OLIVE WASTEWATER BIOREMEDIATION USING A ROTATING

BIOLOGICAL CONTRACTOR (RBC)

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by

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DECLARATION

I, J.S Tapon Njamo, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

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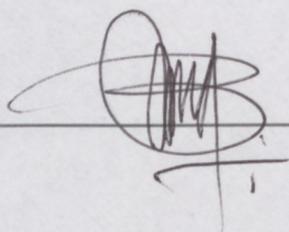
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ABSTRACT

The expansion of the South African olive processing industry has brought an increased threat to the environment. More production activities lead to more wastewater generation that requires more costly treatment. Regulatory bodies concerned with the release of effluents into the environment are imposing evermore-restrictive guidelines. With this information in mind, the South African olive industry is facing a significant challenge of maintaining economic competitiveness while becoming more environmentally benign. To begin addressing this challenge, the olive processing industry must develop and implement new, more effective, tailored treatment technologies to remediate olive wastewater prior to its release into the environment. In this study, the use of indigenous olive wastewater biofilms in combination with a rotating biological contactor (RBC) was investigated for possible bioremediation purposes. The development of microbial biofilms resulted in the breakdown of the hazardous chemical compounds present in the olive wastewater, i.e. reducing the chemical oxygen demand (COD) and polyphenol content. Results showed that indigenous microorganisms within table olive and olive mill wastewater have a strong tendency to form biofilms. Furthermore, when these biofilms are applied to a small-scale RBC system, significant lower levels of both COD (on average 55% for table olive wastewater (TOWW) and 46% for olive mill wastewater (OMWW) and total phenol (on average 51% for TOWW and 39% for OMWW) were obtained. Results from shocking the biofilms with chemicals routinely used during olive processing indicates that most have a negative effect on the biofilm population, but that the biofilms are able to survive and recover in a relatively short time. This study confirms the potential application of indigenous biofilms found in olive wastewater streams for future bioremediation technologies that form the basis for the development of an eco-friendly, easy-to-manage, low cost technologies specifically designed for the small South African olive processing industry.

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Contents

CHAPTER 1

General Introduction and Project Aims

1.1   Introduction

The treatment of liquid and solid wastes from olive-processing industries has received a vast amount of research focus over the last 50 years, due to the serious environmental problems associated with these wastes (Niaounakis and Halvadakis, 2006). By its inherent nature, olive processing, including table olive and olive oil production, requires substantial amounts of clean water often in countries where this resource is scarce and generates significant volumes of wastewaters and solid wastes. The high-polluting potential of olive wastewater (OWW), which is ca. 200-400 fold more than domestic sewage, is as a result of the high organic content, particularly the phenolic fraction, as well as total solids and acidic character of OWW (Cardinali *et al.*, 2010). Unregulated exposure of both aquatic and terrestrial ecosystems to OWW, untreated or partially treated, leads to serious disturbances in the natural ecology of these exposed environments and long-term environmental destruction due the antimicrobial and phytotoxicity effects of OWW. Furthermore, a range of secondary problems introduced by current OWW disposal strategies, including surface water and aquifer pollution, water discolouration and changes in soil fertility when applied as irrigation water, also need to be contended with (Al-Malah *et al.*, 2000; Tezcan Ün *et al.*, 2006).

South African law prescribes that all discharged wastewaters should comply within a certain maximum range of chemical oxygen demand (COD) (75 mg/L) and pH values (Water Act No 36, 1998). Typically, OWW is characterised by COD levels 100-1000 times that of the allowable limit (Davies *et al.*, 2004). As is the case with other high organic wastewaters, OWW is considered a severe polluting waste product as microorganisms in aquatic environments will respire the excess organic content, leading to a rapid depletion of dissolved oxygen with adverse effects on the remaining aquatic life in the receiving water bodies. More importantly, a significant fraction of the organic content within OWW is in the form of simple and polymerised phenolic derivatives, which account for the phytotoxic and antimicrobial effects of OWW, and hampers the application of aerobic or anaerobic microbial treatment strategies (Tafesh *et al.*, 2011). Furthermore, some of the phenolic compounds in OWW are particularly recalcitrant to degradation due to its complex and variable polyphenolic composition, including for example flavonoids and tannins, and imparts a dark black/brown colour to the wastewater (Zouari and Ellouz, 1996). Dilution rates of 1:5000 is typically needed to safely dispose OWW in natural aquatic environments, making this option impractical due to the sheer volume of additional water required (Rivas *et al.*, 2001).

It is estimated that worldwide table olive production has surpassed 1.7 million tons per year, while the annual olive oil production levels are around 2.5 million tons, of which the Mediterranean countries are the leading producers (Niaounakis and Halvadakis, 2006). Furthermore, it is estimated that between 7 - 30 million m^3 olive-mill wastewater (OMWW) is produced annually worldwide, and this figure excludes table olive wastewater (TOWW) for which no accurate account of wastewater volumes is available. The South Africa olive industry is a small but budding industry sector compared to the global olive industry, with the Western Cape the primary producer (ca. 90% of South Africa's total olive production). Currently, ca. 3 500 tons of table olives are processed and 430 tons of olive oil is produced annually in South Africa (Anon., 2000). Although small, the South African olive-related manufacturing industry is considered a rapidly expanding sector as the demand for olive-related products is far in excess of the current supply. Based on the rate at which new olive orchards are being planted, olive farming is growing at a rate of ca. 20% annually, i.e. doubling in size every four to five years, which makes it one of the fastest growing agricultural sub-sectors. This envisioned growth of the South African olive processing industry brings with it an increased hazard to the environment. Increasing olive processing volumes lead to more wastewater, which in turn requires more treatment capacity. Furthermore, regulatory bodies are becoming increasingly more strict with the release of polluting effluents into the environment and are imposing ever-more stringent guidelines for the release of wastewater. In developing countries with a severe water deficits such as South Africa, OWW treatment should focus on the remediation of the water for reuse purposes to minimize environmental effects. In light of the scenario depicted above, it is clear that the South African olive industry will be facing a mammoth challenge in terms of sustainable growth, i.e. maintaining economic competitiveness while remaining environmentally benign.

Various methodologies and technologies for OWW treatment have been investigated and developed to date, either physicochemical, biological methods or a combination of both. The physicochemical methods for OWW treatment include a diverse range of treatments, from simple precipitation (using flocculants and coagulants) to more advanced chemical and electrochemical treatments, ultrafiltration, reverse osmosis, incineration, thermal concentration and cryogenesis techniques. However these methods are generally characterised as being either too expensive and/or unable to completely resolve the pollution problem of OWW (Justino *et al.*, 2012).

In contrast, the biological treatment of OWW, which include both anaerobic and aerobic processes or a combination of the two, have gained considerable acceptance and allure and range from simple methods such as evaporation ponds, composting and co-composting to more complex aerobic activated sludge and anaerobic digestion methods using well-controlled conditions in more or less advanced bioreactors. Biological methods constitute an attractive alternative over physicochemical methods for remediation, as it is generally considered to be less expensive, can selectively achieve complete destruction of organic pollutants and can be used *in situ* for pollutants that are present at low but environmentally relevant concentrations. Various bacterial and fungal species native or alien to OWW have been evaluated either as single species or consortia for their ability to degrade components of OWW (Niaounakis and Halvadakis, 2006).

Importantly, the advanced treatment technologies developed to date have been developed with the large olive industries in mind, and are not as such suitable or feasible for the small South African olive processing industry due to the high monetary and human capital input and technical expertise required. To begin to address this challenge, the South African olive processing industry must develop and implement tailored, more cost-effective, cutting-edge treatment technologies to remediate olive wastewater prior to its release into the environment. Ideally, the treatment methodology must be flexible enough to endure seasonal fluctuating organic loads, require minimal human attention, sustainable and should not require too much land space.

In this study, we assessed the application of a small-scale rotating biological contactor (RBC) in combination with indigenous OWW biofilms as a means to develop a tailor-made treatment option for the South African olive industry. RBC systems represent an excellent option for OWW treatment for several practical reasons. RBC systems are relatively easy to operate, using discs on a rotating shaft that provide a partially effluent-submerged surface that supports biofilm growth responsible for the degradation of the organic compounds in the wastewater. Furthermore, it allows for sufficient retention times, is compact in size, has low energy cost, high process stability and high specific removal rates (Tawfik *et al.*, 2005). The RBC has been already used in the treatment of other wastewaters produced by different industries such as mining (Stott *et al.*, 2001) and domestic sewage treatment (Costley and Wallis, 2001; Gupta and Gupta, 2001; Tawfik *et al.*, 2005). In general, the efficiency of the RBC depends heavily on parameters such as the microbial composition of the biofilms developing

CHAPTER 2

Literature Review

2.1   Introduction

This chapter provides a literature overview of olives and the production practices for table olives and olive oil, with a specific focus on the various water-dependant processing steps that contribute to OWW, the chemical characteristics of OWW (specifically, TOWW and OMWW), the microbial ecology of OWW, the current treatment options available and the benefits of using biofilms in OWW bioremediation.

2.2   History of Olive Tree Cultivation and Olive Processing

Olive trees (*Olea europaea*) are among the oldest known cultivated trees in the world. Although the true origin of the olive tree is highly disputed, archaeological and genetic evidence suggest that its domestication and cultivation is rooted 8000 years ago in the ancient Syrian/Iranian regions of the Middle East (Bartolini and Petruccelli, 2002; Arvanitoyannis *et al.*, 2007; Vossen, 2007; Kaniewski *et al.*, 2012). The olive tree is particularly long-lived, drought and salinity tolerant and has been reliable producers of food and oil for thousands of years. Olive cultivars are clustered in a wide group called the *O. europaea* L. complex, comprising approximately 40 species and sub-species, which have spread to countries such as Asia, Australia, Africa, Europe and the Americas due to its ease of vegetative propagation and cultivation in dry climates (Sweeney, 2006; Kapellakis *et al.*, 2008).

Similarly to the history of the domestication and cultivation of olive trees, the processing of olives for edible olive products and extraction of the oil is one of the oldest and most pivotal agro-industrial activities in the Mediterranean countries (Zamora *et al.*, 2001; Kaniewski *et al.*, 2012). Olive products are a staple of the Mediterranean diet, with widely recognized health benefits, including reduced coronary heart disease and anticancer properties, due to the presence of mono-unsaturated free fatty acids (e.g., oleic acid) and antioxidant polyphenolic compounds (Willett *et al.*, 1995; Samieri *et al.*, 2011; Poole and Blades, 2012).

Today, olives are the most extensively cultivated fruit crop in the world, with 800 million productive olive trees worldwide, 98% of which are located in the Mediterranean biogeographical zone, and a land surface occupation surpassing that of grape cultivation (Kaniewski *et al.*, 2012). The annual worldwide production of olive has been estimated at between 2.8-3.0 million tons and an annual increase in demand of about 2% (Asfi *et al.*, 2012). Although olives are produced in approximately 39 countries worldwide, the majority of world olive production still resides in the Mediterranean areas, including Spain, Italy, Greece, Syria, Turkey, Tunisia and Morocco (Niaounakis and Halvadakis, 2006).

The olive tree was first brought to South Africa in the days of Jan van Riebeeck according to his 18th July 1661 dairy entry, and the first olive oil cultivars were imported by Ferdinando Costa in 1925 (Costa, 1998; Martinaglia, 2011). Olive trees were first cultivated on a commercial-scale in South Africa from the beginning of the twentieth century (Anon., 2004). The local industry is mainly based in the Western Cape with its typical Mediterranean climate and well-drained soils, with the Paarl Valley at the centre of production. Although the typically Mediterranean climate of the Boland area (Paarl, Stellenbosch and Somerset West) makes it ideal as the primary olive growing region, olives are also cultivated in Riebeeck Kasteel, McGregor and Hermanus, as well as in the Karoo and the Northern Cape Province. Currently, it is estimated there are ca. 450 000 olive-bearing trees in South Africa, with the Western Cape accounting for about 90% of the total South African olive production (Anon., 2000). Many of the olive producers in South Africa are primarily wine producers and the olive growing activities are often a secondary or complementary source of revenue and work supply to seasonal labourers during the winter seasons. These farmers produce approximately 430 tons of olive oil and 3 500 tons of table olives annually. Due to an increase in consumer awareness of the associated health benefits of the olive products, the South African olive industry experienced significant growth in the early 1990's. Currently, olive oil production in South Africa is experiencing an approximately 7% growth in revenue per annum, which is higher than the world olive oil growth of 2% per annum, while growth in local table olives is approximately 5% per annum (Anon., 2000).

Many olive cultivars have been imported into South Africa, but only few have proved successful under local conditions (Anon., 2000). The most commonly grown cultivar in South Africa is Mission, from California, although small quantities of Spanish Manzanilla and Gordal cultivars, Kalamata from Greece, and Azapa from Chile, are also cultivated in South Africa (Fernández et al., 1997). The major difference between the South African and Mediterranean olives processing industry is the fact that the European Union subsidises olive growers, processors and packers in order to sustain the olive market. Furthermore, the South African olive markets are threatened by lower-quality olive imports brought into the country at cheap prices. With South Africa's economic policy having moved away from protectionism, the local olive industry now finds itself at a disadvantaged situation (Costa, 1998). South Africa olive producers cannot be price competitive with olive oil for exportation, especially in the face of the worldwide oversupply. However, South African producers are successfully

selling their products on the local market, to some extent replacing imported olive oil (Anon, 2000). Consequently, the olive industry of South Africa is in a much weaker position to spend a large percentage of its revenue on research and implementation of OWW treatment regimens compared to the Mediterranean countries.

2.3 Olive Processing Techniques

Olive fruits can be processed into a variety of edible table olive products or pressed for oil extraction. To ensure good processing, olives are in many parts of the world still harvested by hand when ripe or unripe to prevent bruising or damage to the olives. The time of harvesting is dependent on the type of olive product envisaged. Once harvested, olives are separated from any objects such as leaves and twigs, and washed (to remove pesticides, dirt, etc.) and sorted according to size before processing commences. The sections below provide an overview of the different olive processing procedures, with a specific focus on the steps that generate the most wastewater (Niaounakis and Halvadakis, 2006).

2.3.1 Table olive processing

Two distinctive terms are used to characterise the processes in which table olives have been produced. When olives are processed by the use of alkaline substances, the term "treated" is used, while the term "untreated" is used when the olives are subjected only to salt or brine treatment (Dymiotis, 1990). The main objective of processing table olives is to remove the astringent and bitter taste, which comes from the secoiridoid, oleuropein glucoside, and other complex phenolic compounds in fresh olives (Marsilio and Lanza, 1998; Dymiotis, 1990; Frankel, 2011). Oleuropein (Fig. 2.1) is the major polyphenol that has to be removed in order to improve the edibility of the olive fruit and improve the organoleptic quality of olives.
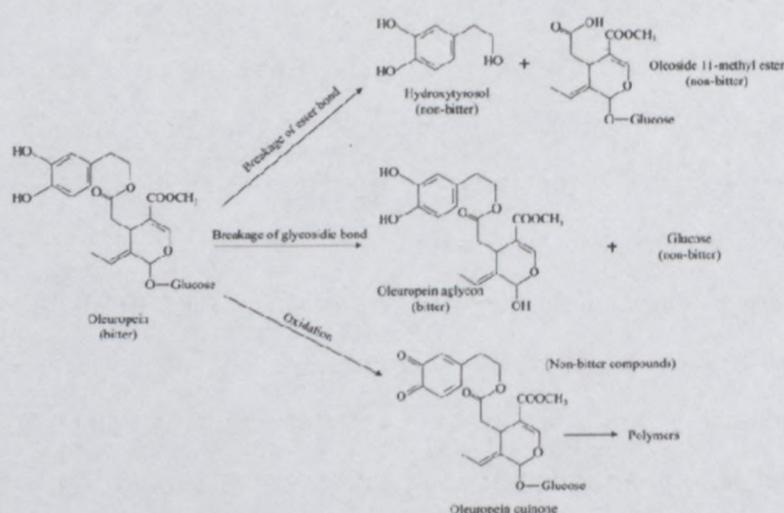
Figure 2.1. Chemical transformations of oleuropein, an ester of hydroxytyrosol and oleoside 11-methylester (García et al., 2008; Frankel, 2011).

Although not poisonous it was found to be an inhibitor of the lactic acid fermentation in some styles of table olive production (Medina *et al.*, 2008). Hydrolysis of oleuropein can be achieved by exposing olives to alkaline conditions (NaOH/lye treatment) during which the ester bond between hydroxytyrosol and the oleoside-methylester is cleaved to release non-bitter products (Fig. 2.1) (García *et al.*, 2008). Similarly, during untreated olive processing, oleuropein can be hydrolysed by endogenous enzymes, such β-glucosidases and esterases, produced by yeast and bacteria during the natural fermentation stage (Restuccia *et al.*, 2011). Lastly, during the process of treated black olives, where the black colour is artificially produced from green olives, oleuropein undergoes oxidation reactions and polymerization reactions where some of the oxidation intermediates are also non-bitter in nature (Fig. 2.1).

In addition to the hydrolysis of oleuropein and other complex phenolic compounds during lye treatment, NaOH also leads to the partial degradation of cell wall polysaccharides (pectin, hemicellulose and cellulose), with the subsequent release of sugars and organic acids (including glucose, fructose, mannitol, sucrose, citric acid, malic acid) from the olive flesh (Mafra *et al.*, 2007). These sugars and organic acids together with the increased skin permeabilization caused by alkali treatment are essential for the successful completion of the fermentation stages of the table olive process.

The fermentation stage of olives is carried out primarily by the spontaneous microbial populations of lactic acid bacteria encountered on olive fruit and in the brine environment. Immediately after brining, fermentable substrates and other nutrients diffuse from the olives into the brine, while NaCl diffuses into the olives (Sánchez *et al.*, 2000). During the fermentation stage, fermentable substrates in olives are converted to organic acids and alcohols (primarily lactic acid, acetic acid, succinic acid, formic acid, ethanol, 2, 3-butanediol) that are released into the fermentation brine. The net effect is an accumulation of acids and a lowering of brine pH, which in combination with the salt content of brine ensures the microbiological safety of the product and at the same time, provides the desirable sensory attributes of the final product (Sánchez *et al.*, 2000; Doulgeraki *et al.*, 2012).

Although there are several table olive varieties that can be produced, the two main types of table olives produced in South Africa are treated green olives, often referred as Spanish style, and the American style (treated black olives), which shares similar processing steps (Fig. 2.2). However, the treated olive styles produce a higher volume of heavily contaminated

wastewaters, including the lye solutions and the further wash waters needed to remove the excess alkali from the olive flesh (Frankel, 2011).

2.3.1.1 Treated green olives - Spanish-style. Green table olives, accounting for ca. 40–50% of the world production (Fig. 2.2), are those exposed to lye (NaOH solution) in the absence of aeration to remain green (Parinos *et al.*, 2007). The olive fruits are initially immersed in a 2% to 2.5% lye solution for 8-12 hours. To avoid blistering of the olive skin, the alkaline solution is prepared one day in advance, while the lye treatment is considered complete when the alkaline solution has penetrated to about $^2/_3$ or $^3/_4$ of the olive fruit's flesh. The remaining part of the flesh not affected by NaOH provides the necessary sugars for subsequent fermentation. If the lye solution does not penetrate sufficiently, bitterness-removal will be incomplete and the subsequent fermentation steps will not commence satisfactorily. However, complete penetration of lye to the stone will result in the excessive softening that often leads to spoilage of the olives.
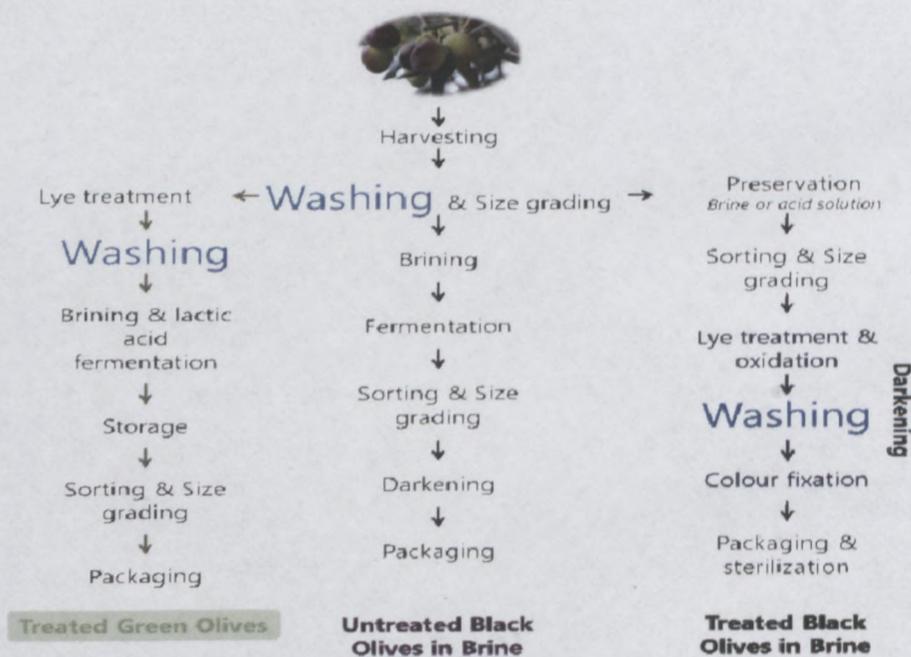
Figure 2.2. Overview of the steps in the three main types of table olive processing (Fernández *et al.*, 1997).

The lye treatment is followed by several rounds of thorough washing of the treated olives with excess amounts of clean water to remove residual lye solution. The washing will last for a few hours and the duration normally depends on the strength of the lye solution. While excessive washing will remove unnecessary levels of the released sugars, insufficient washing will not completely remove the bitterness of the olives, in addition to other problems such as

residual high pH values. When the lye treatment has been completed, the olives are placed in a brine of sodium chloride (NaCl) during which the lactic fermentation commences (Fernández *et al.*, 1997). Although the strength of the brine used is dependent on the cultivar, in general NaCl concentrations of about 5-10% is initially used, followed by reduced values of about 6% during the lactic fermentation. The main fermentation activity is a result of *Lactobacillus* species that convert the available sugars from the olive flesh into lactic acid. The spontaneous fermentation process takes about nine months to a year, depending especially on temperature conditions, and comprise of three successive phases. In the first phase, which generally lasts from 2 to 3 days, the brine has initially a high pH that decreases to a value of approximately 6. The microorganisms that best characterize this phase are *Enterobacteriaceae.* During the second phase (up to 10-15 days), lactobacilli and yeasts generally develop quickly, and *Enterobacteriaceae* decrease until they disappear completely at a pH of around 4.5. During the third phase, which lasts until the fermentable material is exhausted, only species of *Lactobacillus* (mainly *Lactobacillus plantarum*) abound and co-exist with a yeast flora. After the olives have been fermented, the pH of the brine is adjusted to between 3.8 and 4.2 and the fermented olives are packaged with hot brine solution (8% v/w NaCl to avoid further fermentation) into cans, jars or sachets, sealed and pasteurised for one hour (Fernández *et al.*, 1997).

2.3.1.2 Treated Black olives – American style. Black table olives (Fig. 2.2) are those exposed to air during successive lye treatment and aeration steps, which allows for the oxidation and polymerization of phenolic compounds present in the olive fruit, which in turn changes the colour of the olives from green to black. During the production of this table olive style, olives are harvested when their colour starts changing from straw green to purple. These olives may or may not be treated by immersion in brine before lye treatment. Once lye treatment has been completed, the lye solution is drained and the olives are washed with clean water for effective removal of the remaining lye solution. This is followed by placing the olives in a brine solution (8% v/w NaCl), pasteurization and packaging. Since no fermentation takes place, these olives are milder in taste. Untreated black olives (or Greek style) differ from the two methods described above by lacking any lye treatment and thus requiring less washing steps (Bianchi, 2003).

2.3.2 Olive oil production

The olive fruit contains around 12 to 30% oil (on a mass per mass basis), depending largely on cultivar, maturity stage and environmental conditions (Costa, 1998). The process of olive oil production can be subdivided in two distinct phases: the preparation of a homogeneous paste and the oil extraction and purification phase. The first phase of olive oil production involves the washing and milling of the fruit to tear the olive flesh cells and release oil from the fruit tissue (Costa, 1998). The crushed olive pulp then undergoes a malaxation stage (slow mixing at a constant temperature, usually bellow 30°C for 30-90 min, followed by one of three processes to separate the oil, i.e. the press method or a decanter (two- or three-stage centrifugation system) (Costa, 1998; Caputo *et al.*, 2003; Flanagan and Hildenbrand, 2003). These three systems (Fig. 2.3) are nearly similar with respect to the oil yield (except pressing), but they significantly differ in the amount and composition of by-product fractions (solid, liquid) produced.
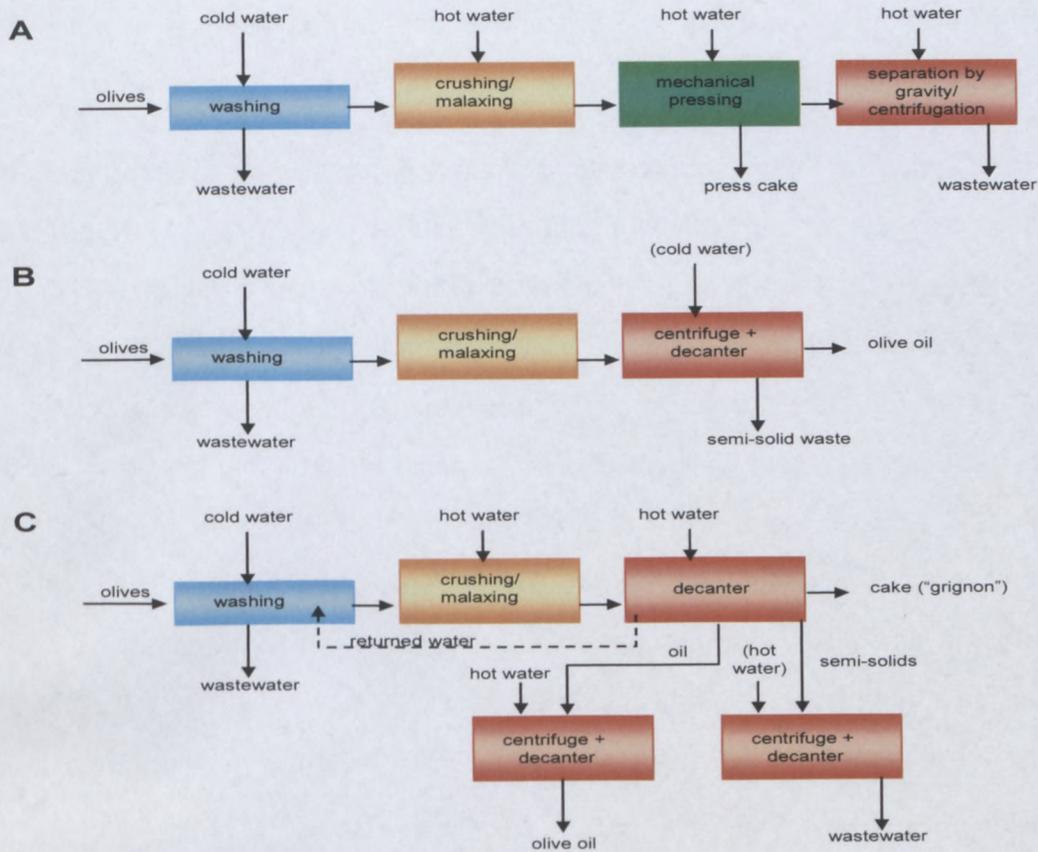
Figure 2.3. Diagrammatical representation of the water consumption during three oil extraction methods showing the (A) classic hydraulic press method, the (B) two- and (C) three-phase centrifugation system (Adapted from Skerratt and Ammar, 1999).

2.3.2.1 The discontinuous (batch) press method (traditional method). One method of extracting the oil from the olive pulp is the discontinuous press method, which is based on hydraulic pressure (Fig. 2.3A). Once the olives are crushed, the paste moves into a cylindrical trough where blades turn the mixture over and over to form a homogenous paste (Costa, 1998). The resulting paste is spread onto round mats of coconut fibre or nylon mesh with holes in the centre. The mats are stacked onto a dowel and under a hydraulic pressure and large quantities of rinsing water; the liquid (water and oil) is pressed out. To aid separation of the liquid phases, water is run down the sides of the discs to increase the speed of percolation. The oil and water mixture released from the olive paste can finally be separated by sedimentation/gravitation followed by decantation or by centrifugation (Niaounakis and Halvadakis, 2006). This method is responsible for generating 40-55 L wastewater per 100 kg of olives (Fiorentino *et al.*, 2003). The traditional process of olive oil extraction by batches has been discontinued in most of the operating olive oil plants, and these days the extraction of

olive oil is obtained by one of two different continuous processes, namely the two-phase or the three-phase system.

2.3.2.2 Continuous centrifugation methods. In this process, olives are crushed and mixed to form a paste with or without the addition of water according to either the three-phase or two-phase system. The three-phase centrifugation system (Fig. 2.3C) is based on the specific weight differences between water and oil and during the process, warm water is added to the olive paste to dilute the olive paste prior to centrifugation. As with the traditional discontinuous press method, the olive paste is separated into three phases, i.e. oil (20%), olive cake (30%, pulp residue and stones, also known as olive mill residual solids [OMRS]), and a vegetative water phase (50%, OMWW, also known as black water or "alpechín" in Spain) (Ergüder *et al.*, 2000; Alburquerque *et al.*, 2004). The major drawbacks of the three-phase extraction system includes (1) oil with reduced phenol content due to the addition of warm water; (2) increased amounts of wastewater, 1-1.6 m^3/tonne of olives; and (3) increased energy requirements (Alburquerque *et al.*, 2004; De Marco *et al.*, 2007). Three-phase extraction systems are still used in some olive mills, mostly in Italy, where they have not yet adapted to the more recent two-phases system, which leads to a considerable reduction in OMWW volumes (De Marco *et al.*, 2007; Roig *et al.*, 2006).

In the 1990's, the olive oil industry adopted a new continuous centrifugation system with a two-phase decanter, which separates the oil from a solid-liquid by-product. In the two-phase system (Fig. 2.3B), the washed olive fruit is fed to a mill for crushing and from there to a mixer to form a paste with a negligible amount of water required. The resulting paste is fractionated into two-phase helical conveyor centrifuge into oil and a solids-liquid mixture (called "alpeorujo" in Spain), which contains 2.5 – 3.5 % residual oil and about 60 % water (Giannoutsou *et al.*, 2004). The two-phase decanter system has advantages over the three-phase system, such as a reduction of the produced waste (0.2 m^3/tonne of olives), higher oil yield and lower energy consumption (Alburquerque *et al.*, 2004). Furthermore, olive oil from two-phase centrifugation systems contains more phenols, tocopherols, trans-2-hexenal and total aroma compounds and is therefore more resistant to oxidation than the oil from three-phase system. However, one disadvantage of the two-phase process is that it is costly (Giannoutsou *et al*, 2004; Niaounakis and Halvadakis, 2006).

2.3.3 Wastewater production during olive processing.

As can be seen from Figures 2.2 & 2.3, in all stages of olive processing (i.e. cleaning, debittering, washing and fermentation steps for table olives, and washing and oil extraction stages for olive oil) large quantities of clean water are consumed. The volume of wastewater produced by the various table olive styles differs according to treatment regime (Table 2.1). In general, untreated olives have relatively reduced wastewater expenditure due to the production of wastewater from only the initial washing and brining steps. However, in comparison, lye-treated olives are always associated with higher volumes of wastewater with higher pollutant content due to the exhausted lye and several washing steps needed to eliminate the excess alkali. The pH of these wastewaters also differs depending on the treatment steps, for example the steps involving lye treatment are alkaline, while wastewater from fermentation steps is usually more acidic (Costa, 1998; Fernández et al., 1997).

Table 2.1. Approximate volume of wastewater produced per kg olives during the main stages of various table olive commercial preparations (Adapted from Fernández et al., 1997).

Commercial preparation	Lye (L/kg)	Washing waters (L/kg)	Holding solutions (L/kg)	Total (L/kg)
Spanish style (Green olives)	0.5	0.5-2.0	0.5	1.5-3.0
California black ripe olives	0.5-2.5	0.5-3.0	0.5	1.5-6.0
Greek naturally black olives			0.5	0.5

Wastewater from the oil extraction process is generated during the washing of the harvested olives and during the extraction of the oil from the olive pulp. Typically OMWW is in a state of a stable emulsion composed of water, olive pulp and residual oil (Lanciotti et al., 2005). The volume of OMWW depends on the method of olive oil extraction used (Mantzavinos and Kalogerakis, 2005), but on average 1.2–1.8 m^3 OMWW per tonne of olives is produced during the milling process (Tabatabaei et al., 2010).

2.4 Chemical and Physicochemical Profile of OWW

The composition of the OWW generated during the olive processing season is not constant, i.e. the chemical and physicochemical characteristics of OWW (TOWW and OMWW) are variable, depending on several factors, including firstly and most importantly the processing methods used as well as a variety of other factors such as the duration of aging steps, the harvesting time, degree of fruit ripening, olive cultivar, climatic conditions, type of soil used for cultivation, and the pesticides and fertilizers used (Niaounakis and Halvadakis, 2006). In general, the basic composition of OWW can be summarised as 83-96% water, 3.5-16%

organic compounds and 0.5-2.5% minerals (Ramos-Cormenzana *et al.*, 1996; Greco *et al.*, 1999).

Table 2.2 indicates a typical chemical composition and comparison of both TOWW and OMWW generated in a South African scenario as studied by Burton (2004). The overall composition of TOWW and OMWW is similar in nature, although TOWW is often weaker in organic strength (Burton, 2004; Parinos *et al.*, 2007).

Compared to other wastewater sources, OWW has a high organic load (BOD$_5$: 20–120 g/L; COD: 40–260 g/L) (Aggelis *et al.*, 2001; Komnitsas and Zaharaki, 2012), as can also be seen in OWW from South African, especially in the case of OMWW (with up to 262 g/L). Although the high COD levels are considered an important negative aspect of OWW, it is relatively easily remediated. On the other hand, the most negative characteristic of OWW is the high concentration phenolic compounds and long chain fatty acids, which account for most of the toxicity effects of OWW. For example, the pollution load of the OMWW is on average a thousand times higher than the domestic sewage with regards to phenolic content (Niaounakis and Halvadakis, 2006). Another important indicator is also the how much of the COD is contributed by the phenolic fraction. From the South African scenario (Table 2.2), it can be seen that although TOWW is weaker in overall organic strength, the ratio of phenolic compounds are considerably higher than in OMWW, which will impact any bioremediation processes of this wastewater.

Table 2.2. Physico-chemical properties of various olive wastewaters

Property[a]	TOWW			OMWW
	Black olive brine	Green olive brine	NaOH treatment	
pH	4.53	4.08	9.91	4.86
Conductivity (mS.cm^{-1})	83.1	79.0	16.3	9.55
Total Solids	114.2	114.3	59.8	119.1
Dissolved Solids	113.9	113.9	58.5	58.8
Suspended Solids	0.3	0.4	1.3	60.3
COD	58.7	50.4	66.3	202
TOC	18.5	18.3	20.6	15.4
Reducing sugar	0.26	1.18	9.31	13.82
Lipids	0.83	1.08	1.39	74
Total Phenols (GAE)	4.05	2.14	1.06	4.07
Simple Phenolics[b]	1.87	1.64	0.51	n.d.
Phenol % of COD (%)		32		11
NH$_4^+$	0.31	n.d.	n.d.	0.03
Na$^+$	22.35	n.d.	n.d.	1.90
K$^+$	3.71	n.d.	n.d.	3.42

Mg^{+}	0.01	n.d.	n.d.	0.23
Ca^{+}	0.71	n.d.	n.d.	0.68
Cl^{-}	30.00	n.d.	n.d.	0.50
F[-]	0.82	n.d.	n.d.	0.12
NO_2^-	0.00	n.d.	n.d.	0.20
PO_4^-	0.62	n.d.	n.d.	0.25
SO_4^-	1.04	n.d.	n.d.	0.78

[a] – all units in g.L^{-1} unless otherwise stated. [b] – as determined by HPLC. COD = chemical oxygen demand, TOC = total organic carbon, GAE = Gallic acid equivalents, n.d. = not determined.

Another important OWW pollutant is the lipid fraction, a problem especially in OMWW (see Table 2.2), that is hydrolysed to glycerol and long-chain fatty acids. The residual oil in OMWW could be considered wasted primary product (oil) and is indicative of inefficient extraction procedures. During the oil extraction procedure, an oil-water emulsion is formed that is not readily separated by the press or centrifuge methods, and ends up as part of the wastewater (Ranalli *et al.*, 2001). High lipid concentrations, specifically the long-chain fatty acids hydrolysis products, have been shown to contribute to the antimicrobial activity of OWW, as well as having a negative impact on physical methods (such as filtration) to clean up OWW (González *et al.*, 1990; Sayadi *et al.*, 2000). Compared to OMWW, TOWW usually has minimal lipid contamination; this is not unexpected as the cells are not disrupted in the fermentation process only during oil extraction.

Both TOWW and OMWW are normally low in nitrogen (N), containing mostly amino acids such as aspartic acid, glutamic acid and arginine (Caputo *et al.*, 2003; Parinos *et al.*, 2007; McNamara *et al.*, 2008). This high C/N ratio should be taken in consideration when designing a bioremediation strategy as N supplementation might be required. Furthermore, as summarized in Table 2.2, the brining wastewater fractions of TOWW and OMWW as a whole are characterised by a relatively acidic pH (4.53 and 4.86), while lye wastewater fractions are alkaline in nature (pH = ±10). Furthermore, brining wastewater fractions of TOWW has significantly higher conductivity values compared to the lye wastewater fraction of TOWW and OMWW, most probably due to the high Na^{+} and Cl^{-} ion concentrations present. Lastly, total solids are a combination of total suspended solids and total dissolved solids and in this analysis seem to be equivalent between the brining wastewater fractions of TOWW and OMWW as a whole, while the total solids value in lye wastewater fractions is significantly lower. A large amount of solids is considered a treatment obstacle for OWW, especially in

OMWW as it has an inhibitory effect on the complete bioremediation of OMWW and TOWW (Tchohanoglous and Burton, 1981; Al-Malah *et al.*, 2000).

Some inorganic compounds such as sodium and chloride (refer to Table 2.2) are also abundant in the TOWW since they are added during the brining stages of olive processing. Kargi (2002) reported that the amount of NaCl influences the removal efficiencies of COD in OWW during biotreatment, because of the inhibitory effect of salt on microbial flora. Salt concentrations of more than 1% cause plasmolysis of cells resulting in reduced biological activity. Paredes *et al.* (2002) also studied the inorganic composition of the OMWW and confirmed that these wastewaters had a high concentration in potassium and notable levels of N, phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca) and iron (Fe) (Komnitsas and Zaharaki, 2012). Arienzo and Capasso (2000) similarly analysed the composition and physicochemical status of the metal cations and inorganic anions present in TOWW. Results obtained from their study show the presences of cationic element such as Na, Zn, Cu, and Mn are the predominant cations. With regard to the anions, the prevailing one proved to be Cl$^-$ followed by the biacid phosphate H_2PO_4, Fe$^-$, SO_4^- and NO_3^- present at very low concentrations.

2.4.1 Organic compounds found in OWW

The organic compounds present in OWW is composed of polysaccharides, sugars, nitrogenous substances (especially amino acids), organic acids, polyalcohols, carotenoids, oil residues, complex phenolic substances and a considerable amount of suspended solids (Balice and Cera, 1984; Ramos-Cormenzana, 1996; Mantzavinos and Kalogerakis, 2005). Specific focus on polysaccharides, sugars, organic acids and phenolic compounds in OWW, as the major contributors to the pollution index of OWW, will now be given.

2.4.1.1 Polysaccharides, sugars and organic acids. The main soluble polysaccharides present in OWW are composed of pectins, although soluble cellulose and hemicellulose polymers have also been reported (Vierhuis *et al.*, 2003). Vierhuis *et al.* (2003) also determined that 5–10% of the polysaccharides present in olive fruit were soluble in water and turned up in the OMWW. The source of these water-soluble polysaccharides in OWW is from the degradation of cell wall polymers (pectin, hemicellulose and cellulose), i.e. the breakage of ester and hydrogen bonds between these polysaccharides within the olive skin during lye treatment, as well as

the mechanical shearing of olive cells in the malaxed paste during olive oil extraction (Vierhuis *et al.*, 2003; Mafra *et al.*, 2007). Interestingly, it was also shown that endogenous olive hydrolytic enzymes had almost no effect on the release of soluble polysaccharides in the OWW during olive processing (Vierhuis *et al.*, 2001).

The amounts of sugars present in the OWW vary and depend on the processing methods, but also the olive cultivar and climatic conditions during growth. Sugars constitute up to 60% of the dry substance of the olive fruit and are comprised of decreasing amounts of fructose, mannose, glucose, saccharose, traces of sucrose and some pentose sugars (McNamara *et al.*, 2008), however arabinose and galactose were found to be the major neutral sugars in OWW (Vierhuis *et al.*, 2003). OWW also contains relatively high levels of organic acids (3-5%), comprising lactic acid, oxalic acid, acetic acid, fumaric, malic, citric, palmitic acid, oleic acid, formic acid and butyric acid (Parinos *et al.*, 2007). Most of these organic acids are present in OWW as a result of the metabolic activity of lactic acid bacteria. Glucose, fructose, mannitol, sucrose, citric acid and malic acid are consumed by these microorganisms present in the OWW to form organic acid products such as lactic acid, malic acid, formic acid and 2, 3-butanediol, whereas acetic acid are primarily formed during the lye treatment steps, due to fragmentation of sugars and other organic molecules by the alkali (Sánchez *et al.*, 2000).

2.4.1.2 Phenolic compounds. Phenol and polyphenols are aromatic molecules containing the benzenoid ring structure with hydroxyl, amide and sulfonic substituting groups (Alemzadeh *et al.*, 2002). Although olive fruits are rich in phenolic compounds, only approximately 2% of the total phenolic content of olives is extracted into the final oil phase, while the remaining 98% of phenols are lost to OMWW (approximately 53%) and in the pomace (approximately 45%) (De Marco *et al.*, 2007). Phenolic compounds can reach levels of 1.5 and 10.2 g/L in OWW and are the main culprits responsible for its polluting impact, characterised by a strong antimicrobial, phytotoxic and recalcitrant nature (Filidei *et al.*, 2003; Nesseris and Stasinakis, 2012).

The "phenolic fraction" typically consists of two major groups. The monomeric or simple phenols of low molecular weight (one single aromatic ring with one or more substituting groups) represent a vast group. The major monomeric phenols in OWW includes compounds such as cinnamic acid and derivatives (caffeic acid, ferulic acid, *o*- and *p*-coumaric acid), benzoic acid and derivatives (vanillic acid, gallic acid, veratric acid, syringic acid,

protocatechuic acid, hydroxybenzoic acid and resorcinol), tyrosol and derivatives (hydroxytyrosol, 4-hydroxyphenylacetic acid) (Justino *et al.*, 2012). These simple phenolic compounds contribute to the phytotoxic and antimicrobial activities of OWW (Capasso *et al.*, 1992; Hamdi, 1993; El-Gohary *et al.*, 2009).

The second group of phenols, the polymeric phenols of different molecular-mass, include a complex of a multitude of flavonoids, tannins and secoiridoids (including oleuropein, dimethyl-oleuropein, verbascoside and olenoside) as well as catechol-melaninic polymers (Capasso *et al.*, 1992; Hamdi, 1993; Cabrera *et al.*, 1996; Di Gioia *et al.* 2002; Damak *et al.*, 2012). The polymeric phenols, the most recalcitrant fraction of OWW, are typically characterised as condensed aromatic rings with several substituting groups resembling a lignin-like structure, and are mainly responsible for the characteristic dark brown colour of OWW. The polyphenols forms as a result of the auto-oxidation and polymerisation reactions of the simple phenolic compounds. The presence of these two phenolic groups constitutes one of the major obstacles in the detoxification of the OWW due to their recalcitrance, strong phytotoxic and antimicrobial activities (Sayadi *et al.*, 2000; De Marco *et al.*, 2007). As mentioned earlier, OWW contains a relatively high concentration of flavonoids. The main flavonoids detected in OWW are apiginin, cyanidin flavonone, anthocyanin, luteolin, luteolin-7-glucoside and quercetin (Damak *et al.*, 2012).

Among the polyphenols described above, oleuropein is the most abundant phenol found in OWW (Damak *et al.*, 2012). As mentioned previously, oleuropein can be readily degraded into break down products (e.g., upon exposure to air/oxygen, certain enzymes/bacteria or lye treatment) that are substantially non-pollutant and non-toxic (Servili *et al.*, 1999). Hydroxytyrosol is worth noting as one of the breakdown products of oleuropein in OWW as it has been shown to have health beneficial properties in humans (Capasso *et al.*, 1992; Manna *et al.*, 1999). Hydroxytyrosol scavenges free radicals (Visioli *et al.*, 1999), inhibits human low-density lipoprotein (LDL) oxidation (Aruoma *et al.*, 1998) and shows *in vitro* antimicrobial activity (De Marco *et al.*, 2007).

2.5 Environmental Effects Caused by OWW

The high volumes and pollution potential of TOWW and OMWW often generate serious environmental problems due to seasonal logistical disposal problems. Removal of the ecotoxicological, phytotoxic and antimicrobial components present in OWW is a prerequisite prior to the disposal of OWW into aquatic and terrestrial systems.

2.5.1   The ecotoxicological effects of OWW

OWW has long been associated with significant toxicity towards aquatic and terrestrial organisms from different trophic levels. Ample evidence of the ecotoxicology effects of OWW compounds on aquatic life has been well-documented over the years (Asfi *et al.*, 2012; Komnitsas and Zaharaki, 2012; Justino *et al.*, 2012). Fish species affected by OWW include freshwater species such as *Gambusia affinis* (western mosquitofish) (Angus, 1983), *Carassius auratus* (gold fish) (Bellido, 1989), *Cyprinus carpio* (common carp) and *Chondrostoma polylepis* (Iberian Nase) (Fiestas, 1977). Marine and freshwater crustaceans (*Daphnia magna* and *Branchiopoda artemia*) have also been used as model organisms to measure the ecotoxicology effects of OWW. Besides the direct ecotoxicological effects to aquatic life forms, OWW have several other negative environmental effects (Justino *et al.*, 2012). In the literature, toxic effects of OWW were also found for amphibian tadpoles and a genotoxic effect for *Drosophila melanogaster* (Yesilada *et al.*, 1999; Capasso *et al.*, 1995).

2.5.2   The phytotoxicity effect of OWW

Phytotoxicity is the inhibitory effect of OWW on plant seed germination and plant growth in general. Many authors have studied the phytotoxic effects of high loads of phenolic compounds both in raw and diluted OWW at different growth stages and on seed germination of several crops, such as maize, radish, cucumber, lettuce, wheat, chickpea, tomato, durum wheat and English cress (Casa *et al.* 2003; El Hadrami *et al.* 2004; Komilis *et al.*, 2005; Ben Sassi *et al.*, 2006; Andreozzi *et al.* 2008; Cardinali *et al.*, 2010; Asfi *et al.*, 2012). It has also been reported that the direct discharge of raw OMWW on plants causes early leaf and fruit abscission (Capasso *et al.*, 1995).

OWW phytotoxicity is a complex phenomenon, since more than one compound is presumably responsible for the effect, but polyphenols are in general regarded as major compounds responsible for phytotoxicity (Sierra *et al.*, 2001). However, the phytotoxicity effect of OWW on plant growth has also been attributed to the simple phenolic compounds (e.g. hydroxytyrosol) and low molecular fatty acids and other non-phenolic compounds (Sierra *et al.*, 2001; Filidei *et al.*, 2003). Research supporting this idea includes the testing of OWW even after total removal of polyphenols (Capasso *et al.*, 1992; Isidori *et al.*, 2005). Furthermore, the phytotoxicity of OWW has also been attributed to some volatile organic acids, alcohols, aldehydes and other smaller molecules according to Tomati *et al.* (1996). Paredes *et al.* (2002) goes further by mentioning that the phytotoxic properties can also be related to low pH and high salts, in addition to phenols. The same authors mentioned that

alteration of soil properties (e.g. competitive sorption effect of certain ions, alteration of cation exchange capacity) after OMW application to soil can result in phytotoxicity.

2.5.3 The antimicrobial effect of OWW

It is also a well-established fact that the simple phenolic, polyphenolic compounds and long-chain fatty acids in OWW display acute toxicity against bacteria (Gonzalez *et al.*, 1990; Yesilada and Sam, 1998; Filidei *et al.*, 2003; Fiorentino *et al.* 2003; Isidori *et al.*, 2005; Mekki *et al.*, 2008). Several studies have demonstrated that OWW have bactericidal activity against both Gram-positive and Gram-negative bacteria, specifically *Streptococcus pyogenes*, *Staphylococcus aureus*, *Vibrio fischeri*, *Bacillus megaterium*, *Pseudomonas fluorescens* and *Escherichia coli* and *Klebsiella pneumoniae* (Isidori *et al.*, 2005; Yesilada *et al.*, 1999; Capasso *et al.*, 1995; Mekki *et al.*, 2008; Tafesh *et al.*, 2011). Capasso *et al.* (1992) have also shown that methylcathecol have toxic effects against some Gram-positive and Gram-negative bacteria. Growth of freshwater unicellular algae such as *Chlorella* sp., *Scenedesmus obliquus*, *Selenastrum capricornutum*, *Ochromonas danica* and *Scenedesmus obliquus*, is also inhibited by OWW (Hodaifa *et al.*, 2012).

Studies investigating the effects of OWW on microbial populations in soil have indicated that the application of OWW to soils results in major microbial ecology shifts (Paredes *et al.*, 2002; Saadi *et al.*, 2007; Karpouzas *et al.*, 2009; Rousidou *et al.*, 2010; Bodini *et al.*, 2011). Mekki *et al.* (2008) used culture-dependent methods to show a decrease in the number of nitrifiers and an increase in fungi and yeasts, actinomycetes and spore-forming bacteria, while Karpouzas *et al.* (2010) used PCR-DGGE methods to show significant microbial changes in the ammonia-oxidizing bacterial population. Significant decreases in plant root colonisation by arbuscular mycorrhizal fungi were also observed in long-term studies by Di Bene *et al.* (2012).

2.5.4 Problems associated with release of OWW into natural aquatic resources

Disposal of both TOWW and OMWW into aquatic environments has a direct environmental impact in that it causes aesthetic disturbances. The most immediately visible effect of OWW pollution is the discolouring of natural waters, i.e. water colour changes to a dark brown colour. Typically, OMWW has an intense violet-dark brown to black colour and TOWW a dark green colour, as a result of the presence of recalcitrant polyphenolic compounds found in OWW, which are often chemically linked to lignin and humic acid (Al-Malah *et al.*, 2000; Filidei *et al.*, 2003). OWW's dark colour characteristic would act as a barrier to sunlight

penetration in rivers and streams, which in turn would reduce the photosynthesis activity and dissolved oxygen concentrations causing harm to aquatic life (Filidei *et al.*, 2003).

As stated, OWW contains a significant amount of biodegradable carbon and when discharged untreated into natural water bodies, will result in a rapid increase in heterotrophic microbial populations that will respire the organic molecules (i.e. sugars etc.) as carbon (C) and energy source in the presence of oxygen. This increase in the microbial respiration will lead to the increased consumption of dissolved oxygen in the water with COD, and thus will reduce the available oxygen for other living organisms and disturbs the balance in the whole ecosystem (Niaounakis and Halvadakis, 2006). Anaerobic zones will rapidly develop due to oxygen depletion, followed by the development of bad smells due to an increase in anaerobic metabolic products (CH_4, various reduced sulphur compounds and several other fermentation end products) (Beltran-Heredia *et al.*, 2000). Furthermore, the high content of phosphorous (P) in OWW will encourage and accelerate the growth of photosynthetic algae, a phenomenon referred to as eutrophication or "algal blooms".

Ultimately, the release of large volumes of untreated OWW has severe impacts on the aquatic life and humans that may come into contact with the contaminated water (Niaounakis and Halvadakis, 2006). Furthermore, due to the high concentration of organic acids (mainly volatile fatty acids), OWW are corrosive to sewer pipes, this being one of the reasons why direct discharge of OWW in sewers has been forbidden in European countries (Rozzi and Malpei, 1996). Other effects of OWW are associated with the lipid fraction that may form an impenetrable film or emulsion on the surface of rivers. This film often blocks out sunlight and oxygen to organisms in the water below, leading to reduced plant growth in the soil and rivers (Niaounakis and Halvadakis, 2006). Moreover, the chelating character of some compounds in these effluents may contribute to keeping some toxic heavy metals in solution, adding new factors of deterioration to the aquatic system (Rivas *et al.*, 2001).

2.5.5 Problems associated with the release of OWW on land

The terrestrial application of OWW has also been studied in detail (Cabrera *et al.*, 1996; Kotsou *et al.*, 2004; Paredes *et al.*, 2002; Di Bene *et al.*, 2012). Many researchers have established that the application of OWW has a high fertilizing value when applied to soil and for that reason; several studies have reported on the application of OWW as irrigation water. OMW is rich in biodegradable organic matter and inorganic elements, notably K, P, Fe and Mg, available for plant growth and thus resulting in enhanced soil fertility. Komilis *et al.*

(2005) reported that the application of OWW onto soil resulted in the enrichment of N-fixing bacteria and the soil acquired inhibition properties against phytopathogenic fungi. According to Komilis *et al.* (2005), N-fixing bacteria (mainly *Azotobacter* spp.) excrete polysaccharides, which behave as natural adhesives and stabilises soil conglomerates, leading to improved physical structure of the soil. It was further demonstrated by Bonari *et al.* (1993) who performed irrigation experiments using OWW, that if OWW is applied to soil 60 days after seeding, no detrimental effects are observed on the newly grown seeds as long as the OWW annual dosage is kept between 4 and 8 tonne per 1000 m^2. Furthermore, Balis (1994) demonstrated that OWW-derived compost could be successfully used as a fertilizer during the cultivation of olive trees, grapes and potatoes.

These positive effects are however significantly out-weighed by the negative impacts of long-term application of OWW on soil, and for this reason disposal of OWW on land always necessitates some type of pre-treatment (Piotrowska *et al.*, 2006). In general, soil exposed to an uncontrolled spreading of OWW, lose their fertility gradually and proportionally to the amount of OWW spread and the number of times of OWW spreading. Long-term irrigation of OWW on soil reduces soil porosity, thereby limiting the exchange of air and water between the soil and atmosphere (Anastasiou *et al.*, 2011). Most evident is the presence of phytotoxic and antimicrobial polyphenols, which have a detrimental impact on crops (i.e. seed germination and plant growth) as well as the natural soil microbial biodiversity (Rinaldi *et al.*, 2003; Komilis *et al.*, 2005).

Although the addition of OWW stimulates microbial communities in the soil, it could also lead to increased phytotoxic effects through the release of phytotoxins from the microbial breakdown of the organic residues (Stroo *et al.*, 1988; Bradow, 1993; Tiquia *et al.*, 1996; Kotsou *et al.*, 2004). Other negative effects recorded on soil properties due the disposal of untreated OWW in the environment, include the immobilisation of the available soil N (Saviozzi *et al.*, 1991) and pollution of aquifers (Sayadi *et al.*, 2000). It is well established that the addition of substrates, with a high C/N ratio (e.g. OMWW) causes net N immobilization in soil, which can lead to competition for N between soil microorganisms and plants. Lastly, OWW application to soils leads to increased soil salinity (Anastasiou *et al.*, 2011).

2.6   Treatment Options for OWW

It is clear that OWW can been regarded as key environmental problem in olive processing countries and could emerge as a potential environmental risk in South Africa as a result of

projected industry expansion (Al-Malah *et al.*, 2000). Pollution reduction for OWW is difficult due to the high organic loading (compounded by the presence of recalcitrant organic compounds), peak seasonal operation and sparse territorial scattering of processing plants (Ergüder *et al.*, 2000).

Efforts to find solutions for OWW disposal have been undergoing for more than 50 years with intensive research efforts mainly tailored for the Mediterranean olive processing countries (Niaounakis and Halvadakis, 2006; Niaounakis, 2011). Substantial research focus has been given to the development of suitable treatment methods for OMWW, while research advances for the treatment of TOWW have been lagging behind (Aggelis *et al.*, 2001). Although several technologically-advanced OWW treatment processes have been developed and applied by the larger olive processing countries, many olive processing plants still discharge OWW directly into sewer networks, central lagoons or small ponds where it is left to evaporate until the next season (El-Gohary *et al.*, 2009).

The small olive industry in South Africa, has not ventured into the large-scale implementation of these technologically-reliant treatment systems due to the exorbitant costs and technical complexity required. As a result, most OWW is subjected to a minimal treatment regime, i.e. the most common treatment technique practiced in South Africa is to treat OWW in lagoons (evaporation ponds). Although lagooning leads to detoxification of OWW, it requires long treatment periods and increases the risk of secondary pollution if not executed properly (Beltran-Heredia *et al.*, 2000).

2.6.1 Physical, thermal and physico-chemical treatment approaches for OWW

Several existing physical, thermal and physico-chemical treatment approaches for OWW are summarised in Table 2.3. These methods vary from rudimentary chemical and physical treatments to complex technology-intensive methods. Most of these physical, thermal and physico-chemical treatments are generally technically or economically unfeasible for small-scale olive processing plants, and require combinations of these treatments in succession to successfully remove OWW pollutants to acceptable levels (Gonzalez *et al.*, 1994).

Table. 2.3. Summary of physical, thermal and physico-chemical treatment approaches for OWW.

Treatment method	Brief description	Advantages/Disadvantages	Reference
Physical treatment processes for OWW			
dilution	• dilution with water from wells, irrigation, streams, rivers, domestic sewage	• lowers COD, phenolic content • large volumes of fresh water and space required • often used prior to biological treatment to reduce toxicity to the microorganisms	Niaounakis and Halvadakis (2006)
sedimentation/settling	• settling of suspended solids through natural process	• large reduction in COD • slow process (min. of 10 days) • costly flocculants • high COD sludge, requires additional disposal	Al-Malah et al. (2000)
filtration	• removal of suspended solids and colloidal solids using filter-press equipment	• unreliable reduction in COD • rapid clogging of filters • more suitable for small olive plants	Bradley and Baruchello (1980)
flotation	• decreasing specific gravity of suspended particles relative to liquid phase using dispersed gas bubbles	• inefficient COD removal	Mitrakas et al. (1996)
centrifugation	• separation of phases using centrifugal force	• highly variable depending on chemical composition of wastewater • inefficient COD removal	Mitrakas et al. (1996)
membrane-assisted technologies	• separation of particles using membrane pore size		
micro- and ultrafiltration	• cut-off of 2 μm (micro) or 0.1 μm (ultra)	• effective removal of suspended solids, but not dissolved organics • small sludge waste amounts • incomplete removal of COD • membrane fouling • high operating costs - not suited for small-scale oil mills	Turano et al. (2002)
reverse osmosis	• membrane separation process in the molecular range	• effective COD removal • large sludge waste volumes • high operating costs - not suited for small-scale oil mills	Canepa et al. (1988)

Treatment methods	Brief description	Advantages/Disadvantages	Reference
Thermal (vaporization) treatment processes			
physico-thermal processes	• aims to reduce water fraction • Combinations of artificial evaporation and distillation	• not a continuous process • highly variable depending on chemical composition of wastewater • requires industrial evaporators • high energy requirements • requires disposal of produced emissions	Niaounakis and Halvadakis (2006)
irreversible chemico-thermal processes			
combustion	• Incineration/burning in the presence of O_2	• OWW requires pre-drying • high energy requirements	Niaounakis and Halvadakis (2006)
pyrolysis	• retorting, destructive distillation (above 430°C) in the absence of O_2	• emission of toxic gasses • costly infrastructure	Di Giacomo et al. (1989) Brunetti et al. (1991)
lagooning/natural evaporation	• large lagoons for solar energy-assisted evaporation and natural degradation of OWW	• requires 7-8 months treatment period • prone to anaerobic fermentation leading to foul odours, insect proliferation • threat of leakage • remaining 'paste' and the distillate need further treatment	Cabrera et al. (1996)
Physico-chemical processes			
neutralization	• addition of H_2SO_4 or $Ca(OH)_2$ leads to destabilization of suspended and colloidal matter	• requirement for additional chemicals • not suited for large OWW volumes	Mitrakas et al. (1996)
precipitation/flocculation	• addition of precipitate-inducing or aggregation-inducing agents to enhance natural process using a variety of organic or inorganic flocculants	• OWW organics are relatively resistant to precipitation • only partial removal of COD • requirement for additional chemicals • resulting precipitate requires additional disposal	Jaouani et al. (2000); Niaounakis and Halvadakis (2006)
adsorption	• attachment of dissolved compounds to solid surface such as granular activated carbon and clays (bentonite) or resins	• low space requirements • limited purification efficiency • expensive adsorbents required	Beccari et al. (2002)
chemical oxidation, advanced oxidation processes (AOP), electrochemical oxidation	• addition of O_2, O_2-derivatives (H_2O_2, O_3), chlorine and chlorine derivatives. • leads to degradation of recalcitrant organic compounds	• effective COD removal only when used in combination, e.g. ultraviolet radiation (photolysis), $FeSO_4$ (Fenton reaction) • O_3 requires on site production on demand • expensive technology and chemicals required	Rivas et al. (2001); Niaounakis and Halvadakis (2006); Tezcan et al. (2006); Arvanitoyannis et al. (2007)

2.6.2 Biological processes

Biological processes are considered the most environmentally compatible, reliable and in many cases, the least expensive treatment methods of OWW (Mantzavinos and Kalogerakis, 2005; Paraskeva and Diamadopoulos, 2006). Biological processes employ the use of microorganisms (individual or consortia) to degrade the organic compounds present in OWW. The actual type of microorganism that is involved depends on the metabolism of the microbes and the conditions in which the OWW is treated, i.e. aerobic or anaerobic (Niaounakis and Halvadakis, 2006). The biological approach can employ either single species microorganisms or enzymes produced by these microorganisms, as well as microbial consortia (biofilms), where the interaction of various microorganisms can be complex and difficult to analyse (Arvanitoyannis, 2007). Several studies have also indicated that the indigenous microbial inhabitants of olive pulp and OWW have the natural ability to effectively degrade the phenolic compounds contributing toward COD in OWW (Fountoulakis *et al.*, 2002; Tziotzios *et al.*, 2007). Furthermore, the presence of many of the phenolic compounds and long-chain fatty acids in OWW, as mentioned earlier, has antimicrobial properties and has a significant impact on the efficiency of these biological processes. In most cases, some physico-chemical pre-treatment, such as dilution or ozonation, which removes/reduces the antimicrobial effect, facilitates the subsequent biological treatment (Aggelis *et al.*, 2001).

2.6.2.1 Anaerobic treatment processes. Anaerobic digestion is increasingly used to remedy OWW because it allows the recovery of the chemical energy from the waste in the form of biogas, has low energy requirements and produces much less waste sludge than other treatment processes (Borja *et al.*, 1993; Paraskeva and Diamadopoulos, 2006). Anaerobic digestion is carried out in the absence of molecular oxygen by a series of anaerobic microorganisms, mainly bacteria, which have slower growth rates than aerobic microorganisms (Rozzi and Malpei, 1996; Hamdi, 1996; Ergüder *et al.*, 2000).

Typically, anaerobic digestion consists of a series of three successive microbiological processes that convert organic compounds to methane (biogas) and carbon dioxide (Rozzi and Malpei, 1996). After a period of acclimatisation for the microorganisms to adapt to OWW inhibitory compounds using stepped organic loading rates, the anaerobic treatment process ensues with hydrolysing bacteria breaking down the complex organic molecules (proteins, polysaccharides, lignin and lipids) into soluble monomeric molecules (amino acids, simple

sugars, glycerol and fatty acids). The soluble monomers are converted by fermentative acidogenic bacteria into organic acids (e.g. acetic, propionic, formic, lactic or butyric acids), alcohols and ketones (e.g. ethanol, methanol, glycerol and acetone), carbon dioxide and hydrogen. Acetogenic bacteria then convert main fermentation end products, volatile fatty acids and alcohols, into acetate, hydrogen and carbon dioxide, which are used by methanogens (genera such as *Methanobacterium*, *Methanococcus* and *Methanosarcina*) in the final methanogenesis step, where acetate, hydrogen and carbon dioxide are converted into methane (Niaounakis and Halvadakis, 2006).

Anaerobic processes are effective in the treatment of high organic load wastewaters and economically practical when implemented as an OWW treatment. Reductions in COD of 70-89% have been reported for anaerobic processes (Borja *et al.*, 1996; Marques *et al.*, 1997; Marques, 2001). In addition to a substantial reduction in COD, Dalis *et al.* (1996) reported large reductions (>75%) in the concentrations of both toxic phenols and volatile fatty acids using a two stage anaerobic reactor with an inoculant obtained from a domestic sewage facility (Tsonis and Grigoropoulos, 1988). Anaerobic technologies currently available include continuous-flow stirred tank reactors (CSTR), up-flow anaerobic sludge blanket bioreactors (UASB), anaerobic filter, expanded or fluidised bed bioreactors and anaerobic baffled bioreactors (Rozzi and Malpei, 1996).

The anaerobic digestion process is, however, not flawless as it is affected by various factors such as temperature, retention time, pH, H_2 partial pressure, chemical composition of the wastewater and the presence of toxic compounds. The toxic composition of OWW could make the process unstable due to the inhibitory effect of polyphenols. For example, the methanogen population have been found particularly sensitive to the presence of long-chain fatty acids and phenolic compounds in OWW (Fountoulakis *et al.*, 2002; Mantzavinos and Kalogerakis, 2005; Rizzi *et al.*, 2006). Alkalinity adjustments are also usually necessary to keep the pH of the diluted OWW at about 7 to prevent the decreased growth rates of the anaerobic microorganisms involved in the process (Beccari *et al.*, 2002). Single stage anaerobic treatments are usually not effective for complete COD or phenol removal and some form of pretreatment, such as the addition of soluble calcium salts, filtration or aerobic pretreatment, apart from simple dilution and alkalinity adjustment, is usually required (Paraskeva and Diamadopoulos, 2006).

2.6.2.2 Aerobic treatment processes. Aerobic treatment processes can be based on the activated sludge principle where oxygen and nutrients are unlimited. During this process, aerobic microorganisms degrade a significant fraction of the carbon-based pollutants in the OWW into free CO_2 through aerobic respiratory metabolism. A significant amount of the available carbon in OWW is also assimilated into biomass and when the microorganisms settle out, this leads to a significant reduction of the COD released (Rozzi and Malpei, 1996). Although the proportion of the carbon respired into the atmosphere as CO_2 and the proportion converted to cell mass will vary (depending on the rate at which organisms grow), a rough estimate is that about 33-40% of carbon is respired and 60-67% converted to cell mass. Aerobic biological treatments require high residence (hydraulic retention) times and often previous conditioning of the wastewater is required, for example dilution (up to at least 70 times), pH adjustment, as well as acclimatisation of microorganisms (Hamdi, 1996; Rivas *et al.*, 2001).

The main advantage of the aerobic treatment process is the inexpensiveness and simplicity of the installations needed (Paraskeva and Diamadopoulos, 2006). Aerobic treatment technologies include the use of biofilms in activated sludge processes, trickling filters, bio-filtrations or fixed-bed bioreactors, RBC, batch reactors in series and even man-made wetlands. Most of these techniques are based on the provision of optimal surface area for microorganisms to develop on. For example, the RBC is a type of fixed-film wastewater treatment system that consists of a series of closely spaced rotating discs partially immersed in the reactor bed through which wastewater flows (Martin-Cereceda *et al.*, 2001).

In general, aerobic biological processes are less attractive for the treatment of the OWW when compared to anaerobic treatment, because it is difficult to reach the required removal efficiency for specific classes of pollutants, such as polyphenols (Rozzi and Malpei, 1996; Niaounakis and Halvadakis, 2006). For this reason, aerobic treatment processes are often regarded as either a pre-treatment or polishing step for OWW.

Research into the use of aerobic remediation of OWW has been carried out using both bacteria and fungi (Anastasiou *et al.*, 2011). Fungal and bacterial strains indigenous to OWW or isolates from other niches have been evaluated for their ability to detoxify the wastes produced by the olive-oil industry (Ntougias *et al.* 2006). In particular, the pre-treatment of OWW with fungal species such as the white rot fungi including *Funalia trogii, Pycnoporous*

coccineus, Coriolopsis polyzona, Coriolus versicolor, Phanerochaete chrysosporium, P. flavido-alba, Penicillium spp., *Lentinula* and *Pleurotus* spp. (*P. cornucopiae, P. ostreatus, P. sajor caju*), *Lentinus tigrinus*, other filamentous fungi including *Aspergillus niger, A. terreus, Geotrichum candidum* (Hamdi *et al.*, 1991; Borja *et al.*, 1995; Sayadi and Ellouz, 1995; Yesilada *et al.*, 1995, 1998; Hamman *et al.*, 1999; Robles *et al.*, 2000; Blanquez *et al.*, 2002; Tsioulpas *et al.*, 2002; Aggelis *et al.*, 2003; Jaouani *et al.*, 2003; McNamara *et al.* 2008; Assas *et al.*, 2000; Garcia-Garcia *et al.*, 2000), and several different yeasts including *Candida tropicalis* (Ettayebi *et al.*, 2003), *Yarrowia lipolytica* (Lanciotti *et al.*, 2005), *Trichosporon cutaneum* (Chtourou *et al.*, 2004) have previously been described (Fakharedine *et al.*, 2006).

Aerobic bacteria belonging to the species *Bacillus pumilus, Arthrobacter, Azotobacter vinelandii, Pseudomonas* spp., *Sphingomonas* spp. and *Ralstonia* spp. proved to be suitable for the aerobic biodegradation and detoxification of OWW (Knupp *et al.*, 1996; Papadelli *et al.*, 1996; Ramos-Cormenzana *et al.*, 1996; Piperidou *et al.*, 2000; Di Gioia *et al.*, 2001, 2002; Ehaliotis *et al.*, 2006). Bacteria can achieve a significant reduction in COD (up to 80%), as well as the decrease of the content of polyphenols and the complete removal of simple phenols. Earlier studies have indicated that the effectiveness of the aerobic bacteria in OWW remediation varied greatly as they appear to be very effective against some phenolic compounds and relatively ineffective against others (Knupp *et al.*, 1996; Ramos-Cormenzana *et al.*, 1996; Di Gioia *et al.* 2001; Sinigaglia *et al.*, 2010). In general, the yeast and fungi evaluated to date are more effective in OWW treatment, compared to the bacterial isolates, in being able to remove phenols, reduce COD and lighten the dark colour of OWW. For example, comparing the reported efficiencies of COD removal, a selection of microorganisms mentioned above can be arranged in order of COD removal efficiency: *Phanerochaete chrysosporium* > *Aspergillus niger* > *Aspergillus terreus* > *Candida tropicalis* > *Azotobacter vinelandii* (Piperidou *et al.*, 2000; Garcia-Garcia *et al.*, 2000).

2.6.3 The rotating biological contactor (RBC)

The Rotating Biological Contactor (RBC) (Fig. 2.5) is a type of aerobic fixed-film wastewater treatment system that consists of a series of closely spaced rotating discs partially immersed in a trough/reservoir through which wastewater flows (Martin-Cereceda, 2001; Kubsad *et al.*, 2004). Its development originated in the late 1920's in Europe (Banerjee, 1997), but the first commercial RBC systems was only installed in the 1960's in West Germany for wastewater treatment (Mba *et al.*, 1999). Since then, the RBC principle has in particular been used

widely in Europe to treat the sewage generated by areas with a population smaller than 5000 people. These days, the RBC process using RBC units from 1.0 m to 4.0 m in diameter, with shaft lengths of up to 4 m are used worldwide for both municipal and industrial wastewater (Surampalli and Baumann, 1992; Mba *et al.*, 1999).
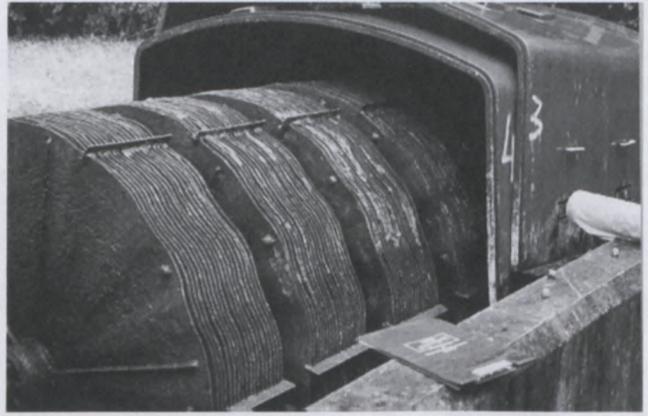
Figure 2.5. Example of a Rotating Biological Contactor (RBC) in an industrial setup (Mba and Bannister, 2007).

The RBC is a proven technology for large-scale wastewater treatment applications with several positive characteristics in terms of the biological process (Guimarães *et al.*, 2005). These include short start-up times, short hydraulic retention times, resilience to toxic substrates and varying concentration loads and effective oxygenation with little sloughing of biomass (Wilson *et al.*, 1980; Israni *et al.*, 2002). Implementation of an RBC system is considered eco-friendly, low on energy consumption, simple to construct with little maintenance, adaptable from small to large scale, low capital and operating costs, minimal technical personnel attention required as well as small land or space requirements (Malandra *et al.*, 2003; Guimarães *et al.*, 2005; Najafpour *et al.*, 2005).

2.6.3.1 Typical Structure and Operation of the RBC. The RBC consists of two main parts called the rotating contactor and the trough (Fig 2.5 and 2.6). The contactor is made up of a central axel on which lightweight cylindrical discs are connected that accommodates biofilm development. The discs are designed to have as large a surface area as possible. In industrial applications, the discs are usually made from corrugated plastics (Mba *et al.*, 1999). The RBC purification mechanism is based on the metabolic activities of the complex microbial communities attached to the disc surfaces (Martin-Cereceda *et al.*, 2001). During the operation of the system, the rotation of the horizontal shaft is usually perpendicular to the direction of the waste flow, while approximately 40-45% of the total disc surfaces area is submerged in the wastewater to be purified. As the discs rotate, by an electrical motor attached to a gearbox for speed regulation (Mba *et al.*, 1999), the microorganisms in the biofilm are alternately immersed in the wastewater and exposed to air, with optimum rotational speed of the discs allowing adequate attachment of biofilm development. Oxygenation is a function of the rotating speed of the RBC (Di Palma *et al.*, 2003; Kubsad *et*

al., 2004), while in practice a greater speeds of rotation tends to increase the detachment of the biofilm from the discs. Due to the alternating contact between air and wastewater, oxygen transfer is achieved by the exposure and renewal of air-water interfaces. Usually, the adherence of microorganisms from the wastewater onto the disc surfaces forms a biofilm ranging from 1 to 4 mm in thickness within 2 to 4 weeks, while the biofilm sloughs off from the discs after reaching a critical thickness. This sloughing process occurs randomly and makes the disc surface non-uniform at any given time. Sloughed-off biofilm and suspended solids are continually washed out of the contactor as the wastewater flows through the RBC (Bishop and Kinner, 1986). Dincer and Kargi (2001) evaluated the performance of a two stages RBC comprising 20 discs per section in treating saline wastewater. Up to 90% COD removal efficiency was obtained with the efficiency decreasing as the number of discs or discs surface area were reduced in the RBC unit.
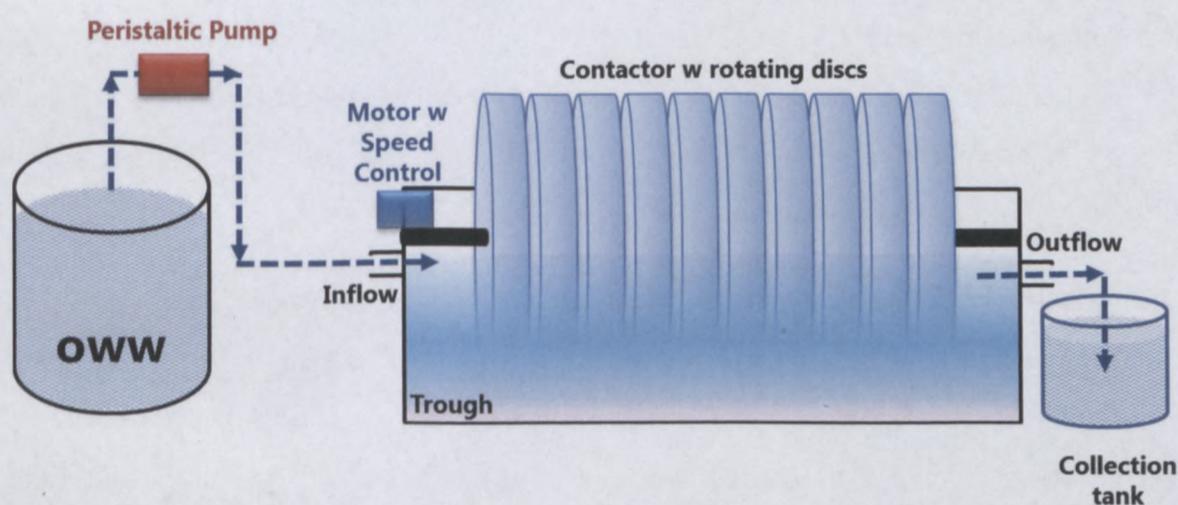
Figure: 2.6. Schematic representation of the set-up for the small-scale evaluation of the RBC (Malandra *et al.*, 2003)

The trough/reservoir is a simple structure that is usually made of cement or plastic material and has an inlet and an outlet. It must have the volume required for the retention time stipulated and must be strong enough to support the rotating contactor when loaded with a fully mature biofilm.

2.6.3.2 The RBC application in wastewater treatment. Numerous research papers have reported on the use of the RBC in treating effluents produced during the manufacturing of a variety of agricultural wastewaters. This includes palm oil mill, sugar refinery, winery, distillery, canning, dairy, slaughter house, meat processing and poultry wastewater as well as OWW (Grady, 1983; Akunna and Jefferies, 2000; Griffin and Findlay, 2000; Nowak, 2000; Teixeira and

Oliveira, 2001; Alemzadeh *et al.*, 2002; Kargi, 2002; Malandra *et al.*, 2003; Hiras *et al.*, 2004; Najafpour *et al.*, 2005; Tawfik *et al.*, 2005; Mba and Bannister, 2007; Acheampong *et al.*, 2012; Emerenshiya *et al.*, 2012; Sauder *et al.*, 2012; Šíma *et al.*, 2012). It has also been suggested that the RBC is best used in combination with an anaerobic digestion process as well as a range of other wastewater treatment initiatives (Mba *et al.*, 1999; Hiras *et al.*, 2004; Tawfik *et al.*, 2005).

Phenol degradation has also been evaluated using the RBC systems. Guimarães *et al* (2005) use of a modified RBC with polyurethane foam discs to increase the area for *P. chrysosporium* immobilization and to investigate the possibility of continuous biodegradation of sugar refinery effluent. Results of this study proved that it is feasible to treat the sugar refinery effluent continuously in an RBC with *P. chrysosporium* immobilized on polyurethane foam discs. This system not only removed the colour of the effluent by 55%, but also reduced total phenols and COD by 63 and 48%, respectively, signifying its possible use in bioremediation of effluents. Malandra *et al* (2003) used an RBC to investigate microorganisms associated with the biological treatment of winery wastewater. Results indicated that the RBC in conjunction with indigenous biofilms growing on the surface of the discs, could be an effective technique in lowering the COD (43% reduction) of winery wastewater.

2.6.3.3 Factors affecting the performance of an RBC plant. Many factors can affect the performance of an RBC treatment plant such as surface organic loading (SOL), hydraulic retention time, rotational speed, number of stages, temperature of water, wastewater specific characteristics (e.g. COD or BOD levels and pH) and biofilm-specific characteristics (e.g. microbial species and biofilm architecture on surfaces) (Klees and Silverstein, 1992; Najafpour *et al.*, 2005; Burns, 2012).

The surface organic loading (SOL) of an RBC system is the amount of COD or BOD in 1 m^3 of wastewater that passes over the total media surfaces per day and is measured in COD / (m^3 x day). The COD removal rate generally increases as the SOL increases, but at a decreasing rate. Therefore, substrate removal efficiency decreases as the SOL increases. The hydraulic retention time is the total volume of the fluid at a specific moment in the RBC divided by the inflow rate. Longer retention times will generally increase the removal rate of COD and BOD from the wastewater and *vice versa* (Kugaprasatham *et al.*, 1991).

A faster rotational speed of the RBC discs will decrease biofilm attachment (high shear stress) and hence decrease the COD removal efficiency of the biofilm. However, if the rotational speed is too slow, the biofilm will become too thick and detach due to weight stress (Hiras *et al.*, 2004). A typical problem that can develop with the RBC system is the development of excessive thickness of biofilms, resulting in the formation of biofilm bridges that links neighbouring discs to each another, resulting in loss of effective surface area for treatment. A higher number of stages present in an RBC system will increase the surface organic loading of the reactor, as well as the surface area of the biofilm in contact with the wastewater and will consequently increase the COD or BOD removal capacity of the plant.

Finally, the temperature of the wastewater also affects RBC performance. Higher temperatures will generally improve the removal rate of COD and BOD, but changes are relatively small between 15°C and 25°C (normal temperatures of wastewater). In colder countries where wastewater temperatures can reach below 1-5°C, removal rates are significantly lower (Pano and Middlebrooks, 1983; Burns, 2012).

A laboratory-scale RBC unit can be used to study and model the efficiency of naturally occurring biofilms to remove COD and to degrade toxic compounds. It can also be used to predict the effect that biocides will have on the ability of larger industrial-scale RBCs to effectively treat higher volumes of wastewater.

2.6.4 Combined and co-digestion biological processes

Due to the typical high total phenol concentration and the high initial organic load, anaerobic and aerobic biological methods do not completely remediate OWW in large scale systems. These biological treatment methods individually cannot achieve acceptable remediation levels, and combined sequential anaerobic and aerobic biological treatments have therefore also been evaluated (Paraskeva and Diamadopoulos, 2006; Chan *et al.*, 2009; Nesseris and Stasinakis, 2012). A further development in OWW involves the co-treatment of OMW with other wastewaters, such as domestic sewage, instead of dilution with clean water (Angelidaki and Ahring, 1997; Marques *et al.*, 1998; Gannoun *et al.*, 2005, 2007; Gizgis *et al.*, 2006; Azaizeh *et al.*, 2010; Jail *et al.*, 2010; Nesseris and Stasinakis, 2012). The additional wastewater stream provides stabilisation of the pH levels as well as nutrients necessary for successful anaerobic digestion. Furthermore, combined biological and physico-chemical treatments have also been reported with improved remediation of OWW (Kyriacou *et al.*, 2005).

2.7 The Ecology of OWW

A substantial body of research has been carried out on the characterization of OWW with respect to microbial composition (Bertin *et al.,* 2004; Rincon *et al.,* 2006; Ntougias *et al.,* 2006; Pozo *et al.,* 2007; Morillo *et al.,* 2008; Vivas *et al.,* 2009). Recent comprehensive molecular ecology studies have revealed the presence of dominant bacterial genera such as *Acetobacter, Gluconacetobacter, Lactobacillus, Prevotella* and members of the order *Clostridiales* in OWW (Kavroulakis and Ntougias, 2011). The variety of carbohydrates released from the olive pulp during olive processing, and organic acids, in particular lactic acid, resulting from the fermentation of sugars by the lactic acid bacteria, favours the proliferation of bacteria that provide a wide range of metabolic capabilities contributing to the bioremediation process of both TOWW and OMWW (Ramos-Cormenzana *et al.,,* 1996).

The prolonged evaporation process in open ponds (lagooning) can be considered a natural biological treatment method that allows a variety of microbial processes (both anaerobic and aerobic) to develop. Studies on the procession of microbial communities in these ponds have indicated an increase of yeasts and moulds in relation to the bacterial population at the latter stages of the biodegradation process (Jarboui *et al.,* 2009). Bacterial populations play a more important role in the beginning stages of OWW's natural biodegradation, since bacteria, with their higher metabolic rates, can outgrow yeasts and moulds as they compete successfully for available nutrients and space. However, as the physiochemical parameters of the OWW changes, bacterial numbers start to decline. Some of the factors responsible for this phenomenon have been identified as rising acidic conditions, the built up of antibacterial activity resulting from higher concentration of polyphenolic compounds, as well as lower water activity levels (Jarboui *et al.,* 2009).

Fusarium, Geotrichum and *Trichosporium* were identified as the dominant fungi in the natural biological degradation process of OWW in open ponds (lagooning) (Jarboui *et al.,* 2009). In another study, Millan *et al.,* (2000) studied the microbial composition of OWW from four disposal ponds and found 12 different genera of fungal species, including *Acremonium, Alternaria, Aspergillus, Chalara, Fusarium, Lecytophora, Paecilomyces, Penicillium, Phoma, Phycomyces, Rhinocladiella* and *Scopulariospsis* are present in OWW. Yeasts isolated from OWW include *Candida boidinii, Pichia holstii, P. membranifaciens* and *Saccharomyces cerevisiae* (Sinigaglia *et al.,* 2010). The yeast and the fungi are active in decay of organic matter, including phenol degradation.

2.8   Biofilms

Biofilms can be described as spatially-distributed mixed-species microbes attached to interfaces in a heterogeneous matrix containing extracellular polymeric substances (EPS), water, as well as "sorbed" substances, interspersed by interstitial voids and channels (Flemming, 2002; Wolf *et al.*, 2002). Biofilms constitute a unique mode of growth that allows protection against desiccation and harsh environmental conditions, antibacterial agents as well as optimal nutrient utilisation in micro-niches through cooperative metabolic activity by differentiated microbial groups within nutritional chains, governed by genetic exchange and quorum sensing phenomena (Blenkinsopp and Costerton, 1991; Elias and Banin, 2012). Nearly all microorganisms (an estimated 99% of all bacteria) are capable of forming biofilms in nature, with the ability to grow on virtually any interfaces (e.g., solid/oil/water/air); a phenomenon considered a dominant form of microbial life (Flemming, 2002).

The presence of biofilms can be both beneficial and detrimental (Wolf *et al.*, 2002). For instance, in wastewater treatment environments, biofilms decompose undesired organic substances, and convert them into harmless products (Nivens *et al.*, 1995). In contrast, biofilms are able to cover practically all accessible wet surfaces and also trap pathogenic bacteria within the biofilm causing serious health risks in drinking water distribution and hospital environments (Schwartz *et al.*, 2003; Lin *et al.*, 2012).

2.8.1   Biofilm development stages

Although biofilms are dynamic and complex microbial environments, their formation and development have been described in detail as a series of successive steps with several models suggested (Evans, 2000; Wuertz *et al.*, 2003; Dufour *et al.*, 2012). As shown in Fig. 2.7 in step 1, the respective substratum is conditioned by the attachment of inorganic and organic macromolecules, thereby providing a nutritious zone for cell settlement (Forsythe, 2000). Nutrients in aqueous environments have the tendency to accumulate at surfaces giving adhering microbes a benefit over free floating, so-called planktonic ones (Andersson, 2009). Different nutrients in aquatic environments absorb onto surfaces to form a conditioning film with different physiochemical properties (Busscher and Van der Mei, 2012). The physiochemical properties of a surface determine how bacteria will attach.

Biofilms form when, in step 2, bacteria move towards the surface by chemotaxis or Brownian motion and adhere (reversible and irreversible) as a monolayer to conditioned surfaces. Microbial movement via flagellar motility, surface translocation, twitching, gliding and sliding

is involved in this process. Excretion of an exopolysaccharide (EPS), glue-like substance, that attaches the cells to the surface and to each other, results in irreversible attachment to the surface (through dipole, hydrogen, ionic, or hydrophobic interactions). Synthesis of exopolysaccharides, which form complexes with the surface material and/or secretion of specific protein adhesins that mediate molecular binding, are known mechanisms for irreversible attachment (Dunne, 2002; Larsen *et al.*, 2007).

The microorganisms start to reproduce (step 3) by binary division and recruitment of other planktonic bacteria results in formation micro-colonies (Trachoo, 2003; Halan *et al.*, 2012). In the course of biofilm development (step 4), a complex three-dimensional architecture with macro-colonies and interstitial voids and channels is built. These voids and channels allow water and nutrients to diffuse through to the deep layers of the biofilm, as well as waste substances to be excreted.

A mature biofilm (step 5) develops after a period of days and maintains a pseudo steady-state in which detachment of cells from the biofilm and re-growth occurs, keeping the biofilm thickness constant. Detachment from biofilms is caused by a combination of processes including, abrasion, shear forces, erosion, sloughing and predator grazing (Horn *et al.*, 2003; Wuertz *et al.*, 2003). Detachment occurs when an external force (e.g. shear force) is larger than the internal strength of the matrix that holds the biofilm together. Mature biofilms are dynamic, spatially and temporally heterogeneous communities which can assume various architectures depending on the characteristics of the immediate environment (nutrient availability, pH, temperature, shear forces, osmolarity) as well as the composition of the microbial consortia (Pulcini, 2001).
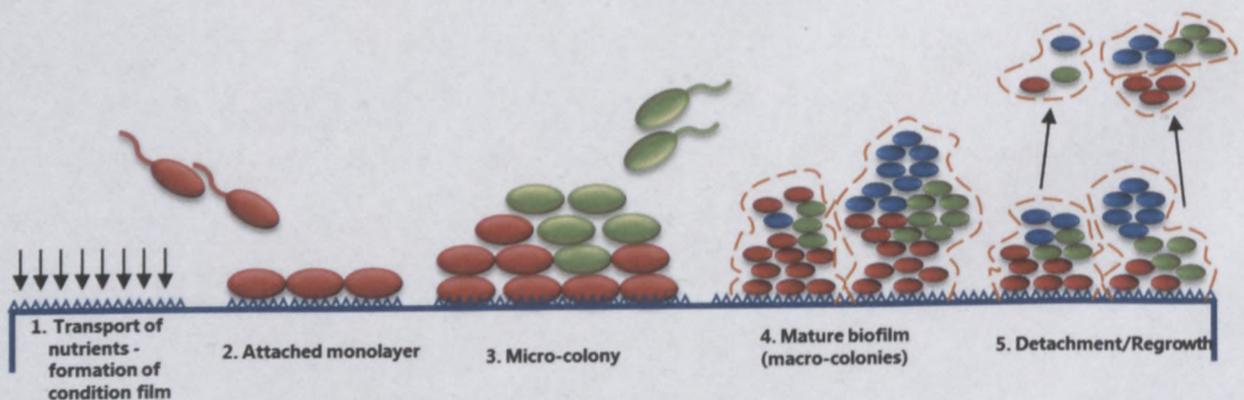
Figure 2.7 Biofilm development steps from the transport of nutrients to the detachment microorganisms (Trachoo, 2003; Halan *et al.*, 2012).

A biofilm can be formed by a single bacterial species, or as in nature, various species of bacteria, fungi, algae, protozoa and debris can make up a biofilm. When biofilms form in a natural aquatic system, each biofilm member has been naturally selected by its cell surface properties including the presence of capsules, fimbriae and cell surface hydrophobicity (Frank, 2001). Biofilms increase in size and complexity over time but it does not mean that all constituent members in the biofilm are actively growing. Biofilms can vary in thickness from a mono-cell layer to several centimetres thick, depending on the biofilm producers and growth conditions (Trachoo, 2003). Detachment from the surface can benefit the bacteria since they can move on to a new growth niche and establish a new biofilm. Factors affecting biofilm detachment include the biofilm thickness, fluid shear stress, fluid velocity and nutrient availability (Elvers *et al.*, 2002).

The EPS, consisting mostly out of polysaccharides (40-95%) and proteins (1-60%), but also lipids (1-40%), nucleic acids (1-10%) and other biopolymers, serves several functions in biofilms, including the facilitation/inhibition of the initial attachment of bacteria to a surface, the formation and maintenance of micro-colonies and biofilm structure and enhanced biofilm resistance to environmental stress and anti-microbial agents (Flemming *et al.*, 2007; More *et al.*, 2012). The EPS and the fimbria on the cell surface of microorganisms can form a bridge between the cell and the surface being colonised (Zottola and Sasahara, 1994; Cloete *et al.*, 1998).

The EPS-matrix changes the surface charge and the free energy for binding to the surface. The EPS also acts as a potential diffusion barrier for disinfecting compounds, while polysaccharides and cellular materials at the periphery of biofilm may react chemically with and neutralise disinfecting agents and thereby further reduce its availability and effectiveness (Evans, 2000; Dufour *et al.*, 2012). The EPS has also been reported to provide protection from a variety of other environmental stresses, such as UV radiation, pH shifts, osmotic shock and desiccation (Flemming, 1993). In addition, the EPS functions as stabilisers of the biofilm structure (Mattila-Sandholm and Wirtanen, 1992). Some microorganisms can bind ions into their EPS, which have an influence on the nature of the biofilm. Finally, the EPS-matrix enables the bacteria to capture nutrients (Costerton *et al.*, 1995).

2.8.2 Advantages and disadvantages of microorganism growing as biofilms

Biofilm formation can have different consequences in different environments. Biofilms in the industry context (e.g. cooling tower systems, process industry, food and beverage industry

and paper industry) are mostly undesirable due to economic losses introduced by biofouling and biocorrosion of surfaces, reducing process efficiencies, product quality and lifespan of industrial equipment (Kolari et al., 2001; Marcato-Romain et al., 2012). Despite effective and regular cleaning regimes, biofilms are often not fully removed and consequently slowly reform in these industries due to the protection mechanisms afforded to microbes by the biofilm environment (Quain and Storgårds, 2009). Cooling towers provide an ideal environment for biofilm growth and dissemination, and the risk of pathogen release in drinking water, such as Legionella, is well-documented, in addition to the energy losses and blockage of pipes (Liu et al., 2009). In the paper industry, the formation of biofilms on machine surfaces leads to economic losses due to increases in cleaning and maintenance time required (Tiirola et al., 2009). Biofilms in food systems lead to hygienic problems and economic losses due to food spoilage and food poisoning, but may be desired in some food processing scenarios (Kumar and Anand, 1998; Batista et al., 2000; Brooks and Flint, 2008).

Despite the negative impacts of biofilms in certain environments, they play a critically important role in natural environments. Microbial cells adapted to a biofilm lifestyle express phenotypic traits distinct from the planktonic population, for example, increased tolerance to antimicrobial agents, altered metabolic or biochemical reaction rates, enhanced degradation ability of toxic chemicals and changed synthesis of biomolecules have previously been described (Stewart and Franklin, 2008). Biofilms are important for water quality maintenance and can be applied in numerous areas such as ground water treatment, municipal and hazardous wastewater treatment (Najafpour et al., 2005).

Biofilms offer a special advantage for nutrient removal processes in wastewater treatment, which traditionally require sequences of aerobic, anoxic and anaerobic conditions. Biofilm systems allow for much more compact reactors, operational flexibility, reduced hydraulic retention times, increased resilience to environment changes and enhanced ability to degrade recalcitrant compounds (Andersson, 2009). Biofilms play an essential role in the bioremediation wastewater either in suspended growth systems such as the activated sludge process, or in attached growth systems using reactors such as trickling filters, RBCs, fluidized bed biofilm reactors, airlift reactors, granular filters and membrane immobilized cell reactors (Gilbert et al., 2002; Andersson, 2009).

Besides primary, secondary and tertiary wastewater treatment, biofilm systems have also been successfully used to treat industrial wastewaters (Singh et al., 2006). Biofilms used in

wastewater treatment employ several mechanisms to remediate wastewater in addition to biological degradation, including biosorption, bioaccumulation and biomineralisation of compounds within the wastewater (Singh *et al.*, 2006). Malandra *et al.* (2003) investigated the bioremediation of winery wastewater by indigenous biofilms in wine effluents using an RBC. This study suggested that the naturally occurring indigenous microorganisms were able to form a stable biofilm and also reduce COD of winery wastewater (on average 43% with a retention time of 1 hour). Bertin *et al.* (2004) studied the anaerobic digestion of OMWW in biofilm reactors packed with granular activated carbon and silica beads. Results showed that both biofilm reactors mediated an extensive OMWW remediation effect under continuous conditions, with a tolerance to high and variable organic loads, while COD and phenolic compound removal rates were significantly higher than what is averagely displayed by most of the conventional and packed-bed laboratory-scale reactors previously proposed for the OMWW digestion.

2.8.3   Detergents used in olive industry

Although the biofilm mode of growth of microbes is well-documented to enhance resistance to antimicrobial agents and disinfectants, the use of a biological process such as an RBC could be negatively impacted by these compounds. Both table olive as well as olive oil processing industries use detergents in order to maintain a clean and sanitised working environment. At Buffet Olives, six cleaning chemicals are routinely used: Robot®, Contrabac®, Alkaliser 485®, Sanitiser HA®, Ordet SC® and Tetrasheen® (trade names). At Vesuvio Estate, two cleaning chemicals, Removil® and Limex®, are routinely used. These detergents used inside the processing plant to clean working areas and surfaces are generally bacteriostatic and/or bactericidal as well as fungistatic and/or fungicidal and could potentially affect the development and stability of indigenous biofilms and therefore influence the efficiency of the RBC system.

On the other hand, exposure to sub-inhibitory concentrations of detergents in the wastewater might also result in increased acquired resistance over long periods of application. The probability of acquired resistance in olive wastewater biofilms is hypothetically high, since washing waters follow the same disposal route as the high volumes of wastewater from olive and olive oil processing stages. This implies that detergent concentrations reaching the RBC system will be diluted and therefore stimulate possible acquired resistance in biofilm microorganisms.

As part of this study, we attempted to quantitatively determine the effect of various concentrations of NaOH and industry relevant detergents on biofilm development and stability in the small-scale RBC setup.

CHAPTER 3

Materials and Methods

3.1 Sources and Composition of Olive Wastewater (OWW) Treated in RBC system

For the purpose of this study, OWW was collected from two olive processing plants situated in the Western Cape, namely Vesuvio Estate, specialising in the extraction and production of extra virgin olive oil, and Buffet Olives, focusing entirely on the production of a variety of table olives. The wastewater from these two sources was chosen because both Vesuvio Estate and Buffet Olives use primarily evaporation ponds as the main treatment regime for their wastewater and they represent the typical chemical composition of OMWW and TOWW, respectively. Initially, 25 L volumes of fresh OWW were collected every second week from May 2004 to November 2004 at the inlet site of the evaporation ponds. During 2005, 100 L OWW volumes were collected monthly from both farms at the evaporation pond sites. The increase in volume was required to operate the RBC bioreactors in a semi-continuous mode.

(A)   **(B)**
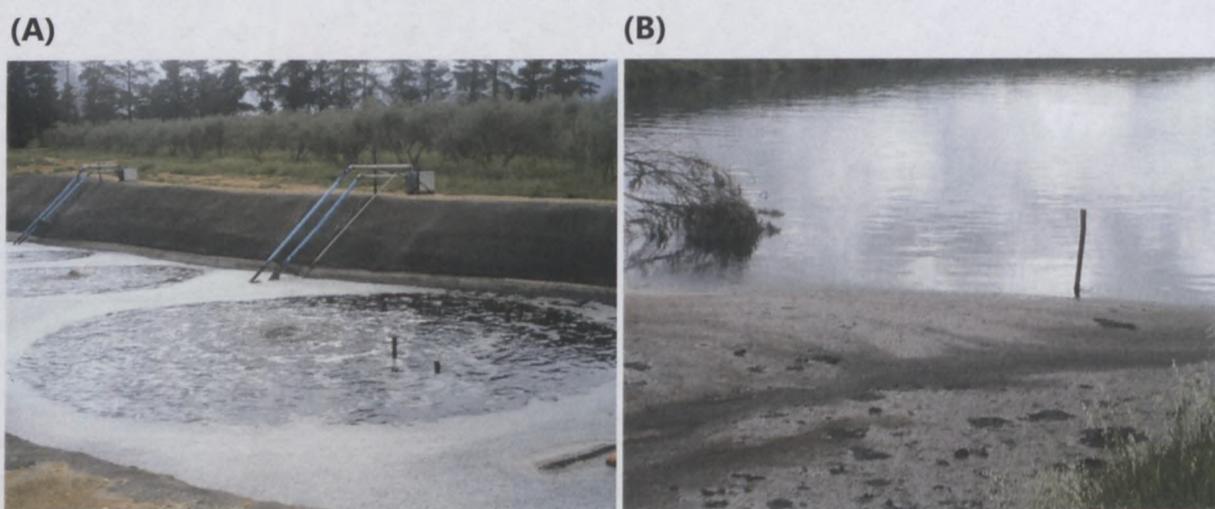
Figure 3.1 OWW collection sites at **(A)** Buffet Olives and **(B)** Vesuvio Estate. Raw wastewater is pumped directly from the processing plants to these evaporation ponds for treatment before disposal. Evaporation ponds at Buffet Olives are clearly more developed (protective lining and aeration pumps) and structured than at Vesuvio Estate, which basically pumps wastewater into a manmade dam, with no clear protection against pollution of underground water resources.

OWW collected from both Buffet Olives and Vesuvio Estate was artificially enriched with the addition of 0.1% (w/v) D-glucose (Merck, SA), 0.15% (w/v) ammonium phosphate [$(NH_4)_3PO_4$] (Sigma-Aldrich, Germany), 0.2% (v/v) commercially acquired olive oil and 0.2% (w/v) homogenised black olive pulp to simulate the peak production effluent composition with high COD and phenol content. The addition of these artificial ingredients ensured COD and phenol levels (Table 3.1) that resemble those reported in literature (Chapter 2, Section 2.4). Olive oil and olive pulp were added to simulate the lipid and phenolic content, respectively, of OWW during active olive production seasons, while the glucose and $(NH_4)_3PO_4$ were added to supply a minimal C and N source to support biofilm growth and development.   Chemical

Oxygen Demand (COD), total phenol content and pH of the OWW's were determined as described below (Section 3.8).

Table 3.1 Initial COD and total phenol content for selected time points of artificially enriched TOWW and OMWW used in the RBC bioreactors.

Samples	Initial TOWW (mg/L)		Initial OMWW (mg/L)	
	COD	Total Phenol	COD	Total Phenol
S1	4800	112	9800	195.5
S2	7985	180	6775	98.4
S3	12320	404	10520	58.6
S4	4875	80	6875	112.5
S5	10474	34		

COD levels of TOWW varied from a low of 4800 mg/L to a high of 12320 mg/L, while COD levels in OMWW ranged from 6775 mg/L to 10520 mg/L. The organic strength of TOWW and OMWW were not normalised by dilution prior to application in the RBC setup, to simulate actual surface organic loading variations which large-scale olive processing plants may experience.

3.2 RBC Setup and Operation

Tailor-made small-scale single-stage RBCs with a total length of 80 cm long were designed and built from PVC material for the evaluation of the indigenous OWW biofilms' bioremediation potential (Fig. 3.2, 3.3; Table 3.2). The structure of the RBCs consisted of a trough with a 4 L total volume capacity, with an estimated effluent depth of 6 cm, fitted with 16 equally spaced polystyrene discs (diameter 17.5 cm and thickness 2.7 cm) mounted onto a horizontal stainless steel shaft, which allowed for at least 40 % submerging of the discs in the OWW at all time. The rotation of the discs was driven by a 12 V motor, while the discs were set at a fixed rotational speed of approximately 15 rpm. Artificially enriched TOWW from Buffet Olives and OMWW from Vesuvio Estate were continuously pumped into separate RBC systems by means of a peristaltic pump (Watson-Marlow Model 323, UK). Furthermore, both types of OWW were sparged with air at the inlet side of the trough to ensure maximum

dissolved oxygen conditions using standard aquarium air pumps and diffuser stones set at the bottom of the trough.

Table 3.2 Summary of RBC specifications.

Description	Measurement
Disc diameter	17 cm
Disc thickness	2.7 cm
Number of discs	16
Length of trough	80 cm
Rotational speed	15 rpm
Total surface area covered by biofilm	8665 cm^2
Submergence of discs	40%
Effluent depth	6 cm
Trough working volume	4 L
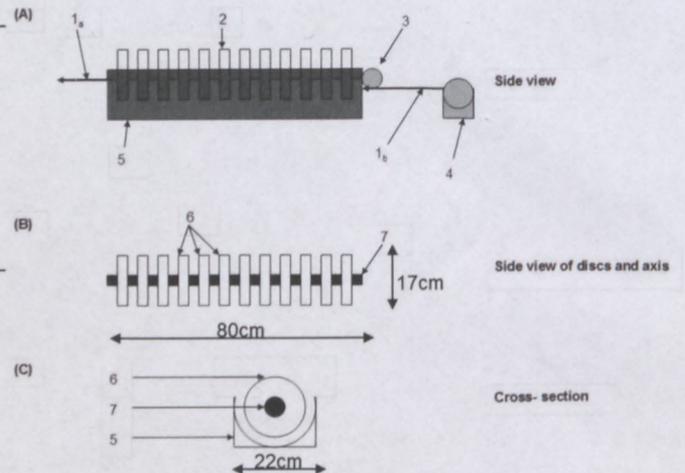
Figure 3.2. Diagramme showing the design of the small-scale RBC. **(A)** Side view; **(B)** stainless steel shaft with discs; **(C)** cross sectional view. **1$_a$**. Outlet, **1$_b$**. Inlet, **2**. Stainless steel shaft with polystyrene discs, **3**. 12V motor, **4**. Peristaltic pump, **5**. Trough, **6**. Polystyrene discs, **7**. Stainless steel shaft
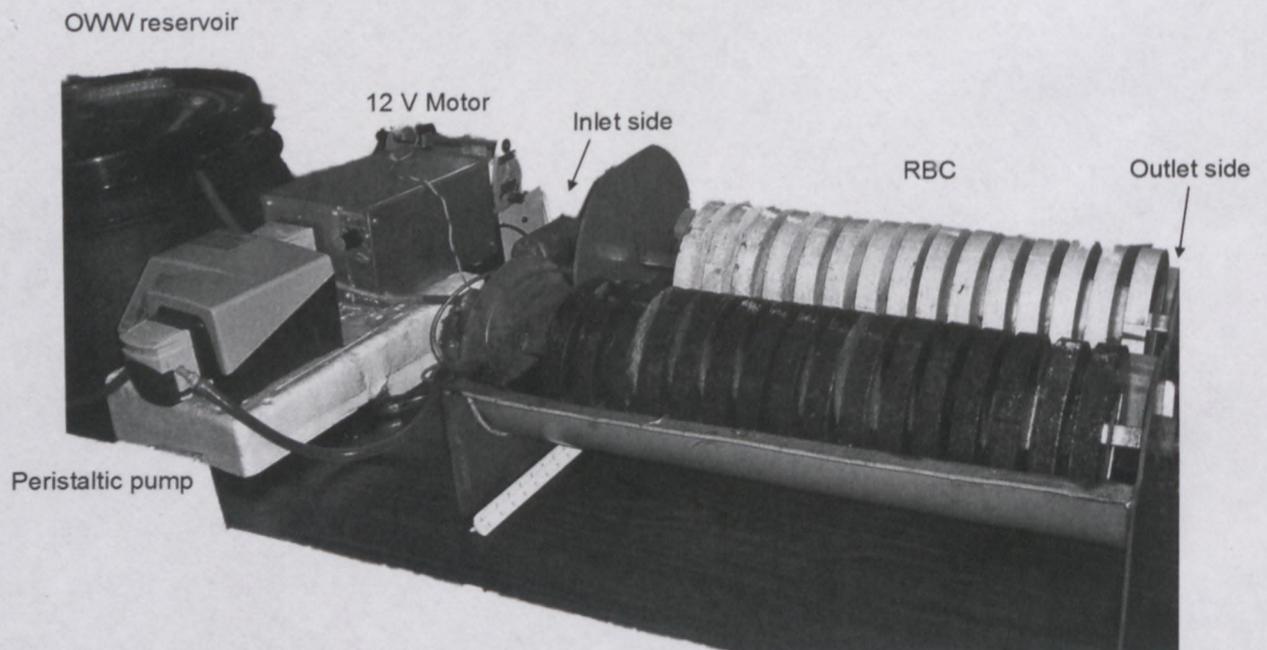
Figure 3.3. Typical setup for RBC operation showing the reservoir tanks, peristaltic pumps(s) and RBC, as well as the 12 V motor driving the rotation of the disc axis.

After initial filling (approximately 4 L) of the RBC trough with OWW, the remaining OWW was continuously fed into the RBCs from the reservoir tanks on the inlet side by means of the peristaltic pump at a constant flow rate of 41.5 mL/min resulting in a theoretical hydraulic retention time of 1 hour and 37 minutes. The RBC discs were allowed to rotate continuously during the course of the study at 15 rpm. The overflowing OWW were collected into a separate collection tank placed at the outlet of the RBC and recycled to the reservoir tanks. Once operational, the RBCs were monitored daily to prevent any mechanical or technical problems that could occur during the operation period.

3.3 Quantification of the Biofilm Development in the RBCs as Percentage Area Coverage

To quantify biofilm development during RBC operation, epifluorescent microscopy combined with digital image analysis was used to quantitatively measure biofilm development in the RBC. A total of 60 sterile standard microscope glass slides were inserted at the bottom of each RBC trough for both OMWW and TOWW, prior to addition of OWW. The biofilms were allowed to develop on the microscope slides for one week. After one week, three slides were removed from each RBC at 2 or 3 days intervals, for a total of 30 days. After eliminating excessive moisture, the material on the microscope slides was stained with 50-200 μL acridine orange (0.1%, w/v; Sigma-Aldrich, Germany) for 1 min at ambient temperature in the dark. Stained microscope slides were washed by gently rinsing the slides three times with double distilled water (ddH$_2$O). Slides were allowed to air dry in a dark chamber.

For quantitative assessment of biofilm development over time, three microscope slides for each time point were examined with a Carl Zeiss-epifluorescent microscope equipped with the AxioCam MRc5 camera (with Peltier cooling) and Axiovision Software (release 4.6) from Carl Zeiss (Germany). Biofilms were observed at 40X magnification and for each microscope slide, 10-12 microscopic fields/slide were captured and analysed using the freeware software, ImageJ (v 1.3u for Windows; National Institutes of Health, USA). Images were analysed for percentage area coverage by converting the image type from RGB colour to 8-bit greyscale. Subsequently, biofilm micro-colonies and cells were discriminated from the background by manual thresholding, yielding binary black and white images. The threshold value for the binary image was set manually to best represent the area of coverage in the original image, i.e. white areas/pixels corresponded to biofilms, while black areas/pixels matched the voids. An ImageJ plugin, Area Calculator, was then executed to measure the percentage thresholded pixels over the total pixels of the image. The percentages obtained were then

averaged for all 10-12 fields to yield the percentage area coverage of the biofilm at that particular point in time.

3.4   Determination of the Dry Biofilm Density/Porosity

In order to determine the density/porosity profile of the biofilm developing on the polystyrene discs in the RBC over time, biofilm samples were isolated every second day from the surface of the polystyrene discs. To ensure accurate density/porosity measurements, sampling commenced only 7 days after the initial start-up of the RBC system, with a minimum weight of 0.1 g biofilm per sample. Wet biofilm samples were obtained by scraping off a ± 4 cm^2 area of the biofilm with stainless steel blades from the temporarily static (not turning) polystyrene discs. To minimise the effect of biofilm removal on the bioremediation effect, only a small area of biofilm was removed from eight discs. Care was also taken not to remove biofilm samples on the same location more than once as density/porosity characteristics rendered from such samples would be reflective of younger biofilms. For accurate biofilm density determination, sampling (eight biofilm samples/time point) was alternatively targeted between the first eight discs at the inlet side of the RBC and the rear eight discs of the RBC on the outlet side. The 8 measured densities were then averaged for that sample time. After sampling, wet biofilm samples were transferred to sterile 2 mL eppendorf tubes, followed by drying at 60°C until bone dry. Due to the oily nature of the OWW and the biofilms, the biofilm samples often exceeded a 7 day period of drying. After drying, the samples were stored at room temperature in airtight glass containers.

The dry biofilm density of the eight samples was determined separately using a helium pycnometer (Accupyc, 1330, Micrometrics Co., USA), which measures density of solid samples. The calibration of the pycnometer was performed by using a steel sphere with a known volume before taking measurements on a series of biofilm samples, as previously described by Ntwampe (2012). After calibration, the accuracy of the pycnometer was checked by using water. A water density of 1.01 g/cm^3 was obtained. The dry mass of the biofilm was determined before the dry biofilm was loaded into the pycnometer sample chamber. The samples were measured by filling the sample chamber with ultra-high pure helium gas. The gas was discharged into a second empty chamber with the difference in volume quantified to determine the density of the dry biofilm. The measurements were repeated in triplicate for accuracy. When measuring density with a helium pycnometer, 0.02% accuracy can be

obtained, otherwise it can fall to 0.1% or less, depending on the material and the variations in the calibration volume.

3.5 Scanning Electron Microscope (SEM) Visualisation of OWW Biofilms

The scanning electron microscopic (SEM) technique was used to monitor and to measure the thickness of biofilm formation on the rotating polystyrene discs. A small sample of 1 cm^2 of polystyrene disc with biofilm growth was removed with a sterile scalpel blade from discs number 1, 3, 5, 7, 9, and 11 after 7 days of RBC operation, while an equal size biofilm sample was removed from discs 2, 4, 6, 8, 10, 12, 14 and 16 for the two week old biofilm. The samples were then subjected to a series of alcohol dehydration steps to replace all the water in the sample with alcohol. These dehydration steps involved placing the biofilm samples in different concentrations of alcohol for at least 10 minutes, i.e. 30%, 50%, 70%, 80%, 90%, 95% and 100%. Once the biofilm samples were in 100% alcohol, they were critical point dried (CPD), a process involving the replacement of the alcohol with liquid carbon dioxide and eventually gaseous carbon dioxide. The samples were then dried and mounted on small aluminium SEM stubs. These were sputter coated with gold/palladium and were ready for examination with the SEM. Samples were examined with a fully analytical Leica LEO S440 scanning electron microscope (Scanning Electron Microscopy Unit, University of Cape Town) and 20 images were captured from each set of samples. The Leica LEO S440 SEM uses a tungsten filament electron source with an accelerating voltage up to 30 kV with capability of secondary electron and backscatter electron imaging. The usual bits used were 10 kV, and the working distance (WD) as well as the magnification of the SEM was varied from 100 to 15000 X magnification to obtain different views of biofilm attachment and structure on the polystyrene surface.

3.6 Measurement of Bioremediation of OWW

3.6.1 Chemical Oxygen Demand (COD) measurements of TOWW and OMWW

To determine the rate of COD removal, 10 mL triplicate samples of OWW were collected in sterile Falcon tubes every 24 hour from the RBCs. For comparative analysis, samples were collected from the effluent reservoir side and from the collection tank of both RBCs. The OWW samples were not pre-treated by filtration or centrifugation prior to COD measurement. COD measurement was executed using the 500-10000 mg/L range COD cell test kit (Cat. no: 1145550001, Merck, SA). Digestion of samples was performed in Spectroquant TR 420 heating block by using Solution-A and Solution-B as standard solutions

supplied by the manufacturer and measured in a Merck Spectroquant Nova 60. After a 10-20 X dilution, 1 mL OWW sample was added to three individual COD Cell test tubes, containing 2.2 mL COD solution A and 1.8 mL COD solution B. Samples were then mixed gently by inversion and inserted into a preheated Spectroquant Nova TR420 apparatus set at 148°C for 2 hours. After digestion, the samples were allowed to cool at room temperature for 10 minutes, followed by photometric analyses using the Spectroquant® Nova 60 according to the manufacturer's guidelines. The COD values obtained was measured in triplicate from each COD cell tube. The average result calculated was recorded as the COD measurement at that specific time point.

3.6.2   Total phenol measurement of TOWW and OMWW

To determine the rate of total phenol removal, 10 mL triplicate samples of OWW from the RBCs were collected in sterile Falcon tubes every 24 hour. For comparative analysis, samples were collected from the effluent reservoir side and from the collection tank of both RBCs. Total phenol concentrations were determined using the phenol cell test kit (0.10 - 2.50 mg/L Cat. no.: 1145510001, Merck, SA) in combination with the Spectroquant Nova 60. Each sample was diluted 10X or 20X prior to analysis, since the apparatus was only able to read accurately within the 0.1 to 2.5 mg/L phenol range. During the analysis, 0.5 mL diluted OWW sample was added into three separate Phenol Cell test tubes, containing 200 µL of concentrated sulphuric acid, 1 g of 3-methyl-2-benzothiazolinon-hydrazonhydrochloride (ph-1K), 2 g of ammonium-cerium (IV) sulphate dehydrate (ph-2K) and 9.5 mL of distilled water. Samples were gently mixed, allowed to undergo reaction for 1 minute, and analysed on the Spectroquant® NOVA 60 photometer according to the manufacturer guidelines. The values obtained were measured in triplicate from each phenol test tube, and the averaged result calculated was recorded as total phenol concentration at that specific time point.

In order to determine the pH and redox potential changes of the OWW during the course of the RBC treatment, approximately 100 mL of OWW was collected every 4 days from the continuous working RBC and measured using the pH meter type BPH 600, model BH electrode BA25 (Boeco, Germany.)

3.7 Evaluating the Effect of NaOH and Other Detergents on OWW Biofilms

In order to observe the effect of NaOH, as a chemical used during lye treatment, and cleaning detergents on biofilm development, a series of artificial shock treatments were conducted on the TOWW and OMWW cultured biofilms. The purpose of this part of the study was to

determine quantitatively the effect of NaOH and the detergents used at Vesuvio Estate and Buffet Olives on biofilm development and stability.

The following description of the detergents used in this study was obtained from the manufacture's information sheets. Unfortunately, the exact composition of some of these detergents could not be obtained from the manufacturers, because the chemical formulations are protected by intellectual property restrictions.

The neutral detergents include: **Ordet SC**® is an all-purpose neutral (pH of 7.4) liquid detergent and foaming additive. It finds a wide range of application in the food industry. Furthermore it is a non-phosphate cleaner which cuts greasy soils. For general cleaning of most hard surfaces encountered in food industry, dilutions of 0.5-1% are used. However, for very badly soiled conditions, solution strength of 2-3% may be required. **Tetrasheen**® is a neutral bactericidal liquid hand soap developed specifically as a broad-spectrum microbiocide. It contains highly effective antimicrobial agents and produces a good cleaning lather that rinses off easily even in very hard waters. **Robot**® is composed of neutralized sulphamic acid effective at a pH of 7-8. **Contrabac**® is a broad-spectrum antimicrobial neutral agent that is generally used as hand soap. It is mostly effective against Gram-positive and Gram-negative bacteria, viruses, fungi and algae.

The alkaline detergents include: **Limex**® a clear, pale yellow liquid of potassium hydroxide and non-ionic surfactants with a pH of 11.8 as a 1% solution. **Removil**® is a high alkaline descaling detergent (pH 12.8 in 1% solution) that contains > 5% sodium hydroxide (NaOH) and 1-2% amino-trimethylene-phosphonic acid. **Alkalizer 585**® is a heavy-duty liquid highly alkaline detergent. It finds a wide range of applications in food industries where an economical heavy-duty product is required. Alkalizer 485 is normally used at 0.5- 3% concentrations.

One acidic detergent used at **Sanitizer HA**® is a peroxide/peracetic based broad-spectrum fast acting sanitizer, which supersedes the conventional chlorinated sanitizers. It may be used as a sanitizer and simultaneously as an acidified rinse due to low pH (2.5) of the working solution. It is very stable and the hydrogen peroxide causes the disinfection action to be rapid. The peracetic acid apart from contributing to the oxidation potential of the product also provides acidity that is essential for the prevention of mineral scale deposits. The product breaks down to water and oxygen when it decomposes and therefore it is not

detrimental to the environment. It is generally used for sanitizing, after washing, at concentrations 0.15-0.2%. Two minutes exposure time is essential for its effectiveness.

3.7.1 Quantification of biofilm detergent sensitivity using % area coverage

Two modified Pedersen's devices containing 12 microscope slides each were inserted into each of the RBCs to allow biofilm attachment onto the slides during RBC operation. After one week of cultivation, two microscope slides were removed and used as negative control. The modified Pedersen's devices containing the remaining slides were removed from the RBC and placed into separate 2 L glass beakers, containing the same OWW spiked with 2.5% final concentration of shock treatment solution. Biofilm shock treatment solutions that were prepared (Tables 3.3 and 3.4) and used in this study included a 2.5% (w/v) solution of NaOH and 2.5% (v/v) detergents solutions of (Contrabac®, Robot® for TOWW, Limex® and Removil® for OMWW, respectively).

Table 3.3 Preparation of Buffet Olive shock treatment solutions used at 2.5% final concentration

Detergent	Volume of NaOH/detergent	Volume of OWW
NaOH (50%)	50 mL	950 mL
Robot®	25 mL	975 mL
Contrabac®	25 mL	975 mL

Table 3.4 Preparation of Vesuvio Estate shock treatment solutions used at 2.5% final concentration

Detergent	Volume of NaOH/detergent	Volume of OWW
Removil®	50 mL	950 mL
Limex®	50 mL	950 mL

After 2.5 hours of each stress treatment, two slides from each treatment were removed and were immediately stained and microscopically analysed (as described above, section 3.3). Control slides were treated similarly, but without shock treatment. The modified Pedersen's devices with the remaining slides were re-inserted back into each RBC to allow biofilm recovery from the stress. Two microscopic slides were removed every 2 to 3 days from the modified Pedersen's devices in the RBC for image analysis. Microscope slides were stained and analysed as previously described (section 3.3).

3.7.2 Quantification of biofilm detergent sensitivity using microtiter plate assay

The microtiter plate method (adapted from Corona-Izquierdo and Membrillo-Hernández [2002] and Pitts *et al.*, [2003]) for biofilm sensitivity to NaOH and detergents was performed for TOWW (Buffet Olives) only. A 96-well, flat-bottom microtiter dish (non-tissue culture-treated PVC) was filled with 150 µL of fresh artificially-enriched TOWW and covered with parafilm to prevent evaporation and contamination. Microtiter plates were incubated without shaking at room temperature for 2, 24 and 48 hours, respectively, to allow microbial adherence to the interior wall of the plates. After incubation, attached cells were stressed with increasing concentrations of NaOH (5%, 15% and 30% [w/v]) and detergents (1%, 5%, and 10% [v/v]) respectively for 24 hours. The detergents used in this experiment are listed in Table 3.5. The first three columns of the microtiter plate were usually kept as negative control, i.e., did not receive any shock treatment. Following the shock treatment, the TOWW and unbound cells were removed from the wells by inversion of the microtiter plate followed by gentle washing three times with 150 µL ddH$_2$O. Plates were air dried in laminar flow for 5 minutes.

The remaining adhered cells after shock treatment were then heat fixed at 80°C for 30 minutes in an oven. The cells were stained by adding 150 µL 0.1% (w/v) crystal violet (CV) solution per well and incubated for 15 minutes (at ambient temperature and without shaking). After 15 minutes, excess CV solution was removed by inverting the plates, followed by gentle washing of the plates with ddH$_2$O and again oven-dried at 37°C for 15 minutes. Subsequently, 150 µL 96 % ethanol was added to each well to assist with the extraction of the intracellular CV from the attached biofilm cells.

Before spectrophotometric measurement, a 10X dilution in distilled water was prepared of each treatment. Dilutions were made by pipetting 135 µL double distilled water into fresh microtiter plates and adding 15 µL of CV ethanol extract of each treatment. Absorbance was measured on the Power Wavex Spectrophotometer (BIO-TEX Instrument, INC) at 595 nm. The spectrophotometer was calibrated with ddH$_2$O prior the start of an assay. The relative biofilm removal (expressed as mean percentage) was determined with the following equation (adapted from Mathur *et al.* [2004]):

$$\text{Percent biofilm removal} = 100 - \left[\left(\frac{A_{595nm} \text{ of treated}}{A_{595nm} \text{ of untreated}} \right) \right] \times 100$$

Each experiment was repeated three times and the mean percent biofilm removal and standard deviation was considered when comparing the results.

Table 3.5 Detergents used to stress the TOWW biofilms in microtiter plate assays.	
Detergents and concentrations tested	**Code**
Tetrasheen® (1%, 5%, and 10% [v/v])	D1
Ordet SC® (1%, 5%, and 10% [v/v])	D2
Sanitizer HA® (1%, 5%, and 10% [v/v])	D3
Alkalizer 485® (1%, 5%, and 10% [v/v])	D4
Contrabac® (1%, 5%, and 10% [v/v])	D5
Robot® (1%, 5%, and 10% [v/v])	D6
NaOH (5%, 15% and 30% [w/v])	NaOH

3.7.3 Flow cell evaluation for viable vs. dead cells in TOWW biofilms after detergent treatment

Two 8-channel flow cells (length = 300 mm, width = 38 mm, depth = 3.5 mm) with disinfected 1.6 mm tubing were connected to a 10 L reservoir filled with artificially-enriched TOWW. The flow cells and tubing were disinfected by pumping 1% (w/v) sodium hypochloride through the cells at 10 rpm (Watson Marlow peristaltic pump, 205U) for 10 minutes. The flow cells were rinsed by pumping sterile ddH_2O through the cells at 10 rpm for 10 minutes. TOWW was then pumped through cells at 1 rpm for 8 days and then increased to 90 rpm for 5.5 hrs. The effluent was not reused, but was discarded into waste collectors. TOWW spiked with 1% Alkalizer 485® and Robot® detergents, respectively, were pumped through flow cells with 8-day old biofilms at 1 rpm for 20 hrs. The flow rate was increased to 90 rpm for 3 hours. The first four channels were used for the controls, (i.e. no detergent added) and last four were stressed with detergent.

Bacterial viability was determined by using the LIVE/DEAD bacterial viability staining kit, (BacLight, Molecular Probes Inc., Eugene, OR). Two stock solutions of stains SYTO 9 and propidium iodide were diluted to a concentration of 3 μL/mL. The flow cell biofilms were stained with 1 mL of the diluted stain solution at room temperature in the dark for 1 hour.

The samples were rinsed twice for 1 min with 1 X PBS. Live SYTO9-stained cells and dead propidium iodide-stained cells in the flow cells were visualized examined with a Carl Zeiss-epifluorescent microscope (100× oil lens) equipped with the AxioCam MRc5 camera (with Peltier cooling) from Carl Zeiss (Germany). The number of viable and nonviable bacteria in the flow cells was estimated from counts of a minimum of 10 fields of view using the Axiovision Software (release 4.6). The intensities of SYTO9 (emitting green fluorescence) and propodium iodide (PI emitting red) were monitored at 480/500 nm and 488-540/617 nm, respectively. SYTO9 stains all cells regardless their viability, whereas PI stains only non-viable cells with damaged membrane integrity.

CHAPTER 4

Results and Discussion

4.1 Biofilm Development in the RBC Setup

4.1.1 General observations and epifluorescent microscopy

The continuous supply of either TOWW or OMWW in an RBC setup resulted in the progressive aerobic growth of biofilms on the surface of the polystyrene discs. Although biofilms developed naturally in both TOWW and OMWW operated RBCs, it was evident that TOWW from Buffet Olives allowed for more prolific biofilm growth when visually compared to the OMWW from Vesuvio Estate (data not shown). The external appearance of biofilms was clearly distinctive among the two RBCs. Biofilms in the TOWW RBC (RBC 1) had a gelatinous/slimy, soft and easily detachable character, while biofilms in the OMWW RBC (RBC 2) appeared to be compact and thin (5 to 8 mm thick) compared to TOWW biofilms.

The indigenously cultured TOWW biofilms reached a maximum thickness of 10 to 12 mm during its development, while the biofilm was also prone to sloughing from the polystyrene discs and accumulated at the bottom in the RBCs trough. The build-up of the detached biofilm in the RBC trough caused a slight reduction in rotational speed of the RBCs discs to less than 15 rpm initially calculated. Detached biofilm mass was therefore removed regularly to ease and maintain the rotational speed of the polystyrene wheels and allow free flow of the TOWW within the RBC system. The sloughing behaviour was not observed in OMWW biofilms. Differences were also observed in biofilm colour; biofilms cultivated from the TOWW in RBC 1 were green to greyish in colour, whereas OMWW (RBC 2) biofilms had an intense dark brown colour.
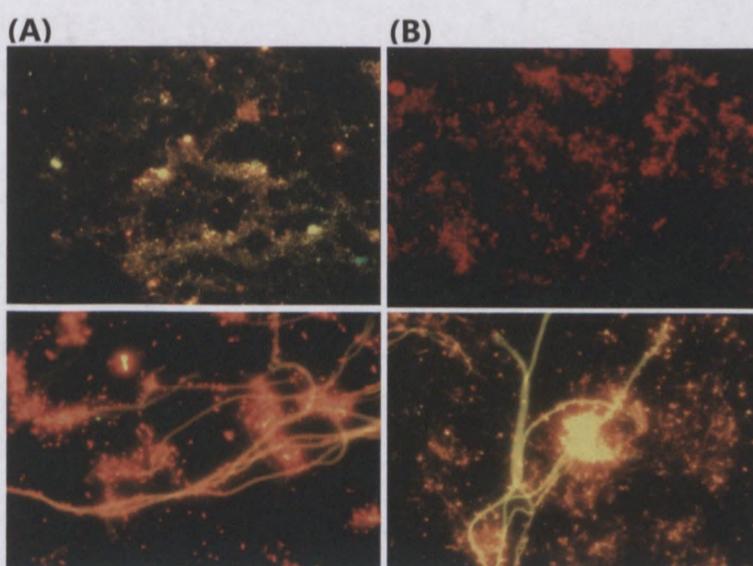

Figure 4.1. Epifluorescent micrographs of representative of 4 day old biofilms in TOWW (A) and OMWW (B) stained with acridine orange indicating early microbial cluster formation.

The differences in appearance could possibly indicate higher proportion of lignin-type polyphenolic compounds in OMWW compared TOWW, although this was not evident from the total phenol assay used in this study.

Microscopic examination of the biofilms stained with acridine orange revealed the presence of typical biofilm structures in both TOWW and OMWW after 4 days of development in the RBC systems. Although isolation and identification of the microorganisms were not performed in this study, micrographs at 40 X magnification (Fig. 4.1), clearly showed the presence of early stage microbial biofilm clusters consisting of a combination of bacteria (cocci and bacilli), yeast cells and fungal hyphae. It was interesting to note the presence of filamentous microorganisms, which play an important role in biofilms as they maintain the yeast concentration within intertwined mycelial-like structures that also act as backbone for yeast and bacterial attachment in the biofilm community (Madoni *et al.*, 2000). The presence of fungi in the OWW biofilms is also not surprising as these microorganisms are part of the normal microflora of olives and OWW, as discussed in Chapter 2. Furthermore, scattered protozoa were regularly encountered in the biofilm (results not shown) in some fields of the microscopic analysis of the biofilms.

4.1.2 Scanning electron microscopy (SEM)

SEM examination of the RBC polystyrene disc surfaces from TOWW confirmed that indigenous biofilms could be readily observed after 6 days of development onto polystyrene discs. The SEM analysis of the 6 days old biofilm in TOWW (Fig. 4.2) showed scattered bacterial cells and microbial clusters entrapped in an exopolysaccharide network or attached to the polystyrene surface. Exopolysaccharides are clearly visible as network (web-like) threads in the young biofilm (Fig. 4.2 A to C).

Although SEM demonstrates a non-homogeneous spatial distribution of biofilms, bacterial cells seemed to be the predominant species in the biofilm community, while early stage microbial cluster formation could be observed in a minority of scanned fields (Fig 4.2 C). This could be due to the fact that the biofilms were still young, 6 days old, and at their early stage of development, or due to the loss of some biofilm structures as a result of sample preparation might also explain the observations. Lazarova and Manem (1995) reported that the main drawback of SEM is the slow and intricate sample preparation procedure, which may provoke specimen damage, distortion or biofilms loss and furthermore have shown that the critical point drying step can result in significant and variable organism's reduction of up

to one half the cell volumes. This could be the phenomenon that has happened with the 6 days old young biofilms organisms.

The SEM analysis of 14 days old biofilms revealed the presence of a complex and dense biofilm community. It appears in Fig. 4.2 (D, E, and F) that biofilms have become dense and compact with no single cells visible, compared to the 6 days old biofilms. In Fig 4.2 (D), fungal mycelium could clearly be distinguished, while Fig 4.2 (E & F) shows the surface of biofilms with clearly distinguishable three-dimensional structures.
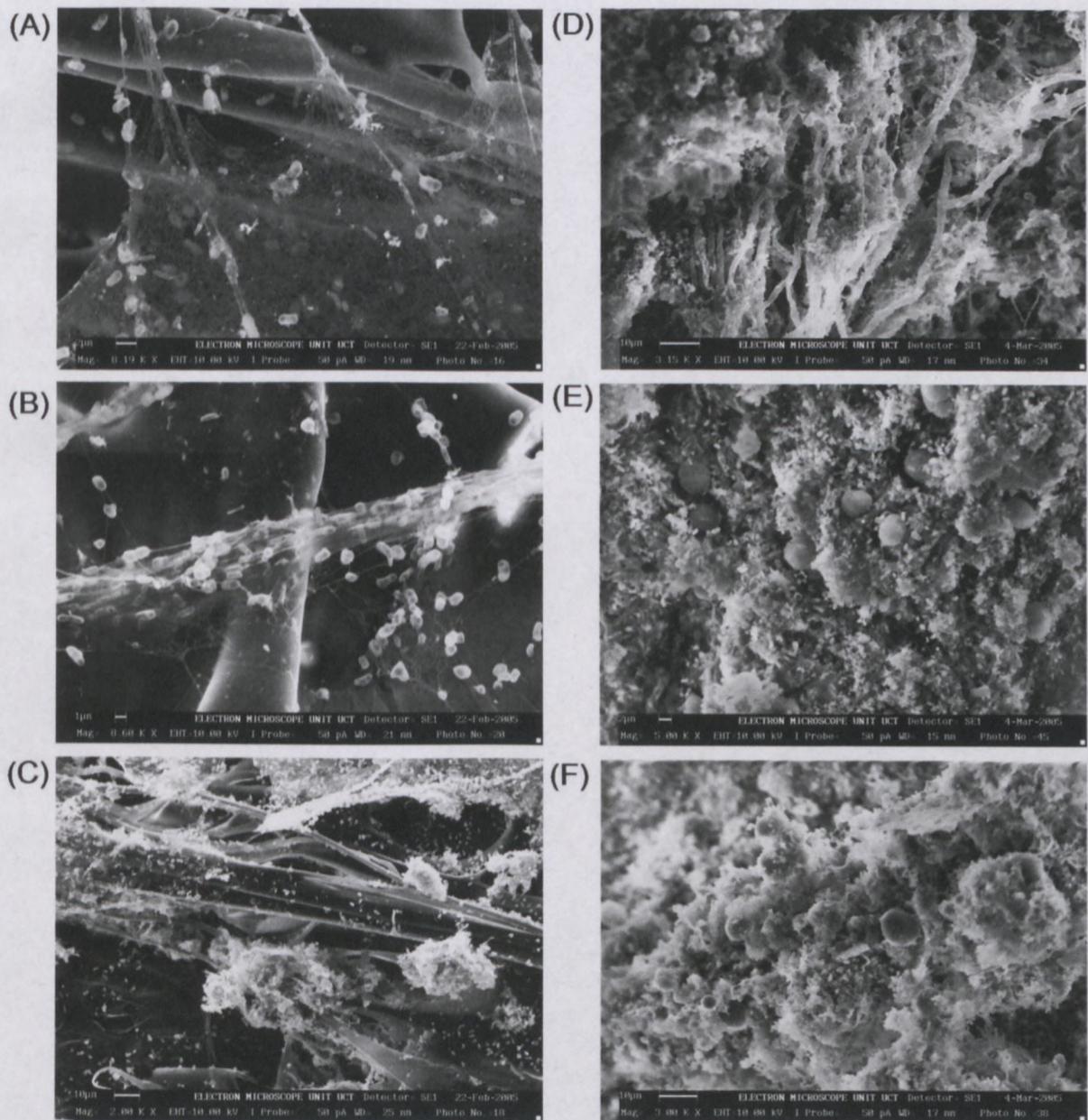
Figure 4.2. Scanning electron micrographs of 6 days old TOWW biofilms (A-C) and 14 days old TOWW (D-F) biofilms from Buffet Olive wastewater.

4.1.3 Biofilm growth in TOWW and OMWW as measured by percentage area coverage

During the percentage area coverage studies, microscope glass slides were used as attachment surface for biofilms and biofilm growth was estimated as percentage area coverage over time. For each time point, three slides were collected and 10 microscopic fields were analysed. Fig. 4.3 shows the column means of 30 data points (10 data points for each microscope slide) with the error bars showing the upper and lower standard deviation.

In TOWW, the area covered by biofilms increased over the first 4 days to ca. 13 % coverage, followed by period of 8 days during which the biofilm coverage stayed relatively constant (Fig. 4.3 A). From Day 13 to 16, a sharp increase in the area covered by the biofilm was observed (ca. 30%), followed by another period where the coverage stayed relatively constant. After 22 days of growing the biofilm in TOWW (RBC 1), approximately 32% of the microscope slides were covered with biofilms. The growth pattern of the TOWW biofilms followed a similar pattern that one would expect for "diauxic growth" in a batch microbial culture, and is perhaps indicative of the population changes in the biofilms as this was continuous growth, not a batch system. It can also be noted that the large error bars for time points indicate a substantial variation in the measurement of percentage area coverage between different microscopic fields of the same time point (i.e. 20-40%).

(A)   **(B)**
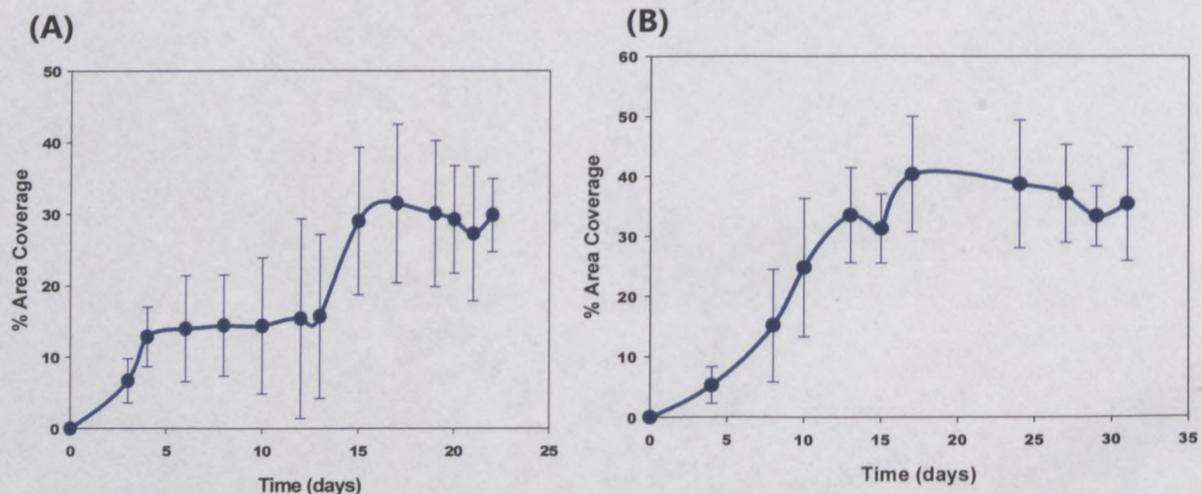
Figure 4.3. Biofilm development as expressed by percentage area coverage in (A) TOWW in RBC 1, and (B) OMWW in RBC2.

The % area covered by the biofilms in OMWW increased exponentially over 15 days, reaching a total percentage coverage area of 40% at Day 17 (Fig. 4.3 B). This could be ascribed to the possible multiplication phase of biofilm microorganisms, following their initial attachment

during which the primary cells may adapt to the environment before they divide, although no lag phase were detected. Furthermore, no "diauxic shift" could be detected, indicating that nutrient availability during the first 16 days was not growth limiting. From day 17, the percentage area covered appeared to be constant and decrease slightly to reach a final percentage value of 38%.

4.1.4 Biofilm density/porosity

The density of the biofilm is an important parameter as it is the single largest barrier the nutrients must overcome to reach the microorganisms in the biofilm. The density can be related to the permeability of the biofilm, thus the denser the biofilm material is packed, the more difficult it is for the long chain sugars and oils to reach the organisms inside the biofilm. Thus, the lower the density, the better the diffusion rate would be in the biofilm. Biofilm density for the TOWW biofilms are shown in Fig. 4.4. However, no clear pattern, i.e. no significant increase or decrease in biofilm density over time, could be established. The same was observed for OMWW biofilms (data not shown).
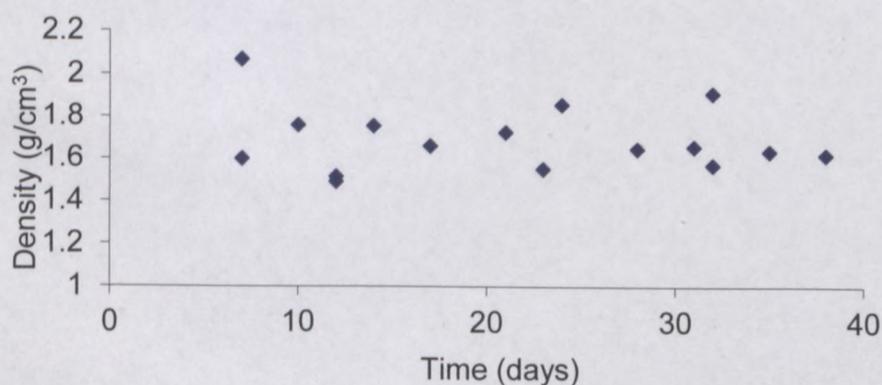
Figure 4.4. Biofilm density over time in TOWW biofilms.

4.2   Evaluation of the Lab-scale RBC for Bioremediation of OWW

If a biofilm-based bioremediation was to be considered as a treatment for olive wastewater (TOWW/OMWW), it is necessary to not only determine whether the naturally occurring microorganisms were able to form biofilms, but also if the indigenous biofilms can reduce the COD and phenol content of the OWW. Two small-scale RBCs, one for TOWW (RBC 1) and one for OMWW (RBC 2), were therefore operated during the olive harvesting season. RBC1 was run for a trial period of eight months between February and October, while RBC 2 was used for four months, i.e. June to October.

4.2.1 Table olive wastewater treatment in an RBC

The first TOWW sample (S1, collected on 01-02-2005) from Buffet Olives' evaporation pond, was initially circulated for two weeks in the reactor (RBC 1). The COD of the freshly collected sample (S1) after artificial enrichment indicated an initial organic strength of 4800 mg/L. During the first two weeks, the COD of the TOWW remained constant, indicating that biofilm development was not directly linked to COD removal. Furthermore, an increased COD (up to 6360 mg/L) was measured on day 13. An increase in COD is difficult to explained as the COD test usually oxidise all organic compounds in wastewater, even those molecules not biologically relevant, such as cellulose. However, an explanation for this could be the breakdown/hydrolysis of recalcitrant polymers such as plant polysaccharides, that would contribute to a spike in COD levels or a possible concentration effect due to some evaporation of the TOWW in the RBC. Addition of the second TOWW sample (S2, collected on 16-02-2005) to the RBC effectively doubled the COD levels to 7985 mg/L on Day 14, (Table 4.1). A rapid COD reduction was observed within the first 48 hours after addition of S2 (ca. 1000 mg/L/day). During the next two weeks, a slow decrease (100 mg/L/day) in the COD levels (down to the initial COD levels of 4800 mg/L) was observed from Day 16 to 27 (Fig. 4.5). At day 29, the final COD level of the TOWW was 5110 mg/L, representing 36% COD degradation in S2.

Table 4.1 COD and pH values obtained per sample for TOWW using RBC 1 (S1 values not included in average).

Sampling period	COD values (mg/L)		%	pH	
	start	end	degradation	start	end
S1	4800	6360	-24.5	8.55	8.10
S2	7985	5110	36	9.27	8.55
S3	12320	6815	44.60	8.01	8.57
S4	4875	1460	70.05	8.78	8.95
S5	10475	3175	69.68	9.00	6.68
Average	8914	4140	**55**		
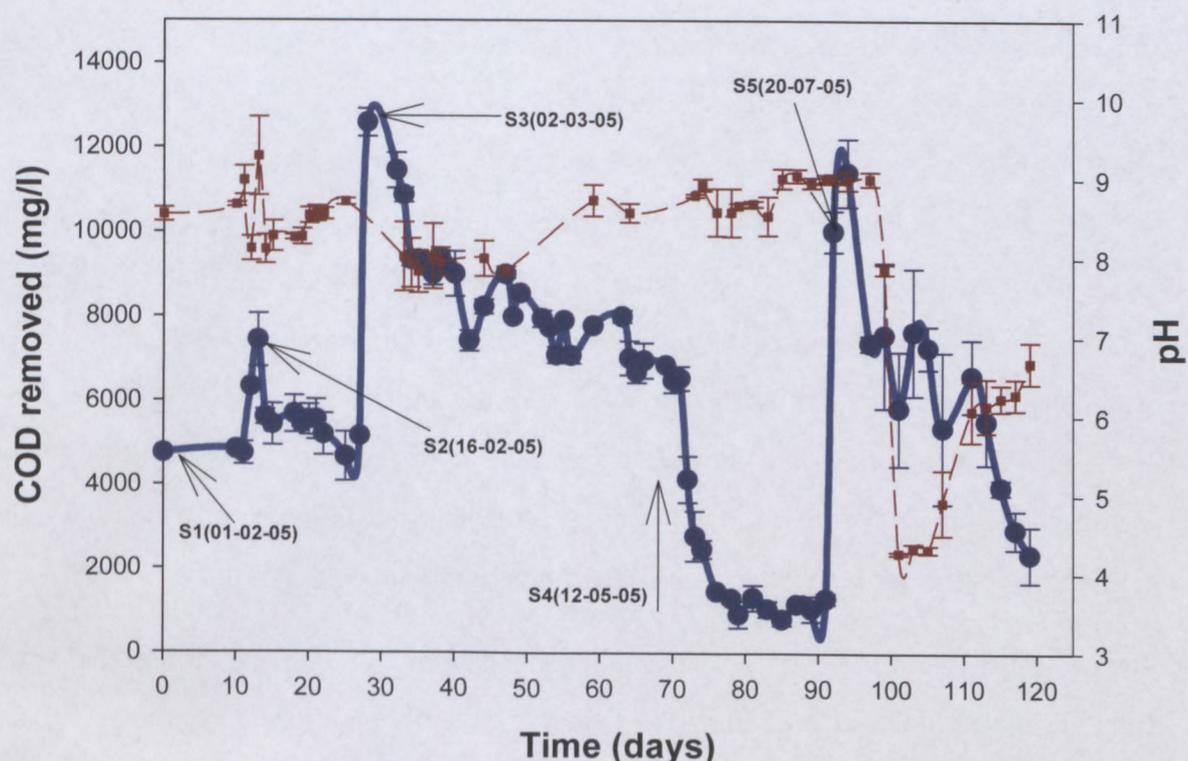
Figure 4.5. COD and pH changes vs. time for treatment of TOWW in RBC 1 (S1-S5 indicates the addition date of fresh TOWW).

The percentage COD removed was calculated by using the initial and final COD values measured at the last day of treatment per period of sample. Again, fresh TOWW (S3, collected on 02-3-2005) with an initial COD measurement of 12320 mg/L was introduced to RBC 1 at this point in time. A rapid decrease of COD was observed during the first 4 days (800 mg/L/day), followed by a period of slower decrease in COD levels (89 mg/L/day) for 33 days. It is interesting to observe that there are fluctuations in the COD at some days, but the overall downward trend in COD was maintained. Prior to the addition of S4, the minimum COD value reached was 6815 mg/L, representing 44.6% COD degradation for S3 (Table 4.1).

The initial COD of the fourth TOWW sample (S4) that was fed to RBC1 was 4875 mg/L. Again, immediately after addition, a rapid decrease in COD was observed (650 mg/L/day for three days), followed by a slow decrease in COD with a 17 mg/L/day removal rate for 15 days. After 30 days of treatment, the final COD value obtained was 1460 mg/L, representing an overall COD removal of 70.05% for S4.

The fifth sample (S5) had an initial COD of 10474 mg/L. During the course of treatment, one can notice a slight increase in COD directly after adding S5 (Day 91-92). This could be due to the hydrolysis of recalcitrant polysaccharides in the TOWW that was not initially measured by the COD test, or due to evaporation of the TOWW. A rapid decrease in the COD values (ca. 4000 mg/L/day) followed between Days 92 to 93. It is clear that between Day 94 and 113 the COD graph fluctuate several times, but kept its downward trend (112 mg/L/day) reaching a final COD level of 3175 mg/L. The percentage COD degradation obtained within that period was 69.68%. Data summarised in Table 4.1 indicate that an average COD degradation of 55.08 % was obtained. It can further also be concluded that the efficiency of COD removal increased with biofilm age, with the youngest biofilms having the lowest percentage removal efficiency, while older biofilms increased the percentage removal efficiency up to 70%.

The pH of the treated TOWW samples remained alkaline (average pH 8) during the 97 days of the RBC experiment (Fig 4.5). After the addition of S5, the pH dropped to pH 4.2 at Day 101 possibly due to fermentative metabolism producing organic acids leading to a reduction in pH.

The trends observed during the COD analysis of RBC 1 for treating TOWW indicate that after addition of fresh TOWW, rapid COD reduction is observed possibly due to the fact that the biofilm first metabolises the easily degradable carbon compounds. The slower degradation stages that follow are indicative of more recalcitrant carbon sources that require longer times to be metabolised by the biofilm microorganisms.

The total phenol content of TOWW was also monitored over the same period and Fig. 4.6 shows the changes in phenolic content of TOWW and the redox potential changes occurring during the RBC treatment of TOWW, while Table 4.2 summarises phenol values obtained during samples analysis.
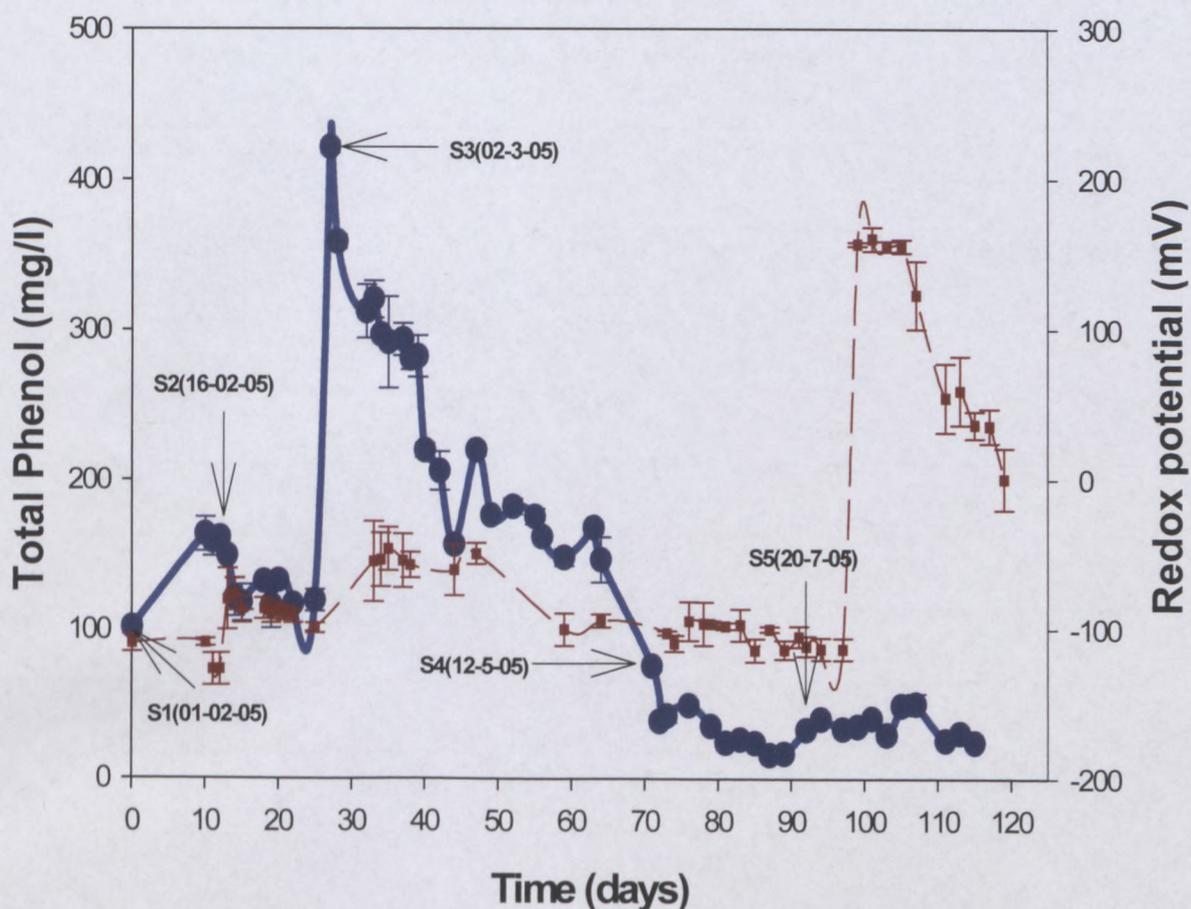
Figure 4.6. Phenol and redox potential changes vs. time for treated TOWW using RBC 1 (S1-S5 indicates addition of new TOWW).

Once again, an increase in phenol content (Fig 4.6) was observed in the first 13 days of RBC operation, from the initial phenol content of 112 mg/L to a final value of 186 mg/L after about two weeks of treating the TOWW in the RBC 1. The second sample (S2) of TOWW started with an initial phenol content of 180 mg/L and after continuous treatment for two weeks, a drop in phenol content could be noticed until a minimum value of 117 mg/L was reached, corresponding to a 35% degradation of the phenol in TOWW (Table 4.2). The third sample had the highest phenol content of 404 mg/L (Fig. 4.6). Between days 30 to 72, a rapid reduction in phenols occurred. The last phenol value obtained after 30 days of treating S3 was 159 mg/L, representing 60% degradation.

The fourth sample was added in the reactor on 12-5-2005 with an initial phenol value of 80 mg/L. The lowest phenol value obtained after 30 days of treating S4 was 37 mg/L, representing 53% degradation. The last sample (S5) was operated in the RBC from 20-7-2005 and started with an initial phenol concentration of 34 mg/L. After 30 days of treatment, the minimum phenol value obtained for S5 was 19 mg/L, with a percentage degradation of 45%.

In general, the average percentage phenol degradation obtained after treatment of TOWW using RBC 1 was 51% (averaging of all values calculated per sample treated) (Table 4.2).

Table 4.2 Total phenol and redox values obtained per sample of TOWW using RBC 1 (S1 values not included in average).

Sampling period	Total Phenol (mg/L)		%	Redox	
	beginning	end	degradation	beginning	end
S1	112	186	- 39.5	-104	-120
S2	180	117	35	-92	-98
S3	404	159	60	-111	-100
S4	80	37	53	-108	-110
S5	34	19	45	110	0
Average	175	83	**51**		

Throughout the treatment procedure, the redox potential of TOWW was monitored (Figure 4.6). The initial redox value of the first sample operated in the RBC 1 was -104 mV. During the treatment, one can observe a decrease in redox potential to a value of -120 mV after two weeks. When the second sample was added to the reactor, the initial value obtained was -92 mV and the redox potential was almost constant for two weeks until it reached a value of -98 mV. The next two samples (S3 and S4) had more or less the same redox values, i.e. -100 mV to -111 mV after every 30 days of treating each sample. The fifth sample had a positive redox value (146 mV) compared to the previous samples.

4.2.2 Olive mill wastewater treatment in an RBC

OMWW (sample 1, S1) collected from Vesuvio Olives was fed in the RBC 2 reactor on 12-5-05 with an initial COD value of 9800 mg/L. During the treatment period of OMWW, a gradual decrease in COD within the first thirteen days (260 mg/L/day) of operating the reactor was observed (Fig 4.7). The COD values then started to increase from Day 15 to Day 20 and reach an ending value of 5560 mg/L before the addition of the newly collected sample (S2). The percentage degradation calculated for S1 after 20 days of treatment was 44% (see Table 4.3).

Table 4.3 COD values obtained per sample for treated OMWW			
Sampling period	**COD values (mg/L)**		**%**
	start	end	**degradation**
S1	9800	5560	44
S2	6775	3860	43
S3	10520	5315	50
S4	6875	3740	46
Average	8493	4618	**46**

The second sample (S2) collected was introduced into the RBC on the 31-5-05 with an initial COD value of 6775 mg/L. COD levels decreases until day 26, with a slight increment Day 28. The average rate of COD removal for this period was 244 mg/L/day. The graph finally indicates a minimum COD value of 3860 mg/L at day 32, which represent a 43% COD degradation. The initial COD value of sample S3 was 10520 mg/L. During the treatment, one can observe a slight increment in COD to an approximate value of 12000 mg/L after a few days of operating the reactor. From Day 34 (Fig. 4.7), there is a considerable reduction in COD levels, whilst the COD increased once more between Day 40-45. Beyond that stage, a continuous decrease can be observed until Day 58, where the final COD value recorded was 5315 mg/L. A 50% COD degradation calculated for S3 after 30 days of treatment was observed (see Table 4.3).

Sample 4 (S4) was added on 31-8-05 with an initial COD value of 6875 mg/L. A progressive decrease in COD levels to 3740 mg/L occurred with 1 day. The percentage COD degraded for sample 4 obtained was 46% (see Table 4.3)
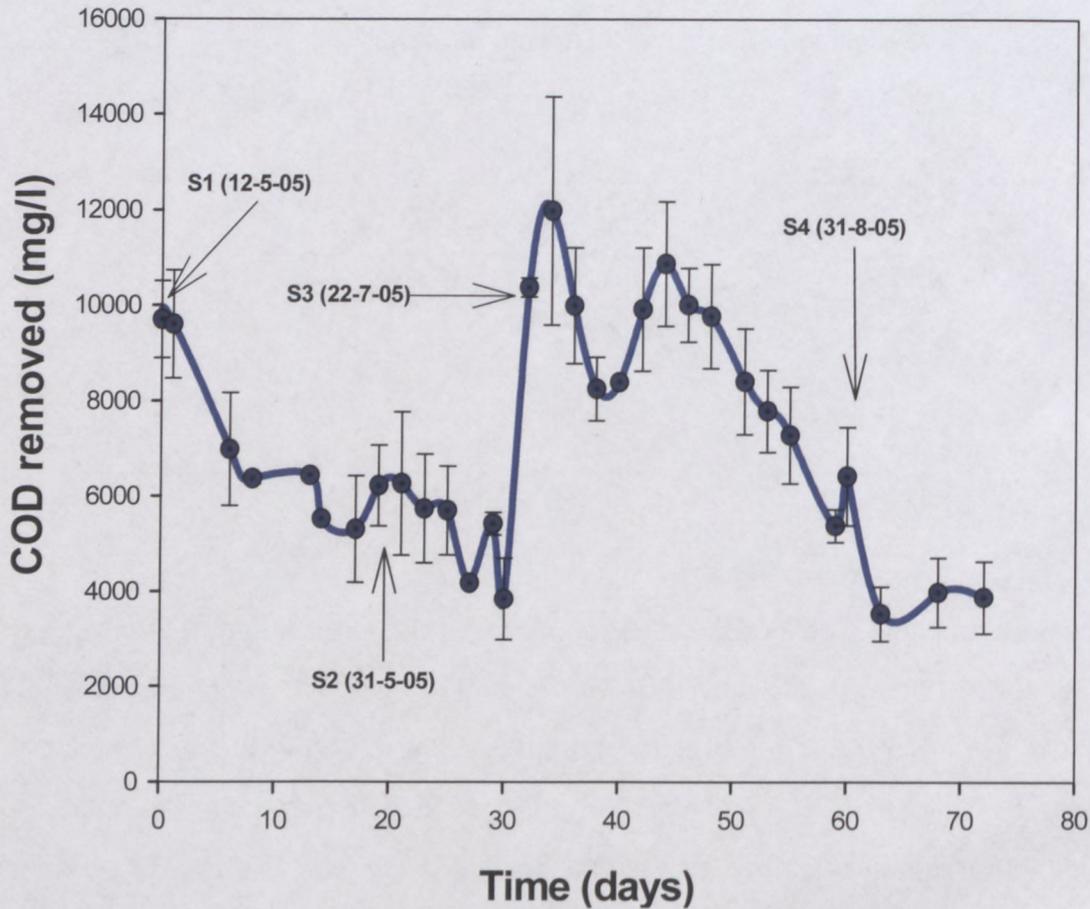
Figure 4.7 COD change vs. time for treated OMWW using RBC 2

Figure 4.8 and Table 4.4 show the phenol degradation data for OMWW in RBC 2. S1 started with an initial phenol value of 195.5 mg/L. After approximately 2 days, a significant drop in phenol value (+/-100 mg/L) representing almost 50% of the initial amount was removed. However, this was accredited to a possible experimental fault. During the continuous operation of the RBC, a gradual decrease in phenol curve can be observed (13 mg/L/day). After 20 days of treating S1, the lowest phenol value obtained was 93.2 mg/L, representing a degradation of 53%. The percentage degradation obtained for S2 was 24% after 10 days of treatment (refer to Table 4.4). S3 started with an initial phenol value of 98.8 mg/L. Between Day 25 and 30, one can observe a slight decrease in phenol levels (Fig 4.8). Beyond day 30, phenol levels stayed constant with little fluctuation until Day 52. A major drop in phenol was observed between Day 53 and 56. The percentage degradation obtained for S3 after 30 days of treatment was 33%.
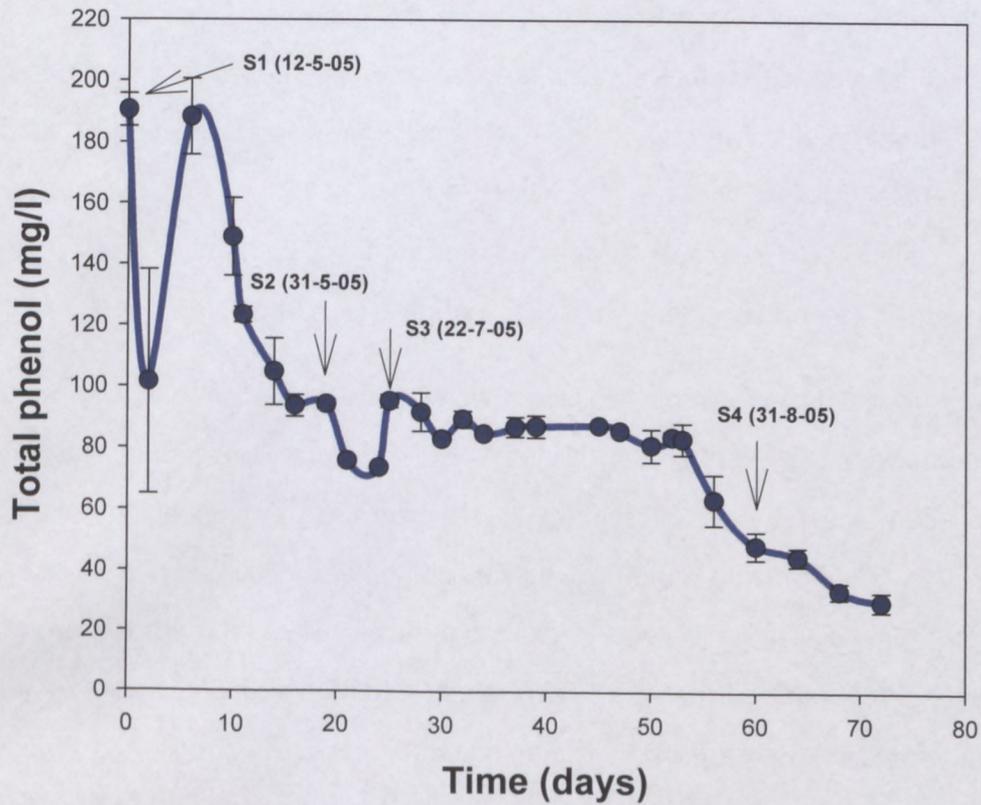
Figure 4.8 Total phenol concentration as function of time for treated OMWW using RBC 2.

The last sample (S4) had an initial phenol contain of 58.6 mg/L. After two weeks of treatment, one can observe a decrease in phenol levels from day 60 to 75. The percentage phenol degradation obtained for S4 was 45% and the average phenol removed for all the 4 samples ran in RBC 2 was 39% (Table 4.4).

Sampling period	Total Phenol (mg/L)		% degradation
	start	end	
S1	195.5	93.2	53
S2	98.4	75	24
S3	98.8	66.2	33
S4	58.6	32.4	45
Average	112.8	66.7	**39**

Table 4.4 Total phenol values obtained per sample of OMWW.

The variation in the COD and phenol values obtained from both samples (TOWW and OMWW) reflected the inconsistency of the olive wastewater. These differences in COD and phenol could be due to factors such as the olive harvest loads and different processing steps/stages of table olive and olive oil extraction. An average decrease in COD of 55% and 46% for TOWW and OMWW was achieved, whereas 51% and 39% was obtained for total phenol reduction. These results suggested that the RBCs system was indeed performing at a reasonable efficiency, considering this was a single-stage RBC setup.

In this study, the OWW samples collected were artificially enriched with selected nutrients, a decision informed by literature, as OWW's composition can vary significantly with regards to organic load (see Table 2.2 in Chapter 2). The purpose of enriching the wastewater was to ensure the continuous operation of the RBCs. Fadil *et al.* (2003) cited that OWW does not generally contain sufficient N and P for an adequate aerobic purification process. Data from Burton (2004) confirmed that both TOWW and OMWW (Table 2.2) were low in N and P. It was therefore necessary to supply the aerobic indigenous biofilms in the RBCs with N and P for optimal growth and an effective aerobic biodegradation of the OWW.

The results of this study are similar when compared to other treatment methods used in olive wastewater bioremediation. For example, Saez *et al.* (1992) obtained 43% reduction in phenols reduction of OWW after 90 days of treatment using evaporation ponds. Borja *et al.* (1995) obtained 63.3% and 65.6% reductions in phenols and COD, respectively, by using *G. candidum* in their study. On the other hand, Hamdi *et al.* (1991) reduced the COD by 52.5% using *A. niger* in aerobic conditions. Hayek *et al.* (1996) reduced the COD of OWW by 75% using up-flow anaerobic sludge blanket (UASB) reactor. Moreover, Scioli and Vollaro (1997) reduced the COD by 80% using yeast, and lastly, Israilides *et al.* (1997) reduced COD and phenols by 41 and 50% after 1 h of electrolysis respectively, while after 10 h of electrolysis, the reduction increased to 93 and 99%.

4.3   OWW Biofilms Response to Detergent Exposure

To evaluate the sustainability of the RBCs biofilms, a number of detergent treatments representative of practices that would occur at an olive production plant were evaluated to determine their effect on the development and survival of the RBC biofilm and the ability of the indigenous TOWW and OMWW biofilms to recover after the detergent exposure. Biofilms were treated with NaOH and several detergents with respective concentrations commonly used at both olives processing plants.

4.3.1 Determination of percentage area coverage using modified Pedersen's devices

Biofilms were allowed to grow for one week on microscope slides within the RBCs and thereafter were subjected to treatment with NaOH, Robot® and Contrabac® at 2.5% concentration (for TOWW) as well as Removil® and Limex® at 2.5% concentration for OMWW, respectively. Fig. 4.9 shows the results obtained after TOWW RBC biofilms have been exposed and allowed to recover from NaOH and detergent treatments over a period of time.

TOWW biofilms covered approximately 25% of the microscope slides inserted in the RBC 1 after ca. 7 days (Controls in Fig. 4.9 A, B and C). Once the biofilms were treated with NaOH, there was a clear drop in percentage coverage (Day 0, Fig 4.9 A). After allowing the stressed biofilms to recuperate from the shock within 3 to 4 days, a further decrease in biofilm coverage was observed to almost 12% coverage compared to the initial percentage area covered. From day 5-8, an increment in percentage area covered was noted, indicating that biofilms were recovering from the shock treatment. After 12 days, the TOWW biofilms almost fully recovered from the NaOH exposure and reached its initial percentage coverage of about 25%. Although the data for NaOH treatment were not statistically significant, the general trend observed was that NaOH initially kills off the biofilm population, but biofilms fully recovered in less than 2 weeks. From this, it can be deduced that NaOH does potentially pose a major threat to the RBC bioremediation process as the biofilms are sensitive to 2.5% NaOH treatment.
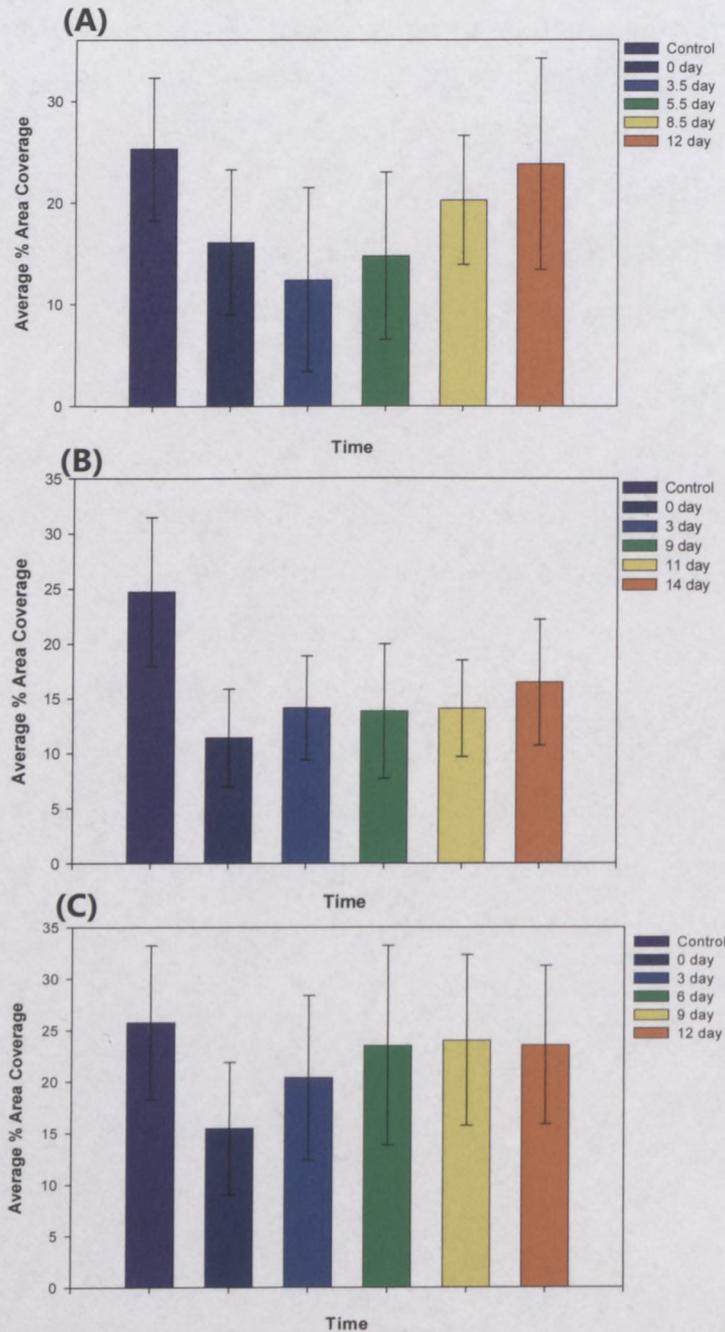
Figure 4.9 Percentage area coverage of TOWW biofilms exposed to (A) NaOH, (B) Robot® and (C) Contrabac® *vs*. time.

Fig. 4.9 (C) shows a similar pattern when TOWW biofilms are treated with Contrabac®. Directly after exposure to 2.5% Contrabac® a drop in % area coverage can be seen (although not statistically significant). TOWW biofilms recovered faster after the initial exposure to Contrabac®, recovering to initial levels of coverage after just 6 days. It could be concluded from these results that Contrabac® had the least destructive effect on TOWW biofilms.

As can be seen in Fig. 4.9 (B), when exposed to 2.5% Robot® detergent, there was a significant drop in biofilm percentage area coverage from the initial 25% to 12% after initial exposure (0 day) to the detergent. From day 3 to 14, the TOWW biofilms recovered only partially to a maximum of 16% area coverage. It would appear from these results that Robot® detergent had the most severe effect on TOWW biofilms, which did not recover to its original percentage area

coverage even after 14 days of exposure. Therefore, discharged TOWW containing low concentrations of Robot® detergent utilized during factory cleaning might influence the RBC bioremediation process of the TOWW.
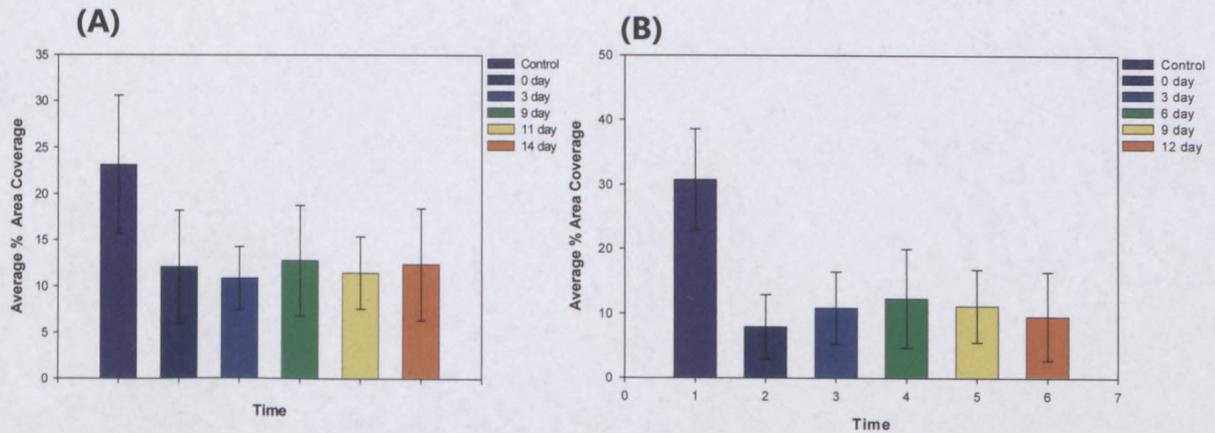
Figure 4.10 Percentage area coverage of OMWW biofilms exposed to (A) Removal® and (B) Limex® vs. time.

The strong alkaline detergents Removil® and Limex® detergents were used to stress OMWW biofilms within RBC 2 and results obtained are represented in Figure 4.10. Figure 4.10 indicates that the initial biofilm formation without stress application (Control) was able to grow and reach a percentage area coverage of approximately 24 -30% within 7 days. After exposure to the Removil® and Limex® detergents, a significant decline in percentage covered area (50% and 80% for Removil® and Limex®, respectively) were observed. Neither the Removil® nor Limex® treated OMWW biofilms fully recovered even after 14 days. The data suggest that both these two strong alkaline detergents could be a threat for the RBC biofilm during the bioremediation processes of the OMWW.

In general, from the percentage area coverage experiments in both TOWW and OMWW, the following general observations can be made: None of the treatments resulted in a 100% removal of the biofilms from the microscope slides. This is an expected result, as it has been shown by other researchers that biofilms show an enhanced resilience to detergents and antimicrobial agents (Flemming *et al.*, 2007; More *et al.*, 2012). Furthermore, the strong alkaline detergents Removil® and Limex® as well as Robot® had a more severe effect on biofilm recovery after exposure, while biofilms treated with NaOH and Contrabac® showed an initial reduction in percentage area coverage, but an almost full recovery within 14 days after exposure.

4.3.2 OWW biofilms detergent exposure using microtiter plate assay

To validate the percentage area coverage data (section 4.3.1), TOWW biofilm's sensitivity to NaOH and detergents (Tetrasheen®, Ordet SC®, Sanitizer HA®, Alkalizer 485®, Contrabac® and Robot®) was also evaluated using and microtiter plate experimental design. The percentage

biofilm removal of 0, 1 and 2 day old biofilms obtained during the assay is tabulated below (Table 4.5) and graphical representation of the data in Fig. 4.11.

Table 4.5 % Biofilm removal after detergent treatment.

Detergent	2 Hour Old			24 Hour Old			48 Hour Old		
	1%	5%	10%	1%	5%	10%	1%	5%	10%
Tetrasheen®	4%	35%	49%	3%	37%	49%	3%	31%	61%
Ordet SC®	33%	24%	59%	37%	6%	28%	2%	33%	37%
Sanitizer HA®	32%	28%	29%	28%	3%	13%	15%	17%	22%
Alkalizer 485®	44%	47%	44%	23%	37%	43%	13%	24%	63%
Contrabac®	78%	76%	104%	32%	37%	43%	46%	30%	26%
Robot®	40%	59%	65%	7%	44%	55%	3%	38%	52%

red text = decrease, green text = increase

The results obtained show that in general, all of the detergents, except Contrabac® had a significant removal effect of TOWW biofilms from the microtiter plate surface. In the case of Contrabac®, almost all the treatment scenarios had a stimulating effect on biofilm development. Furthermore, a trend was observed where two of the detergents (Ordet SC®, Sanitizer HA®) at the lowest applied concentration (1% [v/v]) also showed a stimulatory effect on TOWW biofilm development. This could be an indication that TOWW biofilms have acquired resistance to these detergents, perhaps due to previous exposure to sub-inhibitory concentrations. The biofilm microbes could potentially have evolved through genetic exchange to acquire the metabolic capacity to use some of the compounds within the detergents (at sub-lethal concentrations) as a nutrient source. Lastly, for most of the detergents, except Contrabac®, increased biofilm removal was also observed with increased concentrations of the detergents (from 1-10%).

The age of the biofilm (from 2 to 48 hours) has also, according to our results, no significant effect on the stability or resilience of the biofilm against the detergent exposure. Microbial cells closest to the liquid–biofilm interface of a biofilm will be the least protected by the EPS against antimicrobial agents. The cells in the intermediate regions will be protected by the outermost layers of cells due to the diffusion barrier provided by the EPS matrix. Finally, sub-populations of biofilm cells might exist that express a population-specific resistance mechanisms, increasing their chances for survival. Older biofilms are theoretically more resistant to antimicrobial or detergents due to the increased diffusion barrier provided by EPS (Evans, 2000; Dufour *et al.*, 2012). This was not observed in the TOWW biofilms in this study. Complete biofilm removal by any of the detergents was also not observed.

Figure 4.11 Results of TOWW biofilm removal with 1%, 5%, and 10% of detergents (C= control, no addition of detergent) with (A) 2 hour old biofilm (B) 24 hour old biofilm and (C) 48 hour old biofilms. Tetrasheen® (D1), Ordet SC® (D2), Sanitizer HA® (D3), Alkalizer 485® (D4), Contrabac® (D5) and Robot® (D6), T0= 2 hour old biofilm, T1= 24 hour old biofilm, T2 = 48 hour old biofilm.

4.3.3 Quantification of viable vs. dead cells in TOWW biofilms after detergent treatment using flow cells

TOWW biofilms were cultured in flow cells for 8 days followed by pumping TOWW spiked with 1% Alkalizer 485® and Robot® detergents, respectively. The ratio of viable vs. dead microbial cells within the TOWW was then quantified by fluorescent staining and image analysis (Fig. 4.12, Fig. 4.13). From visual inspection of the microscopic fields (Fig. 4.12), it can clearly be seen that the treatment of both 1% Alkalizer 485® and Robot® detergents had a significant effect on the ratio of nonviable microbial cells (red) over viable microbes (green cells) in the TOWW biofilms.
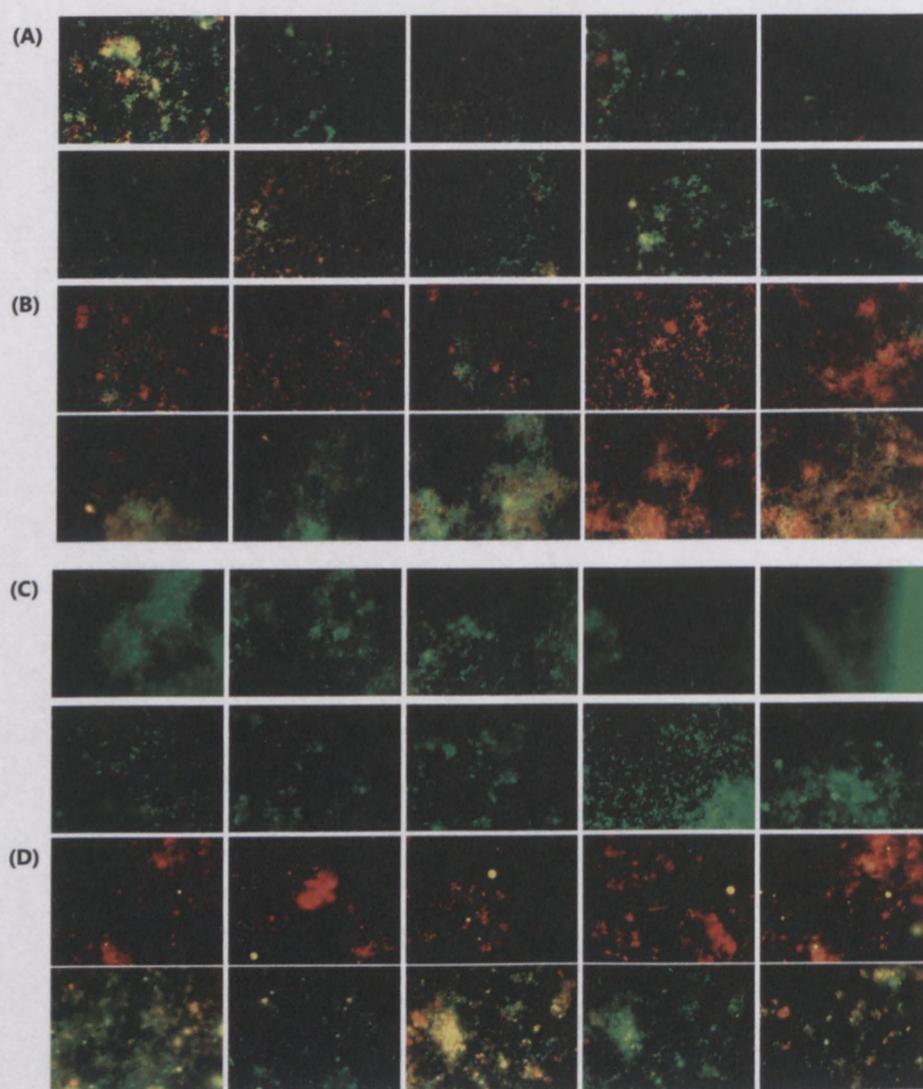
Figure 4.12. Epifluorescent images of 10 fields of flow cell TOWW biofilms prior to detergent treatment (A and C) and after 20 hours treatment with (B) 1% Alkalizer 485® spiked TOWW and (D) 1% Robot® spiked TOWW.

Quantitative analysis of the micrographs (Fig. 4.13) indicated a 4-fold increase in dead microbial cells after treating the TOWW biofilms with 1% Alkalizer 485® and a 9 fold increase in dead microbial cells in TOWW biofilms treated with Robot®. These results also translated

into a 2.79-fold and 16.8-fold increase in the red-to-green ratio of cells with 1% Alkalizer 485® and Robot®, respectively (Fig. 4.13), while both detergent treatments led to a general decrease in percentage area coverage of the TOWW biofilms (however, not statistically significant). It can be concluded that both these detergents had a negative impact on the viability of the microbes within TOWW biofilms. This data confirms the trends observed with the % area coverage and microtiter plate studies for biofilm attachment, indicating that detergents used in the olive processing industry could potential negatively impact on the RBC bioremediation process. In light of this data, consideration should be given by olive processing plants to separate olive processing wastewaters from water streams generated during hygiene practices of the plant if biofilm based bioremediation strategies are considered as a treatment method.

(A)

(B)

Control (Alkalizer 585), 8 days

red	std dev	green	std dev	% area
28.29	21.11	33.51	21.51	17.90
2.04	2.39	6.77	7.01	11.70
3.08	1.95	6.67	3.71	18.30
4.67	7.93	12.65	11.62	12.50
3.13	2.12	3.94	2.72	15.30
3.00	1.71	4.63	2.17	16.70
15.48	9.01	12.19	6.36	25.60
4.05	4.79	11.79	9.22	22.90
11.89	7.71	22.42	13.92	38.00
1.04	1.17	8.18	6.31	8.00
7.67	5.99	12.28	8.46	18.69

Red to green ratio: 0.6246

1% Alkalizer 585 stress

red	std dev	green	std dev	% area
15.33	9.94	3.46	5.18	10.90
8.43	4.50	1.31	1.07	9.10
8.97	6.38	4.33	6.58	9.20
28.13	15.08	4.45	3.96	8.40
44.74	27.22	9.58	8.92	27.10
23.44	14.70	16.58	14.02	16.90
8.11	7.56	21.52	20.68	7.60
42.08	25.62	57.53	30.85	31.10
53.44	22.00	15.20	11.22	13.20
85.33	16.43	51.13	15.30	31.30
31.80	14.94	18.51	11.78	16.48

Red to green ratio: 1.7181

Control (Robot), 8 days

red	std dev	green	std dev	% area
2.01	1.15	46.39	29.59	26.50
1.82	0.69	31.82	13.25	31.90
3.90	3.93	32.42	16.46	36.90
1.16	0.53	17.46	12.62	23.60
2.45	1.79	79.39	85.74	20.70
1.39	0.41	20.51	7.89	24.20
0.86	0.37	13.10	7.52	17.00
1.28	0.38	20.77	9.00	22.80
6.29	5.35	54.42	28.12	48.60
4.88	2.61	39.26	17.13	30.10
2.60	1.72	35.55	22.73	28.23

Red to green ratio: 0.0732

1% Robot stress

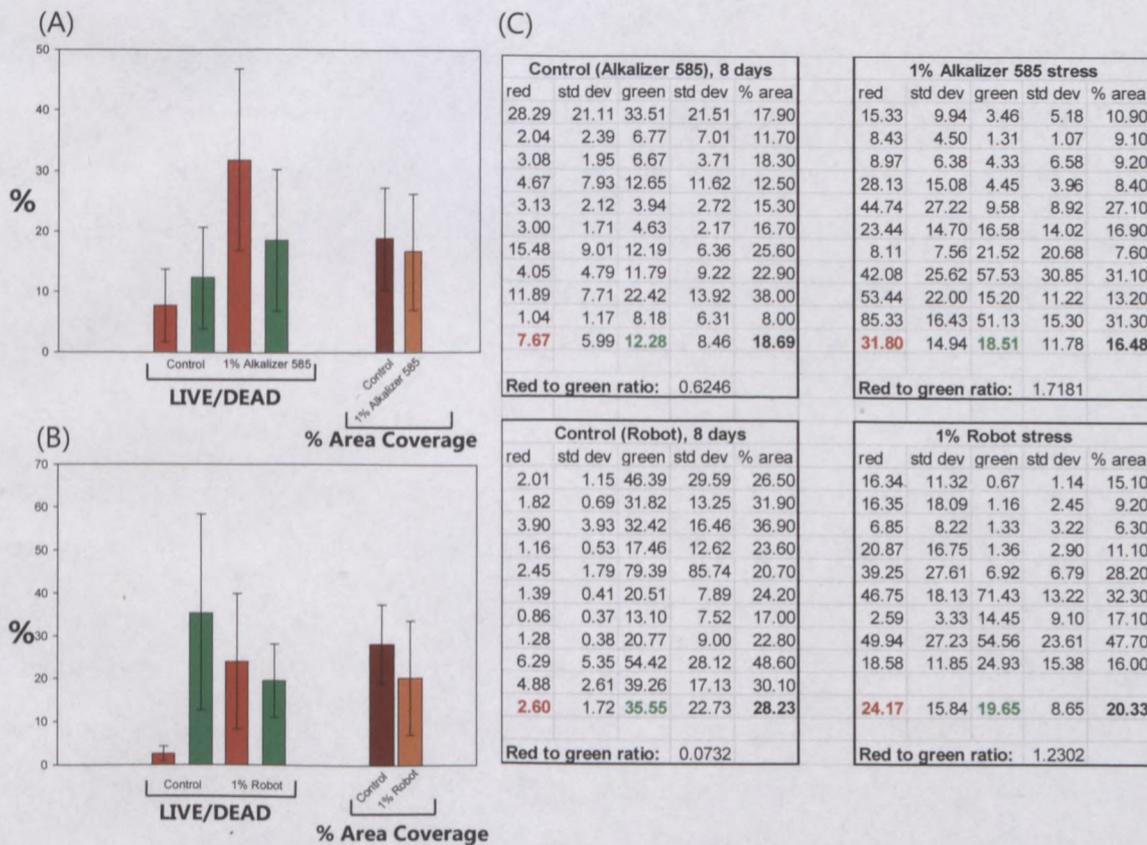| red	std dev	green	std dev	% area
16.34	11.32	0.67	1.14	15.10
16.35	18.09	1.16	2.45	9.20
6.85	8.22	1.33	3.22	6.30
20.87	16.75	1.36	2.90	11.10
39.25	27.61	6.92	6.79	28.20
46.75	18.13	71.43	13.22	32.30
2.59	3.33	14.45	9.10	17.10
49.94	27.23	54.56	23.61	47.70
18.58	11.85	24.93	15.38	16.00
24.17	15.84	19.65	8.65	20.33

Red to green ratio: 1.2302

(C)

Figure 4.13. Data analysis of Live/Dead (green/red) stains and percentage area coverage of TOWW biofilms before and after treatment with 1% Alkalizer 485® and Robot®.

CHAPTER 5

Final Discussion and Conclusions

Olive wastewater (OWW) treatment appears to be a tough and costly problem in South Africa and the rest of the world because of the toxic nature of the effluent. Pollution by OWW is a result of the high organic loads (COD) and the phenolic compounds released during the processing stages of olives. The most prevailing problem with OWW is the recalcitrant nature of some of these compounds, especially the polyphenolic fraction and the severe antimicrobial and phytotoxic effect most of the phenolic compounds in OWW. Various remediation strategies have been developed and investigated for the treatment of table olive wastewater (TOWW) and olive mill wastewater (OMWW). These methods include various physico-chemical and biological methods, including aerobic and anaerobic processes. Many of the physico-chemical and biological strategies have been shown to alleviate the problem of OWW, but there are inherent disadvantages associated with these methods. The most relevant disadvantage is the high cost and technical difficulty of some of these methods that were developed with the large olive industries of the world, mostly in the Mediterranean countries, in mind. Small olive processing plants would not be able to afford and implement some of these remediation technologies, and need tailor-made practical solutions to deal with their OWW.

In this study, the application of a rotating biological contactor in combination with indigenous OWW biofilms were evaluated as such a practical solution for the South African olive industry. The ease of implementation and operation of rotating biological contactors are well-suited for small olive processing plants. Furthermore, biofilms in nature does not develop randomly, rather the species inhabiting biofilms are chosen by a process of "natural selection" that enable biofilms to optimally utilise available nutrients and execute survival mechanisms. Indigenous biofilms that develop in OWW will therefore be the most suitable to survive in the OWW environment with maximised metabolic abilities to use the chemical compounds in OWW as nutrients.

This study investigated a lab-scale RBC in conjunction with naturally grown OWW biofilms as a method to bioremediate OWW from two commercial olive processing farms in the Western Cape (Buffet Olives and Vesuvio Estate). Indigenous biofilms from TOWW and OMWW were shown to develop on RBC discs and reach maturity in relatively short periods of time, using

visualisation techniques such as scanning electron and epifluorescent microscopy. The establishment of biofilms in TOWW and OMWW was also quantitatively measured using image analysis technique, to show percentage area coverage on a microscope slide surface.

The bioremediation potential of the small-scale RBC and natural biofilms was also evaluated for TOWW and OMWW over a period of 120 and 72 days, respectively. During this part of the study, COD and total phenol levels were regularly monitored. Significant reductions in COD and total phenol levels were observed, with bioremediation levels similar to what was previously reported for other biological and physic-chemical methods. COD levels were reduced to an average of 55% and 46% for both TOWW and OMWW, whereas 51% and 39% reduction levels were achieved for phenolic compounds. Taking into account the basic setup of the small-scale RBC in this study, the results obtained are indeed promising and warrants further studies using more advanced RBC configurations.

The resilience of biofilms to certain operations at Buffet Olives and Vesuvio Estate was further evaluated. The effect of specific detergents used for olive plant sanitation and NaOH as lye treatment on the development and stability of biofilms were investigated using quantitative methods, including a percentage area coverage study, a microtiter plate study for biofilm attachment as well as flow cells combined with fluorescent staining. Results from the three different methods confirmed the observation that some detergents and NaOH impede biofilm attachment and survival that could potentially affect the bioremediation efficiency of OWW.

This research makes a contribution to the development of a tailored biological treatment technology for OWW in South Africa. Further research and development of this technology will greatly benefit the South African olive industry by establishing its international status as environmentally benign sector, increased international competitiveness and ensure sustainability as a fast growing agricultural sector.

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