.6**	**11.0**	**2.3**	**4.2**	**413**	**0.2**
15-Oct		978	159	83.7	48.0	4.8	90.1	3378	20.9

Table A2. Continued

16-Oct		939	167	82.2	55.2	3.8	93.1	3104	19.6
17-Oct		1033	159	84.6	45.6	5.4	88.2	3052	19.3
18-Oct		939	124	86.8	43.2	4.8	89.0	2947	19.4
19-Oct		1059	175	83.5	48.0	5.4	88.8	3097	19.8
20-Oct		1018	179	82.5	54.0	6.3	88.3	3372	19.9
21-Oct		986	148	85.0	45.6	6.0	86.9	3191	19.4
22-Oct		958	140	85.4	43.2	5.0	88.4	2116	19.4
23-Oct		921	132	85.7	42.0	6.3	85.0	2811	19.2
24-Oct		899	175	80.6	46.8	4.2	91.1	2895	19.2
Mean	**13**	**973**	**156**	**84.4**	**47.2**	**5.2**	**88.9**	**2996**	**19.6**
Std		**51.6**	**19.1**	**1.5**	**4.4**	**0.9**	**2.2**	**361**	**0.5**
25-Oct		837	152	81.8	49.7	3.3	93.4	2737	19.2
26-Oct		985	149	84.9	52.7	3.7	93.0	3381	19.3
27-Oct		960	156	83.7	50.4	5.4	89.3	2762	19.3
28-Oct		928	152	83.6	45.5	5.4	88.1	2911	19.4
29-Oct		937	182	80.6	50.0	7.5	84.9	3228	20.0
30-Oct		1037	149	85.7	49.5	5.4	89.1	3427	19.3
31-Oct		1044	100	90.4	49.5	5.4	89.1	3103	19.3
01-Nov		1094	126	88.4	46.5	1.2	97.5	3089	19.1
02-Nov		1098	182	83.4	43.5	3.3	92.4	2957	19.1
03-Nov		1119	156	86.0	49.8	8.1	83.7	2804	19.2
Mean	**14**	**1004**	**151**	**84.8**	**48.7**	**4.9**	**90.0**	**3040**	**19.3**
Std		**90.1**	**24.0**	**2.9**	**2.7**	**2.1**	**4.1**	**249**	**0.3**
04-Nov		1178	156	86.8	52.5	5.1	90.4	3341	19.7
05-Nov		1038	172	83.4	54.0	8.4	85.1	2757	19.7

06-Nov		985	172	82.5	51.0	8.2	84.3	2960	19.5
07-Nov		1093	120	89.0	57.0	4.5	93.0	2757	19.4
09-Nov		932	208	77.7	54.0	8.3	85.1	2911	19.6
10-Nov		881	192	78.2	51.1	8.6	84.3	2909	19.8
11-Nov		943	152	83.9	52.5	5.3	90.4	3058	19.7
12-Nov		1005	232	76.9	54.0	8.2	85.1	3329	20.6
13-Nov		1045	136	87.0	52.5	4.5	91.4	3151	19.5
14-Nov		1021	180	82.4	52.5	5.0	90.4	2703	19.6
Mean	**15**	**1012**	**172**	**82.8**	**53.1**	**6.4**	**88.0**	**2988**	**19.7**
Std		**85.1**	**33.4**	**4.2**	**1.8**	**1.8**	**3.4**	**229**	**0.3**

APPENDIX 5

Fixation of gram negative cells (Amann, 1995b)

Introduction:

The hybridisation process exposes the cells to increased temperature, detergents and variable osmotic gradients. Fixation is an essential procedure for maintaining the morphological integrity of the cells. In order to minimize auto-fluorescence, fresh (not older than 24 h) 4% paraformaldehyde in phosphate buffered saline (PBS) solutions are routinely prepared.

Solutions:

- **1 x PBS**

  130 mM sodium chloride (NaCl)
  10 mM sodium phosphate buffer
  pH 7.2

- **3 x PBS**

  390 mM NaCl
  30 mM sodium phosphate buffer
  pH 7.2.

- **4% paraformaldehyde in PBS**
  - In a fume cupboard, heat 65 mL of ddH_2O to 60°C.
  - Add 4 g of paraformaldehyde.
  - Add 2-3 drops 2 M sodium hydroxide solution and stir rapidly until the solution has clarified (usually takes 1-2 min).
  - Remove solution from heat source and add 33 mL of 3 x PBS. Adjust pH to 7.2 with conc. HCl.
  - Filter solution through a 0.45 µm membrane filter.
  - Quickly cool down to 4°C and store in a refrigerator or on ice.

- **98% ethanol**

Procedure:

1. In a 15 mL polypropylene centrifuge tube, add three volumes of paraformaldehyde fixative to one volume of sample and hold for 1½ h at 4°C.
2. Pellet fixed cells by centrifugation (5000 x g) for 10 min and decant fixative.
3. Wash fixed cells twice with 1 x PBS, resuspend to the original sample volume in 50% (v/v) ethanol in PBS and vortex.

Note: Fixed cells may now be stored in the freezer (-20°C) for several months.

APPENDIX 6

Cell dispersion using sonication (Mudaly, 2001)

Introduction:

The activated sludge floc has been found to be highly compact. In order to obtain individual cell counts, it is often necessary to disperse cells by sonication using a Virsonic 100 sonicator (Virtis, Gardiner, NY) at low frequency, after paraformaldehyde fixation.

Procedure:

1. Fill a 2 mL micro-test tube with 1 mL fixed sample.
2. To prevent spillage of the sample, cover the opening of the micro-test tube with parafilm.
3. Pierce a small hole in the centre of the parafilm covering using the tip of the sonication probe.
4. Insert the probe so that the tip of the probe is approximately 1 cm above the base of the micro-test tube and sonicate at 5 watts for 5 min for an MLSS = 2500 - 3000 mg/L.

APPENDIX 7

Preteatment of microscope slides for immobilization of fixed cells

Introduction:

8-Well teflon coated diagnostic microscope slides are routinely used. The teflon coating which is hydrophobic prevents mixing of samples in adjacent wells, allowing samples to be hybridized simultaneously on the same slide. Poly-L-lysine is used as an adhesive; the polycationic nature of this molecule allows interaction with the anionic sites of cells surfaces, resulting in strong adhesive properties.

Solutions:

- **(1:10) Poly - L-Lysine (Sigma diagnostics, U.S.A. Procedure no. P8920)**

  Allow solution to reach room temperature (18-26°C) before use. Dilute 1:10 with dH_2O.

Procedure:

1. Clean slide surface by soaking in a warm soap solution for 1 h, rinse thoroughly with dH_2O and air dry.
2. Immerse clean slides in diluted (1:10) Poly-L-Lysine solution for 10 min.
3. Drain Poly-L-Lysine solution from slides and dry in an oven for one hour at 60°C or at room temperature overnight.
4. Store treated slides in a sealed container or dessicator

APPENDIX 8

Preparation of acid-washed glass beads (Lane *et al*., 1996)

1. Soak glass beads in 6M HCl for 10 min.
2. Rinse with distilled water, checking with pH paper until the pH is equivalent to that of fresh distilled water.
3. Bake the rinsed beads at 180°C until completely dry.

APPENDIX 9

Preparation of buffer-saturated phenol (Maniatis *et al*., 1987)

Liquefied phenol is recommended as it can be used without re-distillation. Most batches of commercial liquefied phenol are clear or colourless. Occasionally, batches of liquefied phenol are pink or yellow, and these should be rejected and returned to the manufacturer. Liquefied and re-distilled phenol should be stored at -20°C in small aliquots. Before use, phenol must be equilibrated to pH >7.8 because nucleic acids will partition into the organic phase at acid pH.

Caution: Phenol is highly corrosive and can cause severe burns. The following precautions must be observed; wear gloves, protective clothing and safety glasses when handling phenol. All manipulations should be carried out in a fume hood. Any areas of the skin that come into contact with phenol should be rinsed with a large volume of water and washed with soap. Do **not** use ethanol.

Procedure:

1. Liquefied phenol should be stored at 20°C. As needed, remove the phenol from the freezer, allow it to warm to room temperature, and then melt it at 68°C. Add hydroxyquinoline to a final concentration of 0.1% m/v. This compound is an antioxidant, a partial inhibitor of RNAse, and a weak chelator of metal ions (Kirby, 1956). In addition, its yellow colour provides a convenient way to identify the organic phase.
2. To the melted phenol, add an equal volume of bead-beating buffer at room temperature. Stir the mixture on a magnetic stirrer for 15 min, and then turn off the stirrer. When the two phases have separated, aspirate as much as possible the upper (aqueous) phase using a 1 mL pipette tip attached to a vacuum line equipped with traps.
3. Repeat the extraction as described above several times until the pH of the phenolic phase is >7.8 (as measured with pH indicator sticks).

4. After the phenol is equilibrated and the final aqueous phase has been removed, add 0.1 volume of bead-beating buffer containing 0.2% v/v β-mercaptoethanol. The phenol solution may be stored in this form in a light-tight bottle at 4°C for periods up to one month.

APPENDIX 10

Determination of the concentration and purity of nucleic acids by UV spectroscopy (Zyskind and Bernstein, 1992)

Both DNA and RNA absorb ultraviolet (UV) light so efficiently that absorbance spectroscopy can be used as an accurate, rapid, and non-destructive method to determine concentrations as low as 2.5 µg/mL. The nitrogenous bases in oligonucleotides have an absorption maximum at approximately 260 nm. Using a 1 cm light path, the extinction coefficient for double stranded DNA at this wavelength is 20. Based on this extinction coefficient, the absorbance of at 260 nm in an OD of 1 cm quartz cuvette of a 50 µg/mL solution is equal to 1. For single stranded DNA and RNA an OD of 1 corresponds to approximately 40 µg/mL and 20 µg/mL for oligonucleotides. Proteins absorb maximally at approximately 280 nm due mainly to tryptophan residues. The ratio of A_{260}/A_{280}, therefore is a measure of the purity of a nucleic acid preparation and should fall between 1.65 and 1.85 for DNA and 1.95 and 2.10 for RNA. A lower value suggests protein contamination. If phenol, which has one λ_{max} at 270 nm, is contaminating the DNA preparation, then the A_{260} will be abnormally high, leading to an overestimation of the nucleic acid concentration.

Materials:

- Matching pairs of 2 mL quartz cuvettes
- Distilled water
- Eppendorf tubes
- UV spectrophotometer

Protocol:

1. Prepare a 1/100 dilution by adding 20µL of the RNA suspension to 1980 µL of distilled water in an Eppendorf tube. Mix well.

2. Allow 20 minutes for the UV lamp in the spectrophotometer to warm up. Set the wavelength of the spectrophotometer to 260 nm. Add distilled water to one cuvette; use the distilled water as a blank and set the absorbance to 0. Measure the absorbance of the dilution.
3. Repeat the measurement at 280 nm and determine the A_{260}/A_{280} ratio.
4. Calculate the concentration of RNA in your dilution, assuming that the concentration of 40 µg/mL has an OD of 1 at 260 nm. Calculate the RNA concentration in your original RNA solution. Store the RNA in the freezer at -20°C.

$$\text{RNA concentration (µg/mL)} = (OD_{260}) \times (\text{dilution factor}) \times \frac{50\ \mu g\ RNA/mL}{1\ OD_{260}\ unit}$$

5. Calculate the total yield:

   # µg RNA recovered = RNA (µg/mL) x total volume in mL

Appendix 11
Dot-blot manifold assembly

1. Prior to assembly, clean and dry the dot-blot manifold using an RNase deactivating agent such as RNaseZAP (SIGMA®, USA) according to manufacturer's recommendations.
2. Place the gasket support plate into position in the vacuum manifold. (There is only one way to slide the plate into the manifold)
3. Place the sealing gasket on top of the gasket support plate. The guide pins on the vacuum manifold help align the 96 holes in the support plate. Visually inspect the gasket to make sure the holes are properly aligned. If the gasket is not centred, pull lightly at the corners until it is aligned.

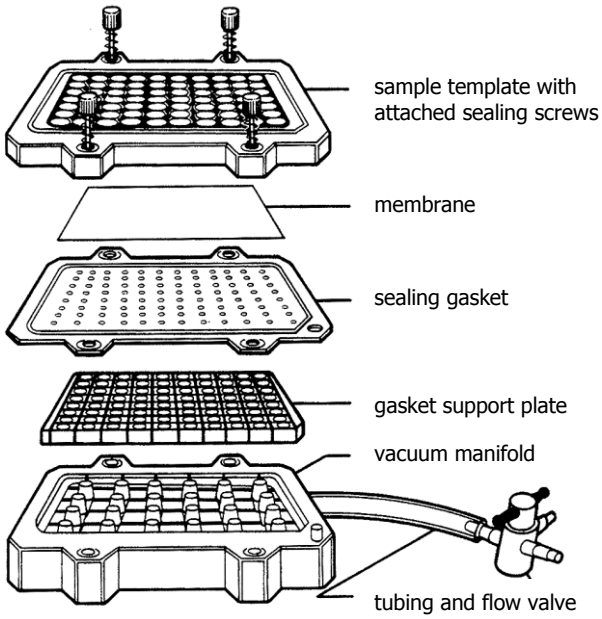

Figure 5.3 Diagram of Bio-Dot apparatus assembly comprising three plastic blocks (sample template, gasket support plate and vacuum manifold) and a rubber sealing gasket which is placed between the blocks. The lower block has an outlet flow valve which is attached to a vacuum pump.

4. Always use forceps or wear gloves when handling membranes. Pre-wet the nylon membrane by slowly sliding it at a 45° angle into sterile distilled water. A 10 minute soak is recommended for complete wetting of the membrane to ensure proper drainage of solutions. Remove the membrane from the wetting solution. Let the excess liquid drain from the membrane. (Touching the membrane to a sheet of Whatman no.1 filter paper is a simple method for removing excess wetting solution.) Lay the membrane on the gasket in the apparatus so that it covers all the holes. The membrane should not extend beyond the edge of the gasket after the Bio-Dot apparatus is assembled. Remove any air bubbles trapped between the membrane and the gasket.
5. Place the sample template on top of the membrane. The guide pins ensure that the template will be properly aligned. Finger-tighten the four screws. When tightening the screws, use a diagonal crossing pattern to ensure uniform application of pressure on the membrane surface (see Figure 5.4).

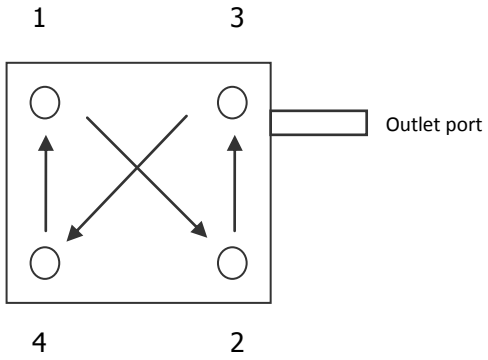

Figure 5.4 Diagonal crossing pattern for tightening screws in the Bio-Dot apparatus.

6. Attach a vacuum pump to the flow valve with a waste trap set up and positioned between the vacuum outlet and flow valve. Turn on the vacuum and set the 3-way valve to apply vacuum to the apparatus.

7. With vacuum applied, repeat the tightening process using the diagonal crossing pattern. Tightening while vacuum is applied ensures a tight seal, preventing cross contamination between slots. Failure to tighten screws during application of vacuum prior to starting the assay may lead to leaking between the wells.
8. Apply 100 µL of sterile distilled water to all 96 sample wells. Use an 8-channel pipette will simplify the process of adding solutions to the Bio-Dot apparatus. Addition of distilled water is necessary to rehydrate the membrane following the vacuum procedure (above step). If this step is not performed prior to applying samples, assay results will show halos or weak detection signal.
9. Gently remove the distilled water from the wells by vacuum. **Watch the sample wells**. As soon as the distilled water drains from all the wells disconnect the vacuum.
10. Apply samples to the wells using an 8-channel pipette and ensure that there are no air bubbles. Air bubbles can be removed by pipetting the liquid in the well up and down. Allow the samples to stand for 10 minutes before applying vacuum. As soon as the sample drains from all the wells disconnect the vacuum.
11. Wash down the sample with 200 mL of sterile deionised water as described in step 8.

APPENDIX 12

Solutions and buffers for dot-blot hybridisation

- **MOPS buffer**

  200 mM morpholinopropanesulfonic acid (MOPS)

  50 mM sodium acetate

  20 mM EDTA

  Adjust pH to 7.0 with NaOH. Sterilise by autoclaving at 121°C for 15 min.

  Note: This buffer yellows with age, if exposed to light or autoclaved. Discolouration does not affect its performance appreciable.

- **20x SSC stock**

  3 M NaCl

  300 mM sodium acetate

  Adjust pH to 7.0 with a few drops of conc. NaOH. Sterilise by autoclaving at 121°C for 15 min.

- **10% Blocking stock solution**

  Dissolve 10% (w/v) Blocking reagent (Roche Diagnostics GmbH, Germany) in Maleic acid buffer. Stir and heat to 60°C for 1 h until dissolved. Sterilise by autoclaving at 121°C for 15 min and store at 4°C.

- **Maleic acid buffer**

  100 mM Maleic acid, 150 mM NaCl

  Adjust pH to 7.5 with conc. NaOH. Sterilise by autoclaving at 121°C for 15 min.

- **10% N-lauroylsarcosine stock solution**

  Dissolve 10% (w/v) N-lauroylsarcosine in distilled water. Filter sterilise through a membrane (0.2 - 0.45µm).

- **10% SDS stock solution**

  Dissolve 10% (w/v) sodium dodecyl sulphate in distilled water. Heat to 68°C to assist dissolution. Adjust the pH to 7.2 by adding a few drops of conc. Filter sterilise through a membrane (0.2-0.45µm).

- **1 M Tris (pH 7.4)**

  Dissolve 121.1 g Tris base in 800 mL of distilled water. Adjust the pH to 7.4 by adding 70 mL of conc. HCl. Allow the solution to cool to room temperature before making the final adjustments to the pH. Make up the volume of the solution to 1 L. Sterilise by autoclaving at 121°C for 15 min.

APPENDIX 13

Chromogenic detection of probes on a blot with NBT/BCIP

Unless otherwise indicated, all of the following incubations are performed at room temperature with shaking. The volumes below are for a 10 cm x 10 cm (100 cm^2) membrane processed in plastic tray. If you are processing smaller membranes, use a smaller container and smaller volumes. Be sure each solution covers the membrane and keeps it from sticking to the container as it is shaken.

Before you start: First prepare the working solutions for the detection procedure:

- **Maleic Acid Buffer:**
  0.1 M Maleic acid
  0.15 M NaCl;
  Adjust with conc. NaOH to pH 7.5. Solution is stable at room temperature.

- **Blocking Solution:**
  Dilute 10x Blocking solution 1:10 with Maleic Acid Buffer. Prepare fresh.

- **Washing Buffer:**
  0.1 M Maleic acid, 0.15 M NaCl; pH7.5; 0.3% (v/v) Tween 20. Solution is stable at room temperature.

- **Antibody Solution:**
  Centrifuge Anti-Digoxigenin-AP for 5 min at 10 000 rpm in the original vial prior to each use, and pipet the necessary amount carefully from the surface. Dilute Anti-Digoxigenin-AP 1:5 000 (150 mU/mL) in Blocking solution. Prepare fresh, stable for 2 h at 4°C.

- **Detection Buffer:**

  0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5 (20°C). Solution is stable at room temperature.

- **TE Buffer:**

  10 mM Tris-HCl, 1 mM EDTA, pH 8.0. Solution is stable at room temperature.

Procedure:

1. Transfer the membrane to a plastic container (e.g. a lunch box) containing 100 mL Washing Buffer. Incubate for 2 min at room temperature, with shaking. Discard the Washing Buffer.
2. Add 100 mL Blocking Solution. Incubate membrane for 30 min, with shaking.

   Tip: This blocking step can last up 3 h without affecting results. Discard the Blocking Solution.
3. Add 20 mL Antibody Solution. Incubate the membrane for 30 min, with shaking. Discard the Antibody Solution.
4. Wash membrane twice (2 x 15 min) with 100 mL portions of Washing Buffer.
5. Equilibrate membrane for 3 min in 20 mL Detection Buffer.
6. Prepare the Colour Substrate Solutiuon by adding 200 µL of the NBT/BCIP stock to 10 mL of Detection Buffer to make Colour Substrate Solution.

   Note: Store the Colour Substrate Solution protected from light.
7. Cover **one** membrane completely with 10 mL Colour Substrate Solution.

   Note: If you are working with more than one membrane, process each membrane separately. Incubate the membrane in the dark **without shaking**.

Note: The coloured precipitate begins forming within a few minutes. The reaction is usually complete after 16 h.

8. When the colour reaction produces dots of the desired intensity, stop the reaction by rinsing the membrane for 5 min in 50 mL of TE buffer.
9. Document the NBT/BCIP result by photocopying or photographing the wet membrane. Dry the membrane at room temperature and store the dry membrane at room temperature. Colours may fade on the dry membrane, but you can renew them by rewetting in TE buffer.