INVESTIGATION INTO THE BIOREMEDIATION AND BENEFICATION OF OLIVE-DERIVED WASTEWATERS FROM THE WESTERN CAPE

By

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good candidates as value-added products that can be obtained from the wastewaters. Natural antioxidants have significant commercial potential, and there is much scope their industrial application, for example, in the pharmaceutical, food processing and cosmetic sectors. The higher molecular weight phenolic components of the wastewaters include tannins, lignins and humic substances, which are particularly resistant to biodegradation and are predominantly responsible for the dark colour of the wastewaters.

The recovery of the phenolic antioxidants from fermentation brines was successfully performed at laboratory scale using a membrane-assisted solvent extraction process. This process has several advantages over conventional extraction processes such as liquid/liquid extraction and solid phase extraction. The process is similar to liquid/liquid extraction, except that the aqueous (wastewater) and extracting (solvent) phases are not mixed; they are separated by a membrane through which the desired solutes are extracted. This means that subsequent separation of phases (as in liquid/liquid extraction) is not required. The overall mass transfer coefficient for the process was determined through the use of an appropriate mass transfer model, which allows for design of such extraction systems. The main product of extraction was the valuable phenolic antioxidant hydroxytyrosol, which can be recovered from the wastewater in quantities of up to 1 g per litre of wastewater.

Because of the high phenolic content and organic load, the olive wastewaters are resistant to biodegradation, although there are numerous microorganisms capable of metabolising phenolic compounds as a carbon and energy source. A preliminary screening process was performed to identify microbial species that would be appropriate for the treatment of fermentation brines. These included wild-type isolates obtained from evaporation ponds, pure cultures obtained from various culture collections, and mixed, acclimatised microbial consortia. The various species were evaluated on the basis of their ability to reduce the organic load and total phenolic content of the wastewaters. Certain of the microbial species investigated were able to reduce total phenol content and organic load of the wastewater by up to 80%, but mixed cultures are perceived to be the best option for wastewater treatment, as these do not require aseptic conditions for cultivation. For the fermentation brines

investigated, phenol degradation generally occurred through a polymerisation and humification process.

A small-scale submerged membrane bioreactor was then developed, hydrodynamically characterised and modelled, and subsequently used for degradation of the brined olive wastewater. The reactor was designed such that the membrane unit was located in the draught tube of a conventional airlift reactor, which resulted in a high liquid velocity past the membrane surface, and thus in minimal membrane fouling. The versatility of the reactor was demonstrated through the cultivation of single-cellular yeast and bacteria, filamentous fungi and mixed microbial culture, while degradation rates were generally better than achieved in the preliminary shake flask degradation experiments. The reactor was shown to be able of treating black olive brine wastewater at an influent total phenol concentration of 800 $mg.L^{-1}$ and an organic loading rate of up to 420 $mg.L^{-1}.day^{-1}$. This was sustained for 9 days, until overload occurred, due to increasing the organic loading rate.

Since this was the first research project into olive wastewaters produced in South Africa, the scope of the project was broad, and recommendations were made for further research in several directions. Most importantly, the development of small-scale treatment systems that could be used on site would be of great benefit to olive and olive oil producers, as they are often remote and do not have access to appropriate treatment facilities. In addition, the combination of unit operations, such as an extraction system and a biological degradation system, would allow for the recovery of a valuable product that would offset the cost of producing a treated effluent.

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OUTPUTS ARISING FROM THIS WORK

Conference presentations:

van Schalkwyk, A., Garcin, C.J., Burton, S.G. and Cowan, D.A. UV-mutagenesis as a means of improving the biotransformation potential of bacteria isolated from olive oil wastewater. SASBMB National conference, Pretoria, 2003.

Garcin, C.J., Burton, S.G. and Cowan, D. Identification, extraction and quantification of polyphenolic antioxidants from olive fermentation brines. SASBMB National conference, Pretoria, 2003.

Garcin, C.J., Burton, S.G. and Cowan, D. Identification, extraction and quantification of polyphenolic antioxidants from olive fermentation brines. Cape Biotech, 2003

Werner, C., Garcin, C.J., Cowan, D. and Burton, S.G. The biodegradation of the constituents of olive fermentation wastewaters using locally isolated microorganisms. Cape Biotech, 2003.

Garcin, C.J. and Burton, S.G. Selective recovery of low molecular weight phenolic antioxidants from olive production wastewaters using membrane-assisted liquid extraction. Water Institute of South Africa (WISA) Biennial conference and exhibition, 2004, Cape Town, South Africa

Papers in preparation:

Garcin, C.J, Cowan, D. and Burton, S.G. Quantification, extraction and antioxidant activity assessment of hydroxytyrosol (3,4-dihydroxyphenylethanol) from olive fermentation brines.

Garcin, C.J., Cowan, D. and Burton, S.G. Extraction of antioxidants from olive wastewaters using membrane-assisted solvent extraction.

Garcin, C.J., Cowan, D. and Burton, S.G. Development of a submerged membrane bioreactor for high strength wastewater treatment.

Werner, C., Garcin, C.J. and Burton, S.G. Development of a bioreactor system for bioremediation of wastewater from olive production.

Technical report:

Garcin, C.J., Werner, C., van Schalkwyk, S., Burton, S.G. and Cowan, D. (2005). Development of a customised bioreactor for the treatment of organic-containing effluents and conversion of constituents to high value chemicals. Report K5/1361 to the Water Research Commission of South Africa.

Business plan for the National Innovation Competition:

Garcin, C.J. Novel Extraction Technology for the Recovery of Valuable Components from Agro-industrial Wastewater Effluents, National Research Foundation, 2004. (Finalist)

CHAPTER 1: LITERATURE REVIEW AND RESEARCH OVERVIEW

1.1 OLIVES AND OLIVE OIL

Archaeological evidence suggests that active management and cultivation of olive trees started during the Bronze Age (c. 3000BC), although their usage and exploitation for firewood dates further back to Mesolithic times (c. 8000BC) (Terral, 2000). Homer's "Liquid Gold", "Tree of Light", Noah's olive branch and anointing oil in the Bible, these and other such historical references to the olive tree and oil are plentiful. It was the embalming oil of choice for the ancient Egyptians. In ancient Greece such was their reverence that only chaste men and virgins were allowed to cultivate, harvest and process olives. The species *Olea europea* (the commonly cultivated olive) is derived from wild olive species *Oleaster*, which occurs extensively throughout the Mediterranean and Africa. Thousands of years of cultivation and crossbreeding have led from the wild type to today's myriad varieties (Flanagan and Hildenbrand, 2003).

Olive oil is considered to have superior organoleptic properties compared to other vegetable oils; this is ascribed to its unique fatty acid composition and low molecular weight aromatic phenolic components. Olive oil has a remarkable shelf life, in terms of resistance to oxidation or rancidity. It is known to have beneficial effects upon human health as in the "French paradox" of a high saturated fat diet yet the relative good health of the nation. Reports on the beneficial aspects of the "Mediterranean diet", and olive oil in particular, have increased dramatically in recent years. In Greece, Italy and Spain, for example, the incidence of cardio-vascular disease and cancer is significantly lower than in other Western countries (Kris-Etherton *et al.*, 2002). Olive oil is renowned in folklore as having numerous medicinal properties and is still used for the treatment of burns, wounds and diseases in some countries.

The Mediterranean diet is largely vegetarian and olive oil is the principal source of fat. It has a unique fatty acid profile consisting of predominantly monounsaturated fats (56-84% oleic acid, 3-21% linoleic acid). However, this alone cannot account for the beneficial aspects of the Mediterranean diet, because oleic acid is also one of the predominant fatty acids in animal foods such as poultry and pork. The percentage of oleic acid in the Mediterranean diet is only slightly higher than in other Western diets such as the American diet (Visioli and Galli,

1998). Abundant research has indicated that it is the high proportion of biologically active aromatic phenolic compounds in olives and olive oil that are at least partially responsible for its beneficial effects, in common with similar compounds in fresh fruit and vegetables and (especially) red wine. These compounds are known to be powerful antioxidants and free radical scavengers, and have several other unique biological activities, as discussed in section 1.3.

Approximately 97% of the global olive industry occurs in the Mediterranean regions, but South Africa has a climate well suited to the production of olives, and is increasingly favoured as a region for future development. Annually South Africa imports one million litres of olive oil and produces approximately 500 000 litres. South African oils are considered to be of high quality and have won numerous international awards, while equipment has already been purchased for increasing production to twice the current amount.

South African olives are often handpicked, which makes for good table olives, as the ripe fruit bruises easily. Mechanical harvesting is known to damage the fruit and results in lower quality table olives. As a consequence the South African table olive industry is large and accounts for approximately 40% of the local harvest; globally this figure is only about 10%, with the rest being pressed for oil. Approximately 4000 tons of tables olives are processed annually, although this is expected to increase to 20 000 over the next ten years.

Most of the local production occurs in the Western Cape, where there are about 50 major producers with 1500 hectares under cultivation, although this is increasing rapidly. The first olive trees in South Africa were planted in the days of Jan van Riebeeck (c. 1652), however the olive industry only took root seriously in South Africa in the late 1800's. Today the industry is burgeoning.

1.2 WASTEWATERS ARISING FROM THE PRODUCTION OF BRINED OLIVES AND OIL

Olives are seasonal fruit, they spoil (ferment) quickly, and hence they are processed as soon as possible after harvest, generally within a few hours. Olives, oil and the associated wastes are thus also seasonally produced which is problematic, because waste treatment plants need to be designed and scaled to meet large temporary waste flow requirements, and then stand

idle for several months. Alternatively, smaller scale systems can be used, but then large storage facilities are required. In addition, wastewater treatment facilities are expensive and are often not economically feasible for small producers. Increasingly stringent wastewater legislation and water costs are, however, forcing producers to confront the issues of treatment, disposal and reuse. This problem has led to the development of regional treatment facilities in the Mediterranean regions, where critical densities of producers permit economy of scale. Such facilities take a holistic approach to waste treatment, and if not profitable, are at least cost-effective (Gurbuz *et al.*, 2004; Vlyssides *et al.*, 2004).

Traditionally, olive oil was (and in some instances still is) produced through a discontinuous filter pressing method. However, most oil is now produced through a continuous centrifugation process. In brief, the process is as follows: after harvesting the olives are washed and then fed into a crusher or mill; thereafter they are soaked in warm water and gently stirred in order to release the oil from the pulp (referred to as malaxation). This mixture is then fed into a centrifuge for separation. Centrifuges are either 2- or 3-phase. Two-phase centrifuges produce oil as one product stream and a mixture of pulp and water as another, while three phase centrifuges produce oil, pulp solids and process water as three separate streams. Two-phase centrifuges use significantly less water than 3-phase, but the waste stream is more concentrated (higher organic load) and is difficult to treat due to its high suspended solids content. The extraction is also slightly less efficient than 3-phase; some of the oil (up to 10%) is lost in the waste stream. In three phase systems the pulp solids (often referred to as olive husk) are relatively dry and are often reprocessed with an organic solvent to recover residual oil; the liquid nature of the 2-phase stream makes this difficult. Nonetheless 2-phase systems are becoming increasingly popular due to the water savings associated with this process. In both cases the wastewater streams contain the cellular vegetation waters from the olives as well as process water, and are commonly referred to as olive mill wastewater (OMW).

The large-scale production of table olives is a relatively simple process: after harvesting the olives are washed and then fermented for several months in a 10-15% NaCl solution. The fermentation brine becomes naturally acidic (pH 4-5) due to the diffusion of acidic phenolic compounds and other organic acids from the fruit into the brine (Brenes *et al.*, 1995); these conditions lead to the spontaneous growth of predominantly lactic acid bacteria, particularly *Lactobacillus plantarum*, and some yeast species (Brenes and de Castro, 1998; Ozay and

Borcalki, 1996; Chorianopoulos *et al.*, 2005; Duran Quintana *et al.*, 1999; Randazzo *et al.*, 2004). The lactic acid bacteria consume the sugars leached from the fruit and produce lactic acid, which maintains the low pH of the brines and prevents the growth of other pathogenic bacteria. After fermentation the olives are rinsed; the combined brine and rinsing waters are referred to as Olive Fermentation Wastewaters (OFW). Green olives are subject to an additional alkali pre-treatment with NaOH to extract and hydrolyse their natural bitterness, followed by one or two water washes (de Castro and Brenes, 2001; Brenes *et al.*, 1995). However, there are many variations of the basic table olive production process, and "tricks of the trade" are not readily disclosed by producers. OMW and OFW are similar in their darkly coloured phenolic nature, yet are different in composition (discussed in Chapter 2). Collectively they will be referred to simply as olive wastewater (OW).

The specific problem in the olive industry is that large quantities of this darkly coloured, phenolic-rich OW is generated during the production of olives and oil, up to 1.5 $m^3.ton^{-1}$ of olives for oil, and up to 20 $m^3.ton^{-1}$ for table olives, depending on the process (Benitez *et al.*, 1999). These wastewaters constitute a serious environmental pollution and disposal problem due to their high organic load, high phenolic content and acidity. The wastewaters have antimicrobial and phytotoxic properties (Sayadi *et al.*, 2000; Visioli *et al.*, 1999; Ramos-Cormenzana *et al.*, 1998; Garcia Garcia *et al.*, 1997; Borja *et al.*, 1996, 1995, 1992; Monteoliva-Sanchez *et al.*, 1996; Hamdi, 1992; Rodriguez *et al.*, 1988), as well as being toxic in aquatic environments (Isidori *et al.*, 2004). The quantity of these wastes produced in the Mediterranean regions was estimated to be of the order 3×10^7 $m^3.year^{-1}$ (Hamdi 1993). Considering that 1 m^3 of this waste has the same organic load as 100-200 m^3 of municipal sewage, the scale of the treatment and disposal problem becomes apparent (Sayadi *et al.*, 2000). The wastewaters are traditionally disposed of in evaporation ponds or, if suitable, are applied to agricultural land as fertiliser (discussed in section 1.4.4).

The wastewater has a very high polluting organic load, expressed as Chemical Oxygen Demand (COD) or Biochemical Oxygen Demand (BOD). The COD and BOD reach 250 and 100 $kg(O_2).m^{-3}$ respectively (Lesage-Meesen *et al.*, 2001; Hamdi, 1993). The main organic constituents responsible for these high values are sugars, volatile acids and polyalcohols, pectins, fats and particularly aromatic phenolic compounds, and higher molecular weight polyphenols or tannins (Rozzi and Malpei, 1996).

1.3 PHENOLIC COMPOUNDS FROM OLIVES: ANTIOXIDANT AND OTHER BIOLOGICAL ACTIVITY

All plants contain phenolic compounds; they are an extremely diverse group derived from the shikimate pathway and phenylpropanoid metabolism. The low molecular weight phenolic compounds form one of the main classes of secondary metabolites. They have a vast range of functions and structures, but are generally represented by an aromatic ring bearing one or more hydroxy substituents (Robards *et al.*, 1999). The phenolic compounds from olives, however, cover a large molecular weight distribution, and include high molecular weight polyphenolics and tannins; these are discussed later.

The low molecular weight phenolic fraction from olives is of particular interest, as these compounds are known to be powerful antioxidants with various biological activities (Ryan *et al.*, 2002; McDonald *et al.*, 2001; Visioli *et al.*, 1999; Baldioli *et al.*, 1996). The phenolics are, in fact, cytotoxic to humans, but only at concentrations far exceeding those attainable by habitual olive and olive oil consumption (Babich and Visioli, 2003). At low to moderate concentrations, the antioxidant properties of these olive-derived phenolics have been implicated as being particularly beneficial towards human health, e.g. in the prevention of cardio-vascular disease and cancer (Kris-Etherton *et al.*, 2002; Tuck and Hayball, 2002; Della Ragione *et al.*, 2000; Visioli and Galli, 1998).

Antioxidants perform many vital functions ranging from phytoprotection in plants, to protecting lipids in foodstuffs, to *in vivo* antioxidant activity in humans, scavenging of reactive oxygen species, acting as antagonists towards oxidative enzymes and influencing the expression of multiple genes (Finley, 2005). Possibly the most important function of antioxidants though, is the scavenging of free radicals which would otherwise have detrimental effects in the body. Primary amongst free radicals are the reactive oxygen species (for example O^{-}, O_3^{-} and OH^{-}). There is recognised involvement of reactive oxygen species in the onset of several human diseases (Aruoma, 1998). The antioxidants function by being able to donate an electron or proton, thereby quenching a radical, while themselves remaining stable, thereby terminating the chain reaction that is characteristic of free radical chemistry and its deleterious effects.

More than 50 aromatic phenolic compounds have been identified in olives, oil and the associated wastewaters and solid residues. Some of the most common are illustrated in Figure 1.1. This is by no means a comprehensive list, and there are many derivatives (glucosides etc.) of these compounds (Ryan *et al.*, 2002, 1999a, 1999c; Bendini *et al.*, 2003).

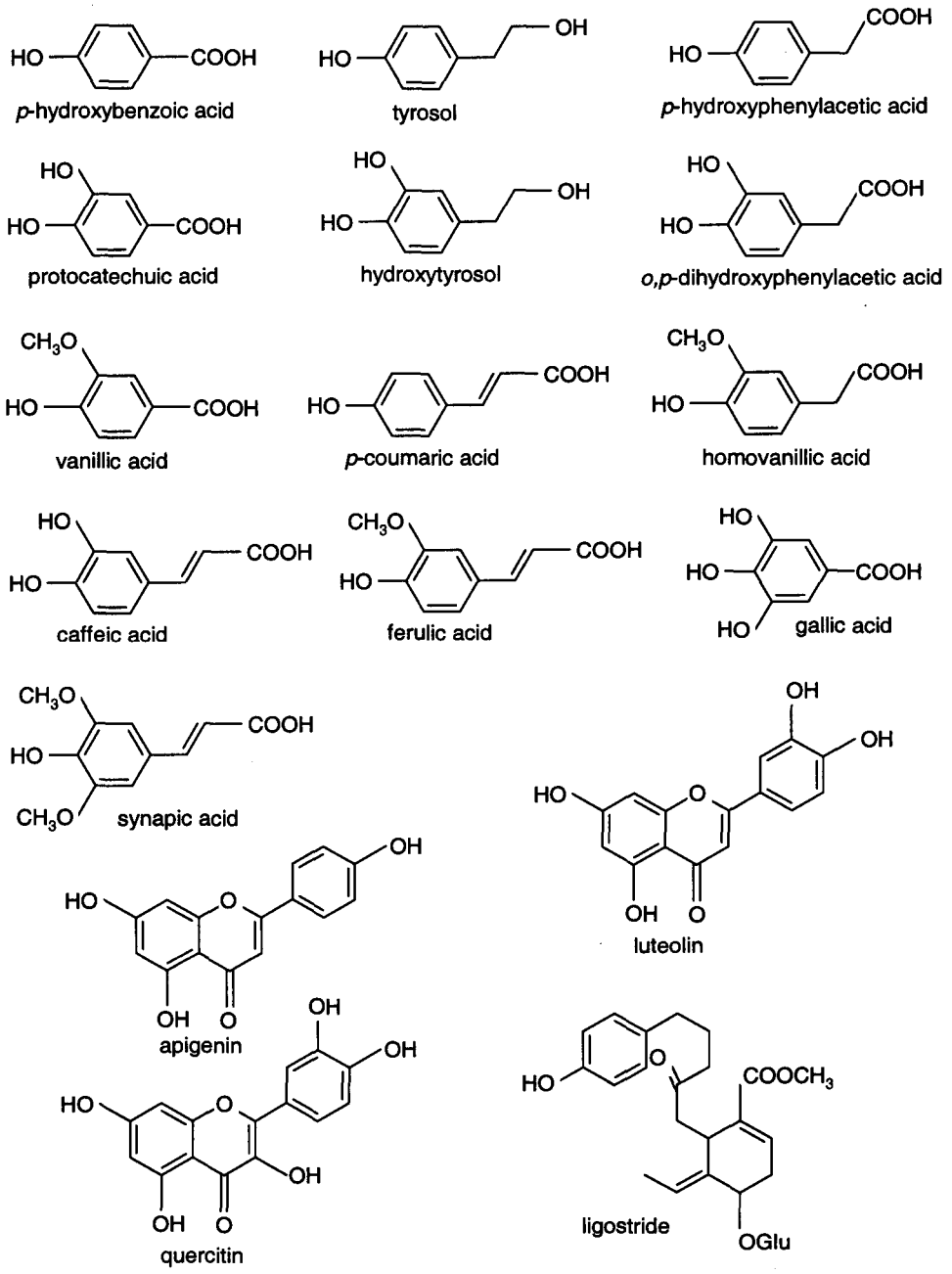


Figure 1.1: Structures of common phenolic compounds from olives.

In terms of abundance, the main low molecular weight phenolic compound occurring in the olive fruit is oleuropein (Ryan *et al.*, 2002, 1999b; McDonald *et al.*, 2001; Brenes and de Castro, 1998; Brenes *et al.*, 1995); it is responsible for the bitter taste of green olives. The concentration of this compound decreases as the olives mature from green to black; it is hydrolysed through breakage of the ester bond into hydroxytyrosol (3,4-dihydroxyphenyl ethanol) and elenolic acid glucoside (see Figure 1.2). Along with α-tocopherol (non-phenolic vitamin E), hydroxytyrosol and oleuropein are the main antioxidants in olive oil (Litridou *et al.*, 1997; Tasioula-Margari and Okogeri, 2001; Mulinacci *et al.*, 2001; Baldioli, 1996).

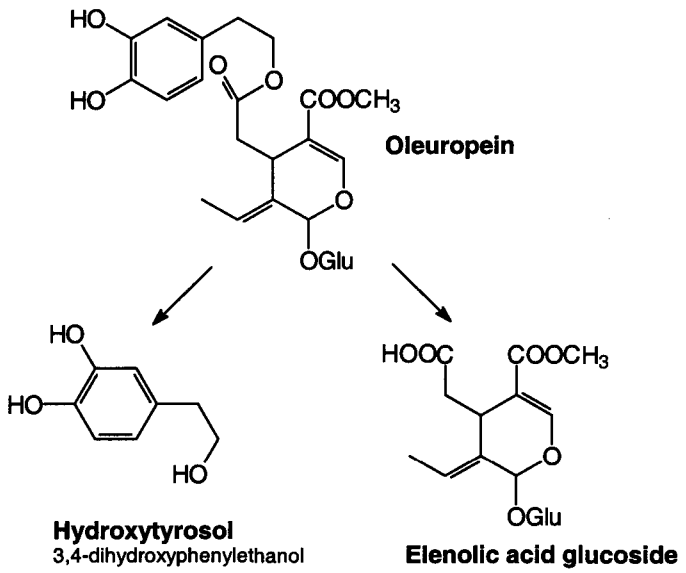


Figure 1.2: Hydrolysis of oleuropein

Hydroxytyrosol is an interesting molecule, due to its three-dimensional structure and associated biological activity. It is a natural metabolite of the compounds dopamine and L-DOPA (3,4-dihydroxyphenylalanine), which have diverse functions in the body, most notably as neurotransmitters (Hashimoto *et al.*, 2003). The *o*-dihydroxy structure confers stability to the radical form, and in conjunction with the ethanolic hydroxyl group participates in electron delocalisation, which is essentially what leads to it being a good antioxidant (Salah *et al.*, 1995).

The radical scavenging ability of hydroxytyrosol (and related compounds) has recently been evaluated in terms of structure-activity relationships by means of quantum chemical

calculations. The bond dissociation enthalpy of hydroxyl groups and ionization potential were calculated in order to predict the H-atom-donating and electron-donating abilities respectively. These results illustrate that due to their structure, catechol derivatives with the *o*-dihydroxy moiety (such as hydroxytyrosol) have lower bond dissociation energies and thus have better antioxidant potential than compounds lacking the dihydroxy grouping (Nenadis *et al.*, 2005).

The antioxidant and other biological properties of (particularly) hydroxytyrosol and oleuropein have thus been subject to intensive biochemical and pharmacological research, some recent reports of which are listed in Table 1.1.

Table 1.1: Pharmacological research related to hydroxytyrosol and oleuropein

Description	Reference
Radical scavenging/antioxidant activity	O'Dowd *et al.*, 2003; Moreno, 2003; Tuck and Hayball, 2002; Mc Donald *et al.*, 2001; Espin *et al.*, 2001; Visioli *et al.*, 1999, 1998; Baldioli *et al.*, 1996.
Antiviral activity	Micol *et al.*, 2005; Lee-Huang *et al.*, 2003.
Antibacterial (mycoplasmal) activity	Furneri *et al.*, 2004, 2002; Capasso *et al.*, 1999.
Anti-inflammatory effects	Miles *et al.*, 2005.
Melanoma/UV irradiative protection	D'Angelo *et al.*, 2005.
Erythrocyte (red blood cell) oxidative protection	Manna *et al.*, 1999.
Lymphocyte (white blood cell) auto-immune response	Palmerini *et al.*, 2005.
DNA oxidative protection	Quiles *et al.*, 2002; Armstrong *et al.*, 1997.
Prostate/urinary anti-mutagenicity	Malaveille *et al.*, 2004; Quiles *et al.*, 2002.
Colon anti-mutagenicity	Della Ragione *et al.*, 2000.
Inhibition of LDL oxidation (arteriosclerosis)	Covas *et al.*, 2000; Visioli *et al.*, 1999; Aruoma *et al.*, 1998.
Metabolism regulation	Polzonetti *et al.*, 2004.
Alleviation of heavy metal intoxication	Casalino *et al.*, 2002.
Nerve cell oxidative cytoprotection	Hashimoto *et al.*, 2003.

Hydroxytyrosol has, however, only recently become available in pure form, and it is prohibitively expensive (around US$1000.g^{-1}, http://www.caymanchemicals.com/). Alternatively, researchers obtain and purify it directly from olives, leaves or wastewaters (Mulinacci *et al.*, 2001; Briante *et al.*, 2004; Capasso *et al.*, 1999, 1994; Fernadez-Bolanos *et al.*, 2004). Several protocols are available for the synthesis of hydroxytyrosol, including: chemical synthesis from 3,4-dihydroxyphenylacetic acid (Capasso *et al.*, 1999), acid or alkaline hydrolysis of oleuropein (Litridou *et al.*, 1997; Garcia *et al.*, 1996), or it can be biochemically produced from tyrosol using tyrosinase as biocatalyst (Espin *et al.*, 2001), or using whole cell cultures of *Pseudomona aeruginosa* (Allouche *et al.*, 2004).

Hydroxytyrosol and other beneficial low molecular weight phenolic antioxidants occur in the olive wastewaters at 10 to 100 times the concentrations found in oil (Mulinacci *et al.*, 2001; Ramos-Cormenzana *et al.*, 1998; Capasso *et al.*, 1992; Rodriguez *et al.*, 1988). The wastes thus represent an attractive source of valuable antioxidants that could be extracted and purified for commercial use. This has been investigated by several researchers (Fki *et al.*, 2005; Fernandez-Bolanos *et al.*, 2004, 2002; Capasso *et al.*, 1999, 1994).

OW also contains many higher molecular weight polyphenolic compounds, particularly tannins and lignans. Tannins comprise a large group of compounds (M_w 500-20 000kDa) of complex oligomeric structure characterized by the presence of phenolic groups. They are natural substances widely distributed among different parts of vascular plants. The basic unit of tannin is tannic acid (pyrogallic acid), $C_{76}H_{52}O_{46}$ or penta-(digalloyl)-glucose. A major characteristic of tannins is their ability to form strong complexes with protein and other macromolecules such as starch and cellulose, and minerals (Chen and Hagerman, 2004; Makkar *et al.*, 1993).

Tannins are classified by their behavior on dry distillation, into two groups, (1) condensed tannins (proanthocyanidins), which yield catechol, and (2) hydrolysable tannins, which yield pyrogallol. Group (2) comprises two groups on the basis of its products of hydrolysis, glucose and (a) ellagic acid or (b) gallic acid (Belmares *et al.*, 2004; Hawley, 1977). An example of a condensed tannin is shown in Figure 1.3.

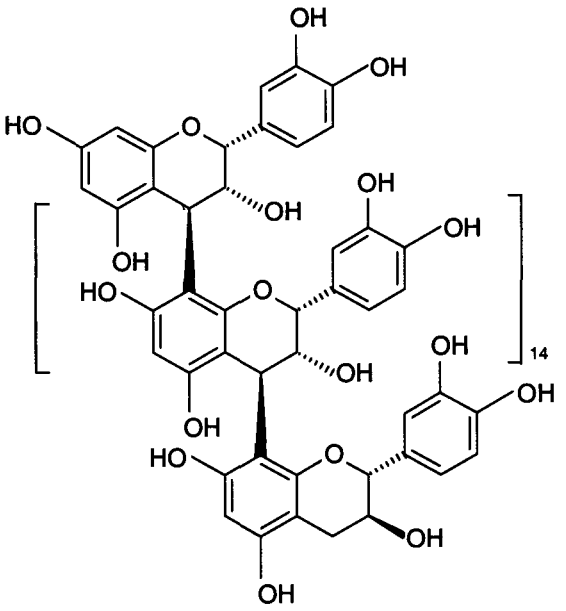


Figure 1.3: Example of a condensed tannin (Procyanidin)

Lignin is the main non-carbohydrate constituent of wood (comprising about one fourth). It acts as a natural plastic binder for cellulose, and is also of random polymeric structure characterized by phenolic groups (Hawley, 1977).

The low and higher molecular weight phenolic compounds occur in the various wastewaters as a result of the production of table olives and oil, and the total phenol concentration can reach up to 10 $g.L^{-1}$. The phenolic compounds can be broadly separated into a low molecular weight monomeric fraction including hydrolysable tannins (< 8 kDa) and a higher M_w fraction consisting of monomer-free polyphenols (> 8 kDa). It is the low molecular weight fraction that is predominantly responsible for the phytotoxic and antimicrobial effects of OW (Sayadi *et al.*, 2000; Ramos-Cormenzana *et al.*, 1998; Garcia Garcia *et al.*, 1997). The higher molecular mass fraction contains pigmented polyphenols, condensed tannins, lignin polymers and humic substances that are responsible for the dark colour, and although not quite as toxic, they are particularly resistant to degradation (Sayadi *et al.*, 2000). The phenolic composition of OW is variable, depending on olive cultivar and region, processing methods or in the case of oil, the extraction system employed (Lesage-Meessen *et al.*, 2001; Belmares *et al.*, 2004).

1.4 TREATMENT OF OLIVE WASTEWATERS

Due to the antimicrobial properties of OW, mainly attributable to the phenolic components, and its high organic load, OW is detrimental and toxic to municipal sewage treatment systems. These systems are capable of dealing with only low concentrations of feed (in the order of 1 kg(COD).m^{-3}. Trickling filters are considered unsuitable for the treatment of OW, as these systems can also only tolerate low concentrations of feed. This leads to unacceptably long hydraulic retention times and high dilution requirements (Rozzi and Malpei, 1996), so that disposal of OW in sewers is discouraged and in most cases it is illegal. The effect of OW on sewers is quite severe due to its acidity (which leads to pipe corrosion) and the high suspended solids content (of OMW) that causes blockages.

In conjunction with the appreciable concentration of slowly biodegradable compounds such as the tannins that give the effluent a dark colour, there are other problems associated with the biological decomposition of OW. In particular, it contains only low concentrations of nitrogenous compounds that are essential for active microbial metabolism (Sayadi *et al.*, 2000). Fermentation brines can also contain high concentrations of dissolved salts (mostly NaCl), which are also detrimental to microbial degradation. On the other hand, concentrations of heavy metals and halogenated organics are negligible in OW.

OW is traditionally disposed of in evaporation ponds, but this is not perceived to be an optimal solution as it does little to reduce the organic load, merely serving to concentrate the waste. There are additional problems associated with this approach, which include leaching into groundwater, overspill from flooding and offensive smells. Thick, phenolic-rich sludge accumulates in these ponds, while the process water is lost to the atmosphere. In fact, autooxidation and subsequent polymerization can make stored wastewaters more resistant to degradation than the fresh wastes (Assas *et al.*, 2002).

Most wastewater treatment systems have been tested for the treatment of OW. Early studies were directed solely towards the degradation (removal of COD and phenolics) of the waste. However, the cost of such treatment and the abundance of the waste has stimulated the investigation of the production of value-added products, facilitating the utilisation of the waste as a resource for further biological fermentations or other purposes. This is discussed in section 1.4.4. Due to their high strength and complex nature, it is considered unlikely that

a single physical, chemical or biological process will be capable of completely degrading these wastewaters. Olive wastewaters are, in fact, one of the most complex agro-industrial effluents (Hamdi, 1993).

1.4.1 Physical and chemical processes

Physical and chemical processes for treating OW are reasonably widely employed in the Mediterranean regions but the results are mostly unsatisfactory. These include thermal processes such as distillation, evaporation and incineration; the drawbacks of these processes are related to energy requirements, post treatment and disposal of produced emissions. Distillation and evaporation serve to concentrate the waste, but the concentrated product is still problematic. In addition, the distillate is not pure; it contains volatile alcohols and acids. Solid olive residues should not be burned to provide the heat required for distillation, as their combustion produces severe aromatic atmospheric pollution (Rozzi and Malpei, 1996). However, this has not stopped research into gasification of olive solid residues for energy production (Topal *et al.*, 2003; Andre *et al.*, 2005), hopefully with concomitant flue gas treatment considerations. Solar distillation is an attractive option for treatment as it allows the recovery of a relatively pure distillate stream for no energy cost, although there still remains the issue of a solid residue (Potoglou *et al.*, 2003). Composting is considered to be the best option for treatment of solid residues resulting from the oil extraction process (Tomati *et al.*, 1996).

For the wastewaters, chemical processes such as coagulation and flocculation are not very efficient because many of the organic components are difficult to precipitate. Liming is extensively employed; however, the efficiency in terms of organics removal of even heavy liming is in the order of 40-50% (Aktas *et al.*, 2001). Other chemical processes that have been investigated are treatment with aluminium sulphate, gelatine, bentonite, activated clay or carbon, and polyelectrolytes (Federici *et al.*, 1991; Flouri *et al.*, 1996; Sarika *et al.*, 2005; Meyssami and Kasaeian, 2005; Al-Malah *et al.*, 2000; Galiatsatou *et al.*, 2002). These processes are never entirely sufficient on their own, and are thus generally employed in conjunction with biological processes.

Advanced oxidation processes (AOP) are becoming increasingly popular for the treatment of OW, but these are also generally performed in conjunction with a biological treatment step.

AOP's include ozonation (Benitez *et al.*, 2001, 1999; Beltran-Heredia *et al.*, 2000), and electrochemical or photocatalytic treatments with H_2O_2 and various catalysts like Fe(II) (Fenton's reagent), TiO_2 and Pyrylium salts (Oukili *et al.*, 2001; Kyriacou *et al.*, 2005; Ahmadi *et al.*, 2005; Kotsou *et al.*, 2004; Benitez *et al.*, 2001; Drouiche *et al.*, 2004; Barakat *et al.*, 2005; Miranda *et al.*, 2002). Electrocoagulation has been investigated using sacrificial aluminium electrodes (Adhoum and Monser, 2004; Inan *et al.*, 2004), as has sonochemical treatment (Atanassova *et al.*, 2005). These processes have all proved to be effective in terms of removal of organics from the water, but there remains the issue of disposal of the precipitate or solid residue, which can subsequently also have increased metal concentrations. In addition, the processes are expensive in terms of both capital and operation, and are thus of limited utility to small-scale producers.

Membrane concentration processes such as microfiltration and ultrafiltration have been investigated but are not widely employed; problems arise from the high suspended solids concentrations (in OMW), the cost and maintenance of such installations and energy requirements for pumping (Borsani and Ferrando, 1996).

1.4.2 Microbial degradation

Despite the toxicity of OW due to its phenolic components, there are numerous microorganisms capable of utilising compounds of aromatic structure for metabolic purposes, and there is an abundance of literature describing the microbial degradation of OW. This is because biological treatment is perceived to be an inexpensive and environmentally friendly method for the removal of organics, with end products being, at worst, easily disposed-of, recyclable biomass (sludge). However, dilution of OW is generally necessary before microbial treatment, as concentrations of phenols and organics in the undiluted waste can be inhibitory or toxic.

In the bioremediation of OW, detoxification and degradation essentially involve the removal of phenolic and other organic compounds, the amounts of which can be broadly expressed as total phenols (TP) and the chemical oxygen demand (COD). Comparisons of results arising from degradation research are complicated by the high variability of wastewaters from different sources, but the evaluation of these two parameters allows for broad comparisons of the effectiveness of different degradation processes. The metabolic fate of the phenolic

compounds is an important consideration during biological treatment, as there are several pathways that can be followed. This depends on the microorganism (or microbial population), and the metabolic state thereof. The different size fractions of phenolic compounds and their relative concentrations have different biological and metabolic effects, and these determine the composition and colour of the effluent after treatment.

Monomeric phenols are generally colourless. Oxidation of these compounds (microbial or otherwise) proceeds through the formation of quinones and catechols (discussed in section 1.4.3). These are then either mineralised through ring cleavage, or otherwise undergo subsequent polymerisation through condensation resulting in the formation of darker coloured polyphenolic compounds and tannins (Hafidi *et al.*, 2005; D'Annibale *et al.*, 2004; Toscano *et al.*, 2003). The end-point of the polymerisation process is the formation of high molecular weight humic substances, which can have molecular sizes up to 2000 kDa.

Humic acids are the brown polymeric constituents of soil and humus. They are not well-defined compounds, but a mixture of random polymers containing aromatic and heterocyclic structures, carboxyl groups and nitrogen. Like tannin, humic acids are chelating agents; they are important in the exchange of cations in soils, and also occur in aquatic sediments as the major organic constituent. Humic acids play an important part in the bioavailability and binding of nitrogen, trace metals and other organics (Redwood *et al.*, 2005; Tomati *et al.*, 2000). Humic acids are not considered toxic; in fact they are essential and desirable components that can be beneficially recycled into agricultural land (Tomati *et al*, 1996). The humification process is a common consequence of mixed culture aerobic treatment of OW, as well as occurring through the action of soil microflora and during composting (Hafidi *et al.*, 2005).

An alternative to the process of polymerisation is the depolymerisation of larger compounds such as lignins and tannins to smaller phenolic and aromatic compounds, and ultimately complete mineralization. This process leads to the removal of dark colour, and is a result of the action of enzymes produced mainly by lignolytic (wood degrading) microorganisms. Primary amongst these are the white-rot fungi, but there are numerous other species of microorganisms capable of such depolymerisation, including bacteria, yeasts and algae. Some examples of organisms capable of the degradation of OW and phenolic compounds therein are listed in Table 1.2.

Most research listed in Table 1.2 has been performed on the mill wastewaters (OMW) as globally, this waste is produced in the majority.

Table 1.2: Microorganisms capable of degrading phenolic compounds in olive wastewater

Organism	Reference
Bacteria	
Pseudomonas spp.	Perez *et al.*, 1990; Di Gioia *et al.*, 2001; Chung *et al.*, 2003.
Ralstonia spp.	Di Gioia *et al.*, 2001.
Azotobacter spp.	Piperidou *et al.*, 2000; Borja *et al.*, 1995, 1992.
Lactobacillus spp.	Ayed and Hamdi, 2003, 2002; Brenes *et al.*, 2004.
Bacillus pumilus	Ramos-Cormenzana *et al.*, 1998.
Yeasts	
Saccharomyces spp.	Hafidi *et al.*, 2005; Giannoutsou *et al.*, 2004.
Yarrowia lipolytica	Lanciotti *et al.*, 2005.
Candida spp.	Yan *et al.*, 2005; Fadil *et al.*, 2003; Ettayebi *et al.*, 2003; Fialova *et al.*, 2004; Giannoutsou *et al.*, 2004.
Algae	
Ankistrodesmus braunii,	Pinto *et al.*, 2003.
Scenedesmus quadricauda	Pinto *et al.*, 2003
Fungi	
Geotrichum candidum	Borja *et al.*, 1995, 1992; Fadil *et al.*, 2003; Assas *et al.*, 2002.
Aspergillus spp.	Vassilev *et al.*, 1998; Martinez Nieto *et al.*, 1993; Fadil *et al.*, 2003; Borja *et al.*, 1995, 1992; Hamdi *et al.*, 1991; Kotsou *et al.*, 2004; Kyriacou *et al.*, 2005.
Phanerochaete spp.	Sayadi *et al.*, 2000, 1995, 1993, 1992; Blanquez *et al.*, 2002; Dias *et al.*, 2004; Garcia Garcia *et al.*, 2000.
Panus tigrinus	D'Annibale *et al.*, 2004.
Penicilium spp.	Robles *et al.*, 2000.
Pleurotus spp.	Flouri *et al.*, 1996; Aggelis *et al.*, 2003; Fountoulakis *et al.*, 2002; Jaouani *et al.*, 2003; Martirani *et al.*, 1996; Setti *et al.*, 1998.
Pycnoporus spp.	Jaouani *et al.*, 2005, 2003.
Lentinus spp.	Jaouani *et al.*, 2003.
Mixed cultures	Hafidi *et al.*, 2005; Isidori *et al.*, 2004; Benitez *et al.*, 1999, 1997; Tay *et al.*, 2005; Aggelis *et al.*, 2001; Beltran-Heredia *et al.*, 2000.

In terms of microbial degradation, there are important distinctions between OMW and the brined fermentation wastewaters (OFW): Firstly, the OFW has a high salt concentration (10-

16% w/v) from brining; this salinity is inhibitory to many microorganisms. Secondly, OMW contains large quantities of readily available carbohydrates (from the olive pulp), which aids in the growth of microorganisms and co-metabolism of phenolic compounds. The OFW does not contain these, because lactic acid bacteria and yeasts responsible for the fermentation/brining process consume any carbohydrates and sugars that leach out of the olive fruit during the brining process. The alkali pre-treatment wastewater from the production of green table olives does contain sugars (from the hydrolysis of oleuropein), but obviously also has high pH (10-11) that must be neutralised before biological treatment.

Most OW degradation research has been directed towards the degradation by mineralization of high molecular weight polyphenols, resulting in a removal of colour from the wastewater. A problem with this approach is that it is only achieved through the cultivation of a microbial monoculture under carefully controlled conditions. This is somewhat impractical for a wastewater treatment processes, as the required sterility of operation significantly increases the cost and complexity of a bioprocess. Therefore, if this approach is followed it is desirable to produce some valuable product, for example by harvesting of (valuable) degradative enzymes. On the other hand, non-sterile mixed culture degradation generally results in humification, the products of which can be used for fertilisation or anaerobic digestion to produce methane (section 1.4.4).

1.4.3 Enzymes involved in olive wastewater degradation

There are many enzymes involved in the metabolism of phenolic and other organic compounds in OW, the most relevant of which are summarised below.

Phenol hydroxylase (EC 1.14.13.1) and catechol oxygenases (EC 1.13.1.1 and 1.13.1.2) commonly work in conjunction and are responsible for oxidation and ring cleavage of monomeric phenols respectively (see Figure 1.4). Phenol hydroxylases incorporate one atom of molecular oxygen into the aromatic ring, resulting in dihydroxylated compounds. The second oxygen atom is reduced to H_2O by a suitable hydrogen donor (e.g. NADPH). Phenol hydroxylase enzymes can also act upon cresols to form methylcatechols. Catechol oxygenase is then responsible for ring fission in either the 1,2 position or the 2,3 position of the aromatic ring. Cleavage in the 1,2 ring position results in *cis,cis*-muconic acid which is then typically

further metabolised to succinate, while 2,3 ring cleavage results in the formation of α-hydroxymuconic semialdehyde and ultimately acetaldehyde and pyruvate.

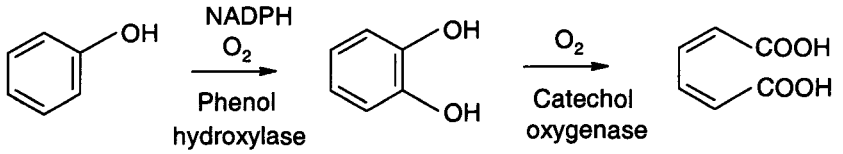


Figure 1.4: Enzymatic hydroxylation and ring cleavage of phenol

The above enzymes are produced predominantly by yeasts (*Saccharomyces* spp., *Candida* spp., *Trichosporum cutaneum*) and bacteria (*Pseudomonas putida, Bacillus stearothermophilus*), and are also known to occur in mixed cultures. The enzymes are sensitive to inorganic salts, especially chloride, which is known to be deleterious (Neujahr and Gaal, 1973; Buswell, 1975; Yang and Humphrey, 1975; Wang and Loh, 1999; Alexievaa *et al.*, 2004; Fialova *et al*, 2004).

Laccases (EC 1.10.3.2 benzenediol: oxygen oxidoreductase) are multicopper phenol oxidase enzymes that use molecular oxygen as a terminal electron acceptor. They catalyse the removal of a hydrogen atom from hydroxyl groups resulting in the reduction of oxygen to water, and have broad substrate specificity. They can oxidise polyphenols, methoxy-substituted phenols and benzenes, aromatic amines and non-phenolic compounds, resulting in the generation of free radicals that are capable of undergoing further polymerisation or depolymerisation, demethylation or quinone formation. In particular they are capable of oxidising higher molecular weight phenolic compounds such as tannins and lignin. Laccases are predominantly produced by white-rot fungi, which are the primary degraders of wood (Robles *et al.*, 2000b; Torres *et al.*, 2003; D'Annibale *et al.*, 1999; Casa *et al.*, 2003; Dias *et al.*, 2004; Blanquez *et al.*, 2002; Jaouani *et al.*, 2005, 2003). Laccases have diverse applications in biotechnology due to their broad substrate range and stability. These include: delignification of cellulosic fibres for paper, polysaccharide cross-linking, textile dye bioremediation, as well as medical, biosensor and analytical applications (Galhaup *et al.*, 2002).

Lignolytic white-rot fungi also produce peroxidase enzymes that are capable of degrading a wide range of phenolic compounds. Lignin peroxidase (LiP, EC 1.11.1.14) catalyses a one-electron oxidation resulting in the formation of aryl cation radicals, which spontaneously decompose by various pathways. Manganese peroxidase (MnP, EC 1.11.1.3) catalyses the oxidation of Mn(II) to Mn(III), which can then in turn oxidise several phenolic substrates. These enzymes require hydrogen peroxide as a co-factor, and this is enzymatically produced in conjunction with LiP and MnP during secondary metabolism, as a response to carbon or nitrogen starvation (Sayadi and Ellouz, 1995, 1993, 1992; Sayadi *et al.*, 2000; Blanquez *et al.*, 2002; Jaouani *et al.*, 2003). Laccase, LiP and MnP have been the subject of much research because of their ability to metabolise the higher molecular weight phenolic compounds in OW, resulting in the decolourisation of these wastewaters.

Tyrosinase (EC 1.14.18.1) is a phenol oxidase enzyme capable of converting phenolic compounds, although this is restricted to low molecular weight monomeric phenols. It acts through two distinct enzyme cycles: firstly monophenols are hydroxylated by molecular oxygen to *o*-diphenols, and then these are oxidised to form quinones. The quinones spontaneously react to form other oligomers (Ikehata and Nicell, 2000; Dec and Bollag, 1990). Tyrosinase is widely distributed among fungi, bacteria, plants and animals.

Tannase (tannin acyl hydrolase EC 3.1.1.20) has also been reported to be responsible for decolourisation of OW, as a consequence of the (anaerobic) growth of *Lactobacillus plantarum* (Ayed and Hamdi, 2003, 2002), and *Aspergillus* spp. (Belmarez *et al.*, 2004). It catalyses the hydrolysis of ester bonds present in hydrolysable tannins.

β-Glucosidase and esterase enzymes play a role in the physiology of olive trees and as a result sometimes occur in OW. They are also produced by yeasts and *Lactobacilli* during fermentation of table olives, and assist in the extraction of bitterness through the hydrolysis of oleuropein (although this also occurs abiologically). Many of the olive-derived phenolic compounds are bound to polysaccharides (Greco *et al.*, 1999; Bouzid *et al.*, 2004; Marsilio and Lanza, 1998).

Enzymatic treatment, although possible, is not currently extensively employed for the treatment of olive wastewaters, compared to whole-cell biological treatment. The cost of

commercial enzymes is currently too high for such treatment to be economically feasible. The wastewaters can, in fact, be used as a substrate to produce such enzymes for commercial purposes (Fenice *et al.*, 2003).

1.4.4 Value-added products from olive-derived wastes

The simplest and most obvious use of OMW is for land treatment, and this is extensively employed where possible, as biofertilization for agricultural lands. This is an inexpensive and beneficial method of disposing of OMW, and simply involves irrigating agricultural land with OMW (although it cannot be done with OFW as the salinity is too high). Natural microorganisms within the top 2 m of soil can almost completely remove the organic components of OMW at a limited annual dosage (~5000-10000 $m^3.ha^{-1}.year^{-1}$). This can benefit soil fertility by increasing the soil organic matter content, which in turn stimulates microbial activity, as well as increasing available nitrogen, potassium and phosphorus. There is a concomitant increase in soil electrical conductivity, but generally below values indicating salinization (Caberra *et al.*, 1996). However, care must be taken not to irrigate around the bases of trees or too soon before sowing, and it is not recommended to irrigate growing crops due to the phytotoxicity of the raw effluent. The relatively low concentration of nitrogenous organic compounds in OMW and richness in carbon sources provides favourable conditions for free-living dinitrogen fixing microorganisms, predominantly members of *Azotobacter* species. This can be particularly beneficial to soil fertility (Balis *et al.*, 1996).

A recent study has shown that polyphenol oxidase enzymes occur naturally in olive husk. These enzymes are effective in significantly reducing the monomeric phenolic content of OMW, and incorporating a simple 'filtration' type process of OMW through the husk in order to detoxify it makes subsequent biofertilization safer (Greco *et al.*, 1999). In another study, OMW was fermented with *Azotobacter vinelandii* to produce a high quality, nitrogen rich liquid fertilizer that could be applied directly to crops and showed promising results (Chatjipavlidis *et al.*, 1996).

Composting the solid components of OMW with the addition of other agricultural waste products is another method of recycling the waste with beneficial effects on soil fertility. The composting process eliminates the toxicity and degrades the OMW, and experiments have shown that yields with these composts are similar to, and sometimes even better than, results

achieved with (costly) mineral fertilizers (Cegarra *et al.*, 1996; Monteoliva-Sanchez *et al.*, 1996; Tomati *et al.*, 1996).

Anaerobic digestion of OMW for the production of methane is an attractive idea for olive producers, as this allows the recovery of some of the energy consumed in the oil production process. Types of processes used include anaerobic filters, anaerobic contact and upflow anaerobic sludge blankets. Anaerobic digestion results in less sludge production than aerobic fermentations, and digesters can be easily restarted without re-inoculation after several months of standing idle.

The disadvantages of using anaerobic digestion for the treatment of OMW are that phenols and lipids are inhibitory to the action of activated sludge microbial consortia. There is thus a danger of organic overloading, and long hydraulic retention times are required. Therefore, many of the aerobic biological treatment processes (section 1.4.2) have been investigated as pre-treatment for anaerobic digestion, with significant improvement of performance. The aerobic pre-treatment reduces the total phenolic load, which makes the wastes less inhibitory for subsequent anaerobic digestion.

Influent concentrations for anaerobic digestion range from 15-70 kg(COD).m^{-3}, loading rates vary from 0.75-3 kg(COD).m^{-3}.day^{-1} and hydraulic retention times are of the order of 20-25 days. Yields for untreated OMW range from 250-400 ml(CH_4).g^{-1}(COD), and for pre-treated OMW this improves to around 350-1000 ml(CH_4).g^{-1}(COD). In effect, enough methane can be produced to cover the energy needs of both an olive oil production plant and the anaerobic reactor. Various pilot plants are in operation in the Mediterranean regions (Bertin *et al.*, 2001; Erguder *et al.*, 2000; Borja *et al.*, 1996, 1995; Fiestas Ros de Ursinos and Borja-Padilla, 1996; Hamdi, 1996, 1992; Fiestas *et al.*, 1990). OMW has also been anaerobically fermented for butanol production using a solventogenic *Clostridium* species (Waehner *et al.*, 1988).

Undiluted OFW brines are unfortunately unsuitable for anaerobic fermentation because of their high salinity and lack of fermentable carbohydrates. The de-bittering wastewaters from green table olive production have, however, been anaerobically treated. In these cases the wastewater was generally chemically adjusted (to neutral pH) and aerobically pre-treated (Aggelis *et al.*, 2001; Brenes *et al.*, 2004; de Castro and Brenes, 2001).

For OMW, there are many aerobic fermentations that have useful by-products that occur in conjunction with detoxification and biodegradation of the wastewater. There is, however, very little (if any) published work about aerobic fermentation of OFW resulting in useful by-products. The white-rot fungi, mentioned previously, produce enzymes capable of degrading OMW as well as significant quantities of biomass. Thus OMW can be considered as a substrate for the production (and possible commercial exploitation) of these enzymes or biomass. *Pleurotus* sp. (oyster mushroom) and *Lentinula edodes* (Shiitake mushroom) have been extensively studied in this regard due to the impressive suite of enzymes produced when they are grown on OMW, and the fact that they produce edible biomass. These fungi produce large titres of extracellular polyphenol oxidase and peroxidase enzymes, and also produce xylanases, proteases and cellulases (D'Annibale *et al.*, 2000, 1999; Setti *et al.*, 1998; Zervakis *et al.*, 1996; Sanjust *et al.*, 1991). The peroxidases and polyphenol oxidases are of particular interest for enzymatic industrial bioremediation of phenolics and other aromatic compounds, while the other enzymes are of use in the food processing industries.

These enzymes are readily produced in submerged fermentations, although there is growing interest in solid-state fermentations (SSF). Recent studies have shown that it is possible to achieve about 75% reduction in toxicity and 80-90% removal of phenolics from OMW within only two days of SSF. Significantly higher laccase production was reported in SSF, compared to parallel submerged fermentations with the same organisms (Setti *et al.*, 1998; Zervakis *et al.*, 1996; Sanjust *et al.*, 1991). One method employed was the immobilisation of mycelia on expanded clay or perlite, with sparging of recycled OMW over the surface; another involved inoculating olive husk press-cake supplemented with liquid OMW. Both these methods produced *Pleurotus* fruiting bodies of high quality and yield after 15 days, while *Letinula edodes* required about twice this time for fruiting. Comparisons with commercially grown mushrooms showed no appreciable difference in taste, texture or form, and phenolic accumulation was not detected in the fruit. Laccase from *L. edodes* has been extensively characterised, and methods for the production of this enzyme in SSF have been well documented (D'Annibale *et al.*, 2000, 1999).

Another interesting application for the recycling of OMW is the production of enzymes for use during the oil extraction process. Pectinase was produced by *Cryptococcus albidus* grown on OMW, supplemented with sunflower calathide meal (to increase pectin concentration). The enzymes so produced were added to olives during the malaxation

(softening) stage of the oil production process, with a resultant significant improvement in oil yield and quality (Montedoro *et al.*, 1993; Federici *et al.*, 1991; Federici, 1988).

OMW has been tested as a substrate for a variety of other bioprocesses, *e.g.* production of single cell protein for animal feed supplementation using *Saccharomyces chevaliere* and *S. rouxii* (Gharsallah, 1993); and production of microalgae (Pinto *et al.*, 2003; Sanchez-Villasclaras *et al.*, 1996). Several processes reported recently are of interest because of their novelty: Xanthan has been produced using OMW as substrate by the organism *Xanthomonas campestris*. Xanthan is the most commercially accepted microbial polysaccharide, and is used for food and non-food purposes as thickener or viscosifier. *X. campestris* can grow on OMW as sole nutrient source. However, yields were improved by the addition of nitrogen and mineral salts (Lopez *et al.*, 2001; Lopez and Ramos-Cormenzana, 1996).

Azotobacter chroococcum has been used to produce polyhydroxyalkanoates or 'bioplastics', in particular poly-β-hydroxybutyrate (PHB), using OMW as a feedstock. PHB has physical properties very similar to polypropylene, but is completely biodegradable to CO_2 and H_2O by many bacteria, fungi and algae. There is a lot of interest in degradable 'bioplastics', but they are not currently competitively priced due to the use of glucose as feedstock. An alternative inexpensive feedstock (such as OMW) would alleviate this problem. In addition, yields were high when OMW was used as substrate: (intracellular) PHA's were produced up to 50% of cell dry weight after 24 hours. This is ascribed to the natural high carbon low nitrogen ratio of OMW (Gonzalez-Lopez *et al.*, 1996). As a final example, OMW was used for photobiological hydrogen production using the algae *Rhodobacter sphaeroides* (Eroglu *et al.*, 2003).

Unlike OMW, OFW has not been subject to extensive research for the purpose of obtaining biological value-added by-products. This is most likely due to two reasons: firstly, OFW is produced in lesser quantities than OMW. Globally most olives (approximately 90%) are pressed for oil, and therefore the problem of OFW, although equally severe, is not as large and has thus received less attention. Secondly, OMW is a more appealing substrate for microbial growth, as it contains readily available carbon sources (carbohydrates) from the olive pulp, and it does not have the high salinity that occurs in OFW.

1.5 BIOREACTORS FOR WASTEWATER TREATMENT

Biological degradation is a cost-effective and environmentally friendly method of wastewater treatment, and is extensively employed for the treatment of municipal and other wastewaters. However, conventional wastewater treatment plants have several disadvantages such as large surface area requirements, low influent concentrations (dilution requirements) and transport to remote locations due to offensive odours. Conventional treatment systems include technologies like activated sludge, algal ponding and sequencing batch reactor type systems. For high strength wastewaters there is a need for small-scale intensive bioreactor systems that can be operated as stand-alone "end of pipe" treatment systems for localized wastewater sources, before suitable discharge or re-use of effluent. This discussion is limited to aerobic bioreactor systems.

There are several technological options for small-scale systems, including stirred tank reactors, airlift reactors, jet loop reactors, packed or fluidized beds, and increasingly, membrane bioreactors. Stirred tank reactors are commonly used in biotechnology research laboratories, however they are not ideally suited to scale-up. This is mainly because there is a large power requirement for stirring, which increases with tank volume and fermentation broth viscosity. Expensive mechanical shaft seals are necessary to ensure sterility, as the motor is located externally to the tank. Stirring impellers cause high shear rates in the fermentation broth, which can be detrimental to the microbial culture, this being especially so in the case of filamentous organisms such as fungi.

Airlift devices operate on the principle of mixing and mass transfer due to an introduced gaseous phase, e.g. bubble columns and draught tube devices. The use of a draught tube causes recirculation of the fermentation broth in a reactor. Highly convective mass transfer regimes are possible within such reactors, with minimum associated shear stress. These are good conditions for microbial growth and metabolism. Other advantages of airlift devices include their simplicity (no moving parts), and low energy requirements (compared to mechanical agitation). The versatility and efficacy of airlift devices has been extensively studied and demonstrated (Chisti, 1989, 1992; Chisti and Moo-Young, 1987, 1993; Jin and Lant, 2004; Quan *et al.*, 2003; Couvert *et al.*, 2004).

Jet-loop bioreactors are a relatively new technology. These operate on a similar principle to airlift draught-tube reactors, except that a mixed gas/liquid phase is injected into the draught tube at high velocity. The gas separates from the liquid in the reactor headspace, while the liquid phase is recycled by means of a pump. These reactors have excellent mixing and mass transfer characteristics, and have been used for the treatment of high-strength (synthetic) wastewaters with COD of up to 54 $g.L^{-1}$ (Yeon *et al.*, 2005; Yildiz *et al.*, 2005). Disadvantages of these reactors include high energy requirements, and high shear rates. The reactors are also significantly more complicated than airlift devices.

Fluidised and packed bed reactors use some form of solid matrix as immobilisation support for microorganisms, which then form biofilms. Liquid and gas phases are passed through the matrix, resulting in wastewater degradation. The liquid phase is often recycled through the bed. Such systems have been reported to be capable of high degradation efficiencies (Bertin *et al.*, 2004, 2001). Advantages of packed and fluidised beds are that they can tolerate high concentrations of organics in wastewaters and are resistant to shock loads; this relates to the nature of biofilms, which offer a measure of protection towards the microorganisms. The main problem with such systems (particularly packed beds) is excessive microbial growth, which can lead to blockages and channelling of the wastewater through the bed.

Membrane bioreactors have recently been receiving considerable attention as a technology for both small- and large-scale wastewater treatment. These come in three different forms: firstly, a membrane unit can be used in conjunction with a biological process for separation of solids (e.g. biomass and sludge). In this way the membrane unit replaces processes like sedimentation, however unlike sedimentation biomass can be immediately and continuously recycled back into the process stream. Secondly, a membrane can be used for gaseous mass transfer within a bioprocess, to provide the necessary oxygen for metabolism (Casey *et al.*, 2000, 1999). Thirdly, membranes can be used for controlled transfer of nutrients to a bioreactor, or pollutants can be selectively extracted from a wastewater stream. Such extractive membrane bioreactors (eMBR's) operate on the principle of selectively permeable membranes, which allow for the transport of certain species across a membrane and retain the rest. Examples of such are the silicon tube-based reactors of Brookes and Livingston (Brookes and Livingston, 1995, 1994; Livingston, 1992), and supported liquid membrane (SLM) bioreactors (Bressler *et al.*, 2002). The silicon tube based reactor allows for the diffusive transport of volatile low molecular weight aromatics (from a waste stream) across

the membrane into a separate biomedium, where biotransformation occurs. The biomedium can be contained in any desired type of bioreactor. The advantage of this approach is that the organisms used for biotransformation are not in contact with the bulk waste medium (which could be inhibitory or unsuitable for the biological process), and undiluted or "aggressive" wastes can be treated in this manner without pre-treatment.

Of the different forms of MBR, the use for separation of solids is by far the most common. Such systems can have the membrane unit located externally to the bioreactor, or otherwise the membranes can be immersed directly within the fermentation tank, commonly referred to as a submerged membrane bioreactor (sMBR). This has several advantages: process air/oxygen (required for microbial metabolism) can be sparged over the membranes to help alleviate membrane fouling, and it reduces the footprint of the process as fermentation and separation operations are combined into a single unit process.

There are numerous other advantages of membrane bioreactors. Hydraulic and solids retention times are separated; while the recycle of biomass leads to high cell density. This means that high organic loading rates are possible. Complete solids removal results in high quality effluent, and there are several reports of the operation of sMBR's with zero sludge discharge (Yoon 2004, 2003; Pollice *et al.*, 2004). Modular or retrofit design options add to the appeal of sMBR's, and they are also more energy efficient than conventional treatment systems (Stephenson *et al.*, 2000; van der Roest *et al.*, 2002). sMBR's have been shown to be more cost effective than activated sludge systems (Cote *et al.*, 2004).

sMBR's can be considered to be in the growth to development stage of their lifecycle; there is increasing acceptance of this technology because of escalating interest in water reuse and recycling, as well as increased demand for advanced wastewater treatment solutions (Howell, 2004). There are several well-established commercially available systems e.g. Kubota® and Zennon®. Zennon has recently supplied the world's largest membrane bioreactor, capable of treating 144 000 $m^3.day^{-1}$ of municipal sewage. Kubota systems have been used on ships for many years because of their small footprint and high volumetric productivity. Table 1.3 lists some interesting recent research into the application of MBR's for treatment of wastewaters from different sources.

Despite their suitability for small-scale systems, and their ability to treat high strength wastewaters, membrane bioreactors have to date not been investigated for the treatment of olive-derived wastewaters. Bioreactor configurations that have been investigated for these wastewaters include: stirred tank reactors with suspended or immobilised biomass (Raposo *et al.*, 2004; Brenes *et al.*, 2000; Borja *et al.*, 1995; Martinez Nieto *et al.*, 1993), packed bed reactors (Bertin *et al.*, 2004, 2001), bubble column reactors (Kotsou *et al.*, 2004; Assas *et al.*, 2002, 2000; Hamdi and Ellouz, 1992), and draught-tube airlift reactors (Kyriacou *et al.*, 2005).

Table 1.3: Membrane bioreactor applications for wastewater treatment

Application	Reference
Municipal wastewater/sewage with low/zero sludge discharge	Hasar and Kinaci, 2004; Zhang *et al.*, 2003; Holler and Trosch, 2001; Cote *et al.*, 1997.
Hospital wastewater	Wen *et al.*, 2004.
Anionic surfactant wastewater (detergents)	Dhouib *et al.*, 2005.
Food industry wastewater	Katayon *et al.*, 2004; Wang *et al.*, 2005.
Chromium reduction	Konovalova *et al.*, 2003.
Kraft pulp mill effluents	Dias *et al.*, 2005.
Electroplating/degreasing wastewater	Blocher *et al.*, 2004.
Dairy industry wastewater	Bae *et al.*, 2003.
Dyehouse wastewater	Kim *et al.*, 2004.
Polluted drinking water	Li and Chu, 2003.

1.6 RESEARCH OBJECTIVES

The overall objective of the project was to investigate the olive-derived wastewaters produced in the Western Cape, and subsequently to investigate possible uses for and treatment of the wastewaters, as they pose an environmental disposal problem. The predominant current procedure for the disposal of these wastewaters (into evaporation ponds) is considered to be unsatisfactory.

The first objective was therefore to determine the composition and chemical properties of various local olive wastewaters. Following on from this, the second objective was to identify

possible value-added products that could be obtained from the olive wastewaters, and attempt to produce these.

The third objective was to investigate biological treatment of the wastewaters, as this is perceived to be the most environmentally prudent and cost effective method of treatment. Comprehensive wastewater treatment is unlikely to be achieved in a single biological process, but experience in Mediterranean countries has shown that a significant reduction in polluting organic load is possible with such systems.

The fourth objective was to investigate and develop a small-scale biological treatment system that could be used by the olive producers for treatment of their wastewater. The olive farms and production facilities are often isolated, and hence do not have access to appropriate wastewater treatment facilities.

1.7 THESIS STRUCTURE

The first step was to collect and analyse wastewaters from various sources. These included black- and green olive fermentation brines, green olive pre-treatment wastewater, and some mill wastewater samples. Analyses included physical composition and chemical properties of the wastewaters. This is the subject matter of Chapter 2. At this stage it was decided that the project would focus on the black table olive fermentation brine wastewaters, as these are produced in large quantities at a central local facility, and of the different types of wastewater associated with the olive industry, these are the least investigated.

In terms of value-added products, the low molecular weight phenolic antioxidants that occur in the olive wastewaters appeared to have the greatest potential. The table olive fermentation brines were shown to contain high quantities of the valuable antioxidant hydroxytyrosol as the main low molecular weight aromatic component. Methods for recovery of the phenolics from the wastewaters were thus investigated. These included adsorption by polymeric resin, liquid/liquid solvent extraction and a membrane-assisted solvent extraction process. This work is described in Chapter 3.

Microbial degradation of the wastewater was then investigated. This involved isolation of wild type strains from wastewater sources, which were compared, for degradative ability,

with several commercially available strains, and activated sludge mixed microbial cultures. Degradation experiments were performed on both raw wastewaters, and wastewaters that had been previously extracted (to recover the antioxidants). This work was performed in collaboration with co-workers, who investigated the improvement of a wild type bacterial strain's degradative capacity through mutagenesis, and compared the degradative capacity of the wild type bacterial strain to a fungal strain. Due to the harsh nature of the wastewater (in terms of high total phenol concentration, COD and salinity) and time constraints, only microbial degradation was investigated. Biotransformation into value-added products was not investigated, although this will no doubt be a future avenue of inquiry. Microbial degradation of black olive brines is the subject matter of Chapter 4.

In work discussed in Chapter 5, a submerged membrane airlift bioreactor was designed, modelled and tested for the purpose treating the olive wastewaters. Degradation of black olive brines in the bioreactor was compared to that of the same cultures cultivated in shake flasks, and to published literature where possible. The effect of influent concentration and organic loading rates were then determined by performing fed-batch and continuous degradation experiments.

Finally, in Chapter 6 overall conclusions are drawn. Suggestions and observations are made concerning the current state of the olive wastewater situation in South Africa, and recommendations are made about the possible way forward towards comprehensive treatment/beneficiation systems.

CHAPTER 2: ANALYSIS OF OLIVE WASTEWATERS

2.1 INTRODUCTION

Olive-derived wastewaters are complex agro-industrial effluents, containing many organic components, as discussed in Chapter 1. In work described in this chapter, olive wastewater samples were subjected to analyses to determine their composition and properties. Particular attention was paid to black olive brines, but green olive brines, green olive de-bittering (NaOH) wastewaters and mill wastewaters were analysed for comparison. The results were compared to some published data from Mediterranean regions.

Wastewaters were collected periodically from various local olive processors over a period of three years. These included one large (commercial) and one small (traditional) table olive producing facility, and two producers of olive oil. Black and green olive brine wastewaters were collected from fermentation tanks at the end of the fermentation period, while the green olive de-bittering wastewaters were collected before discharge. Mill wastewater samples were collected directly from the centrifuges after separation. Table 2.1 lists the different wastes collected over a three-year period.

Analyses were performed according to standard scientific methods available in the literature, and many of these methods were applied throughout subsequent chapters. Firstly, general chemical properties and composition of the wastewaters were determined. The various parameters that were analysed are described below.

The pH of a liquid is a fundamental property that affects chemical and biological reaction, and this was measured routinely. Electrical conductivity is an approximate and convenient measure of dissolved ionic species in a liquid. This is an important parameter in terms of wastewater treatment and disposal, especially in the case of fermentation brines that have a high NaCl content.

Table 2.1: List of wastewaters analysed

Sample name	Description
OW-01	Black olive brine, *Calamata* olives
OW-02	Evaporation pond, mixed and diluted brines
OW-03	Evaporation pond, mixed and diluted brines
OW-04	Black olive brine, *Calamata* olives
OW-05	Evaporation pond, diluted mill wastewater
OW-06	3-phase mill wastewater, *Manzanilla* olives
OW-07	3-phase mill solid waste (husk)
OW-08	Black olive brine, *Calamata* olive
OW-09	Green olive de-bittering wastewater, *Manzanilla* olives
OW-10	Green olive brine, *Manzanilla* olives
OW-11	Black olive brine, *Calamata* olives
OW-12	Green olive brine, *Mission* olives
OW-13	Black olive brine, *Mission* olives, traditional process
OW-14	As above, diluted wastewater tank
OW-15	Black olive brine, *Calamata* olives
OW-16	3-phase mill wastewater, *Frontoio* olives
OW-17	3-phase mill wastewater, *Mission* olives
OW-18	Black olive brine, *Calamata* olives

The total solids (TS) of a wastewater sample includes suspended solids (portion of solids retained by a filter) and dissolved solids (the portion that passes through the filter); it is a weight measure of all species contained in a waste stream when all the water and other volatiles have been removed. Suspended solids in this work included microbial and vegetative biomass, and some colloidal phenolic precipitates (humic acids), while dissolved solids were mostly accounted for by salts and soluble phenolics.

Chemical oxygen demand (COD) is defined as the amount of a specified oxidant that reacts with a sample under controlled conditions. It is used as a measure of pollutants in wastewater and natural water. Both organic and inorganic components of a sample are subject to oxidation, but in most cases it is the organic component that predominates and is of the greater interest. Most types of organic matter are oxidised by a boiling mixture of chromic and sulphuric acids.

A standardized laboratory biochemical oxygen demand (BOD) procedure is commonly used to determine the relative oxygen requirements for the microbial degradation of wastewaters, effluents or polluted waters. BOD testing measures the molecular oxygen utilised during a specific incubation period for the biochemical degradation of organic material, and the oxygen used to oxidise inorganic material such as sulphides and ferrous iron. BOD testing is commonly performed over a period of five days (BOD_5). The method consists of filling an airtight bottle of specified size (to overflowing) with sample, and incubating at specified temperature for 5 days. Dissolved oxygen (DO) is measured initially and after incubation, the BOD is the difference between these two values. However, due to the antimicrobial nature of olive wastewaters and relatively small indigenous microbial population therein, the BOD test was found to be inconsistent and results are therefore not reported; the COD test was preferred.

Total organic carbon (TOC) is a convenient and direct expression of total organic content. However, it does not measure other organically bound elements such as nitrogen and hydrogen. It is independent of the oxidation state of the organic matter, and as such is normally performed in conjunction with COD.

The nitrogen content of the wastewaters was measured, as it is essential for biological processes. Olive wastewaters are known to be deficient in nitrogen, and are often supplemented with a nitrogen source before biological treatment (Sayadi *et al.*, 2000).

Reducing sugars are a readily available carbon source, and thus need to be quantified as they affect microbial growth. Low levels of sugar can be beneficial for co-metabolism of aromatic compounds, but an excess thereof can be detrimental to microbial metabolism of phenols, as the sugar is preferentially consumed (Galhaup *et al*, 2002). Mill wastewaters are expected to contain reducing sugars due to the olive crushing process and hence cell disruption, while the brines are not expected to contain any significant quantities of sugar, as it will most likely have been consumed by microbes during the fermentation process.

Residual lipids are known to occur in the mill wastewaters as a result of incomplete oil extraction. Since there is no cell disruption in the brining process, the brines are not expected to contain any significant quantities. Lipids would in any case separate from the brines

during fermentation, unlike the mill wastewaters, where the lipids occur as an emulsion with the aqueous phase.

Organic acids that naturally occur in olives also occur in the mill wastewaters as a result of cell disruption. In the brines, organic acids are leached out of the olive fruit, as well as being produced during the (lactic acid) fermentation.

The phenolic components of the wastewaters were analysed in some detail, as these account for a significant proportion of the organic load, and are primarily responsible for the resistance of the wastewaters to degradation. The total phenolic content of the wastewaters were thus quantified using a suitable colourimetric assay. Size exclusion chromatography was then performed to determine the molecular weight distribution of phenolic compounds. Thereafter, high performance liquid chromatography was performed to identify and quantify low molecular weight phenolic compounds.

The low molecular weight phenolic compounds were extracted from the wastewaters using liquid/liquid extraction, and their contribution towards the total phenol content was measured. The antioxidant activity of the extracts was then evaluated by using a free radical scavenging method, and was compared to reference compounds of known activity. The low molecular weight phenolics from olives are known to be powerful antioxidants, as discussed in section 1.3. There are many methods of determining antioxidant activity. These include direct methods of measuring radical scavenging ability such as the DMPD and DPPH methods (Briante *et al.*, 2004; Bondet *et al.*, 1997; Sanchez-Moreno *et al.*, 1998), and oxygen radical absorbance capacity (ORAC, Prior *et al.*, 2005) for aqueous systems. There are also many methods reported in the literature for lipidic systems, e.g. antiperoxidative ability by the ABAP method (Fogliano *et al.*, 1999), amongst may others. However, methods for the determination of antioxidant activity of natural compounds have not yet been standardised.

2.2 MATERIALS AND METHODS

2.2.1 General procedures

Wastewaters were stored at –20°C for long term or otherwise at 4°C for short term or during analyses. Standard compounds and reagents were all of analytical or HPLC grade as

required, and were obtained from Merck, Fluka, Sigma-Aldrich or BDH chemicals companies. Distilled and de-ionised reagent water (ddH_2O) was obtained from a Millipore Elix 3 purification system. All analyses were performed at ambient laboratory temperature (25±2°C), unless otherwise stated. Analytical determinations and experiments were performed in triplicate where possible, with results presented as the mean. Standard deviation was in general less than 5%, unless otherwise stated with results. Least-squares correlation coefficients (r^2) for all standard curves were greater than 0.99. Common and routine analyses were performed according to the American Public Health Association's (APHA) Standard Methods for the Examination of Water and Wastewater (1998).

2.2.2 Analytical Equipment

A Cyberscan 1000 was used for pH measurements. The instrument was calibrated with appropriate buffers before use. A Lutron CD-4301 conductivity meter was used to measure total salinity. All spectrophotometric analyses were performed on a Unicam Helios α UV/Visible spectrophotometer. A Buchi R200 Rotavapor was used for *in vacuo* solvent removal at 35°C.

2.2.3 Total, suspended, volatile and dissolved solids

Total solids were determined by evaporating 100 ml of well-mixed wastewater samples in an oven at 80°C, until constant weight was reached. The samples were evaporated in pre-weighed aluminium dishes, and were cooled to room temperature in a desiccator before weighing. Dissolved solids were determined in the same manner after a sample filtration (Millipore 0.45μm). To determine suspended and volatile suspended solids, 100 ml sample volume was filtered through a glass microfibre filter (Whatman), the filter and residue were dried to constant weight at 103-105°C, giving the suspended solids. The filters were then ignited in a furnace at 550°C. The loss of mass is the portion of solids that was volatile (including both organic and inorganic matter). The residue after igniting is commonly referred to as ash.

2.2.4 Chemical oxygen demand (COD)

COD measurement was originally performed according to the reflux method described in APHA standard methods. A sample was refluxed in a strongly acid solution with a known excess of potassium dichromate ($K_2Cr_2O_7$). After digestion the remaining $K_2Cr_2O_7$ was titrated with ferrous ammonium sulphate to determine the amount consumed, and the oxidizable matter was calculated in terms of oxygen equivalent. However, this is a time-consuming and tedious process, and thus the Merck COD reagent set (1.14555 HR) was subsequently used in conjunction with a digestion block and a Nova Spectroquant photometer. Potassium hydrogen phthalate in distilled water was used as a standard, at concentrations of 425 and 850 $mg.L^{-1}$ in distilled water, corresponding to COD values of 500 and 1000 $mg.L^{-1}$ respectively. Distilled water was used as reagent blank.

2.2.5 Total organic carbon (TOC)

Total organic carbon was measured using a SGE Anatoc Series II analyser, after filtration and appropriate dilution of samples. Organic carbon in samples was oxidised using TiO_2 catalyst and UV light. Resulting CO_2 was quantified using infra-red detection. Potassium hydrogen phthalate in the range 0 – 100 $mg.L^{-1}$ was used as standard. TOC analyses were performed courtesy of the Minerals Processing Research Unit at UCT.

2.2.6 Nitrogen

Total nitrogen was measured using a Hanna reagent set (HI 93767 low range) in conjunction with a Hanna C214 bench photometer.

2.2.7 Reducing sugar

Reducing sugar was determined using the dinitrosalicyclic acid (DNS) colourimetric method according to Miller (1959). DNS reagent solution contained 10 $g.L^{-1}$ dinitrosalycylic acid, 2 $g.L^{-1}$ phenol, 0.5 $g.L^{-1}$ sodium sulphite, 10 $g.L^{-1}$ sodium hydroxide. 3 ml of this reagent was added to 3 ml of sample in a capped test tube, which was heated at 90°C in a water bath for 15 minutes. 1 ml of 40% potassium sodium tartrate was added to the solution which was then allowed to cool to room temperature, and absorbance was the measured at 575 nm. Distilled

water was used as a reagent blank, while glucose in the range of 0 – 1 000 $mg.L^{-1}$ was used as a standard.

2.2.8 Lipids

Lipids were determined by extraction with chloroform/methanol (2:1) using the Folch general procedure (Folch *et al.*, 1957). A 10 ml sample was added to 200 ml chloroform and 100 ml methanol and was agitated for 15 min. The combined mixture was filtered (Whatman #1), mixed with an equal volume of 0.9% NaCl solution, and shaken to wash the organic phase. The aqueous phase was decanted and discarded, and the organic phase was placed in a pre-weighed round bottom flask and removed *in vacuo*. The dried residue was then weighed, giving the lipid percentage.

2.2.9 Ion chromatography

Ion chromatography was performed on a Dionex® AI-450 chromatography system. The anion column was an AS14 (4 mm) column, and the mobile phase was 2 mM Na_2CO_3 and 1 mM $NaHCO_3$ at a flow rate of 1.2 $ml.min^{-1}$. For cations a CG12 (4 mm) column was used, while the mobile phase was 2.2 N H_2SO_4 at a flow rate of 1 $ml.min^{-1}$. Appropriate standards were used in the range of 0 – 50 $mg.L^{-1}$. Ion chromatography was performed courtesy of the Department of Geology, UCT.

2.2.10 Organic acids

Organic acids were determined by HPLC on a Varian® chromatography system. The column was a Waters® Fast Fruit Juice 7u (150 mm), which was held at 60°C, while the mobile phase was 0.1% phosphoric acid at a flow rate of 1 $ml.min^{-1}$. UV detection was at 215 nm. Standards were prepared in the range 0 – 1 000 $mg.L^{-1}$. Organic acids analyses were performed courtesy of the Main Analytical Laboratory, Chemical Engineering Department, UCT.

2.2.11 Total phenol content

Total phenol content of wastewater samples was determined colorimetrically using the Folin-Ciocalteau reagent according to the procedure of Garcia *et al.* (2001). The method was scaled down so it could be performed directly in 4ml cuvettes. 400 μL each of sample, Folin-Ciocalteau reagent (Merck) and Na_2CO_3 (20%) were added to 2.5 ml ddH_2O and mixed. This was left to stand for 90 minutes in the dark to allow the reaction to go to completion, and then absorbance was read at 765 nm. Gallic acid in the range 0 – 100 $mg.L^{-1}$ was used as a standard, and all total phenolic assays are thus reported as gallic acid equivalents (GAE). Samples were diluted where necessary to fall within the standard range and distilled water was used as a reagent blank.

2.2.12 Size exclusion chromatography for determination of molecular weight distribution of phenolic compounds

Size exclusion (gel permeation) chromatography with fractionation of the eluent was performed to obtain the molecular weight distribution of phenolic compounds using an AKTA Prime fraction collector (Amersham Biosciences). A 700x26 mm column was packed with Sephadex G-50 C (Amersham Biosciences) as the stationary phase; the mobile phase used for elution was water at $1mL.min^{-1}$. The packed column bed was equilibrated with 3 bed volumes of mobile phase prior to use. Eluting phenolic fractions were measure by UV detection at 280 nm. 2 ml sample was loaded onto the column and eluent was collected in 10 ml fractions. Dextran blue (M_w = 2 000 kDa), dextran sulphate (M_w = 5 kDa) and gallic acid (M_w = 170 Da) were used as retention time calibration standards. Humic acid (2 $g.L^{-1}$, Merck) was also run through the column as a comparison for high molecular weight polyphenolics in the wastewaters.

2.2.13 Liquid/liquid solvent extraction of low molecular weight phenolic compounds

Organic solvent extraction was performed to recover the low molecular weight phenolic compounds from wastewater samples, which were then quantified by total phenolics assay (as above). Wastewater samples (100 ml) were extracted three times using equal volumes of ethyl acetate. The aqueous wastewater and organic phases were thoroughly mixed, left to

settle and then separated using a funnel. The separated organic phases were pooled and dried over anhydrous Na_2SO_4, and then filtered. The combined organic phases were removed *in vacuo*, and the residual dried extract was resuspended in 10 ml of HPLC grade methanol for analysis.

2.2.14 High performance liquid chromatography (HPLC)

Reversed phase HPLC was used to identify and quantify of individual low molecular weight phenolic compounds. This was performed on filtered and centrifuged wastewater samples, and on the wastewater extracts. A Merck Hitachi L-7000 series machine was used. Monophenolics were separated by isocratic elution, using a mobile phase of 80:20:2.5 ddH_2O:Methanol:Acetic acid at a flow rate of 1 ml.min^{-1}. For separating the more complex phenolics and glucosides, gradient elution was performed. The mobile phase was 5% formic acid (solvent A) and methanol (solvent B) at 1 ml.min^{-1}, with solvent ratios as described by Vinha *et al.* (2005). The column was a Waters Spherisorb® S5 ODS1 4.6x250 mm, fitted with a Phenomenex guard column. UV detection was used to measure compounds at 280 nm. Individual compounds were identified by comparison of retention times against both internal and external standards. Standard curves of concentration vs. peak area were used to quantify components.

2.2.15 Synthesis of hydroxytyrosol

Alkali hydrolysis of oleuropein was performed to obtain a hydroxytyrosol standard, according to Garcia *et al.* (1996) as follows: 100 mg Oleuropein (Extrasynthese, Gamay, France) was added to 6 M NaOH (20 ml) and placed in the dark for 5 hours under N_2. The resulting solution was adjusted to pH 3 with HCl and then extracted 3 times with diethyl ether (1v/v). The combined extracts were mixed with 10 ml 0.1 M HCl, and the organic solvent was removed *in vacuo*. The remaining acidic aqueous solution was treated with 30 mg activated carbon for 20 min., and filtered to give a purified hydroxytyrosol solution at a concentration of 752 mg.L^{-1}.

2.2.16 Radical scavenging activity of olive wastewater extracts

The antioxidant activity of wastewater extracts was determined using the DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazil) free radical scavenging method according to Sanchez-Moreno *et al.* (1998). DPPH$^{\bullet}$ stock solutions were made up in methanol at 25 mg.L^{-1}. 3.9 ml of this solution was reacted with 0.1 ml of various concentrations of antioxidant standards (ascorbic and gallic acids) and extracts, in the range 10 - 1000 mg(antioxidant).g^{-1}(DPPH$^{\bullet}$). Extract concentrations were determined using the total phenols assay (section 2.2.11), and by HPLC peak areas. Radical scavenging was measured as a decrease in absorbance at 515 nm, using a reagent blank DPPH$^{\bullet}$ standard curve at 0-50 mg.L^{-1} concentrations. The ratios of antioxidant to DPPH$^{\bullet}$ were plotted against the DPPH$^{\bullet}$ concentration remaining at steady state at the end of reaction. From this plot it was possible to determine the ratio of antioxidant to DPPH$^{\bullet}$ that caused the initial DPPH$^{\bullet}$ concentration to decrease by half, which is referred to as the EC_{50} (effective concentration) and gives a comparative measure of the antioxidant activity of various compounds (Brand-Williams *et al.*, 1995).

2.3 RESULTS AND DISCUSSION

2.3.1 General wastewater analysis

General analysis indicated that all the local olive-derived wastewaters can be considered to be high strength wastewaters (in terms of COD and total phenols), unsuitable for discharge into sewers, watercourses or the ocean. Table 2.2 presents some of the data collected over 3 years in comparison to other published data from Mediterranean regions.

A wide variation in values was observed between the different types of wastewater, and also between the same types of wastewater from different sources. The organic load (measured as COD) in all cases exceeded the discharge limits stated in Section 39 of the National Water Act (> 0.075 mg.L^{-1}). Comparatively, brine wastewater data in Table 2.2 reported by other researchers had significantly lower concentrations of COD and total phenols than wastewaters analysed in this work. This could be due to two reasons: either these wastewaters come from storage facilities that are mixed with rinsing wastewaters, or they could be from traditional style processes where the brining water is changed regularly and is therefore more dilute.

Table 2.2: Data from physical and chemical analyses of olive wastewaters

	pH	Cond. ($mS.cm^{-1}$)	TS ($g.L^{-1}$)	DS ($g.L^{-1}$)	SS ($g.L^{-1}$)	COD ($g.L^{-1}$)	TOC ($g.L^{-1}$)	N ($g.L^{-1}$)	Sugar ($g.L^{-1}$)	Lipids ($g.L^{-1}$)	TP ($g.L^{-1}$)	SP ($g.L^{-1}$)	K^+ ($g.L^{-1}$)
Black olive brine (OFW)													
OW-04	4.5	83.1	114.2	113.9	0.30	75	18.35	0.20	0.26	0.83	4.91	2.89	3.71
OW-08	5.2	76.1	129.2	128.8	0.38	81	16.50	0.19	0.31	0.56	5.28	3.31	
OW-13	4.6	97.7	92.1	91.2	0.82	55	6.25	0.03	0.22	9.36	1.78	1.04	
OW-15	5.2	81.8	139.0	137.9	1.07	82	15.55	0.18	0.34	0.89	4.45	2.92	
Benitez *et al.* (2001)	12.6		8.16		0.34	3					0.24		
Beltran-Heredia *et al.* (2000)			5.72	5.52	0.20	6		0.03			0.18		
NaOH wastewater (de-bittering)													
OW-09	9.9	16.3	59.8	58.5	1.30	81	19.90	0.43	9.31	1.39	2.21	1.80	
Kotsou *et al.* (2004)	12.3	34.0	20.3	16.8	3.54	10					0.32	0.03	
Kyriacou *et al.* (2004)	11.0					22		0.002					2.5
Green olive brine (OFW)													
OW-10	4.1	79.0	114.3	113.9	0.40	57	12.70	0.02	1.18	1.08	2.61	1.65	
Owen *et al.* (2003)												1.36	
Piga *et al.* (2005)	4.8										0.50		
Mill wastewater (OMW)													
OW-06	4.9	9.6	119.1	58.8	60.3	202	15.40	0.46	12.6	74.0	4.07		3.42
OW-17	5.1	11.10	88.9	61.8	27.1	101	19.80	0.39	14.7	58.3	3.78	2.92	
Fadil *et al.* (2003)	5.2		92.4	6.2	86.2	124		0.15	12.8		8.2		
Assas *et al.* (2002)	4.9		109.0	94.0	15.0	120		0.08					
Borja *et al.* (2003)	5.1		124.0	15.8	106.6	113					1.83		
D'Annibale *et al.* (2004)	5.3				38.0	85		1.3	8.75		5.50		0.78
Raposo *et al.* (2003)	4.9		16.7	2.7		22	6.28	0.21			0.06		

TS = total solids, SS = suspended solids, DS = dissolved solids, COD = chemical oxygen demand, TOC = total organic carbon, TP = total phenols, SP = simple phenols, K^+ = potassium. All values are the mean of n = 3, standard deviation < 5%

The COD of the mill wastewaters were generally found to be higher than that of the brines, most likely because of pulp solids and olive vegetation waters that are released during the milling process. The olive vegetation waters contain carbohydrates and other cellular components (e.g. pectins and lipids), which do not occur in the brines.

The wastewaters were generally found to be acidic, due to the presence of phenolic and other organic acids. In the case of high pH this was because of the addition of NaOH during processing (debittering). The black olive brine of Benitez *et al.* (2001) is a notable exception; it had an alkaline pH, and this is most likely because an NaOH post-treatment is sometimes performed to enhance and fix the colour of black olives. The brines were also highly saline (as indicated by conductivity and dissolved solids) from the salt used during processing.

Dissolved solids accounted for almost all total solids in the brines. This increases the resistance of these wastes to biological degradation, as both phenol- and halotolerant

microorganisms are required. Suspended solids in brines were generally low compared to mill wastewaters, as the mill wastewaters contain large amounts of pulped olive solids. Suspended solids in the brines were predominantly a result of residual biomass from the brining process, and were mostly volatile upon firing.

The residual oil content of the mill wastewaters analysed in this work was of the order 6 – 7%. This is due to the formation of a stable oil-water emulsion that is not readily separated by the centrifuges used for oil extraction. The brines contained minimal lipids, with the notable exception of OW-13, which was wastewater from the traditional small-scale producer. In this case oil was added to fermentation barrels as part of the brining process, in order to prevent contamination of the olives by pathogens.

The mill wastewaters had a high concentration of sugar compared to the black and green olive brines. The lack of sugar in the brines is due to a) the absence of cell disruption and b) the fact that microorganisms consume sugars during the brining process (Brenes and De Castro, 1998; Benitez *et al.*, 2001). An exception was the NaOH de-bittering wastewater (OW-09); it had a high sugar content due to the hydrolysis of oleuropein, which results in the production of elenolic acid glucoside (see section 1.3, Figure 1.2).

Olive wastewaters are known to be deficient in nitrogen (Hamdi, 1993; Sayadi *et al.*, 2000). Analysis results confirmed this to be the case for local wastewaters; total nitrogen in all cases was less than $1 g.L^{-1}$. Nitrogen is essential for microbial action and therefore supplementation is commonly performed before biological degradation. Ion chromatography showed that there were appreciable concentrations of other trace elements, particularly potassium (at 3.7 $g.L^{-1}$ for OW-04), phosphorus, and sulphur.

The total phenol concentrations in the brines were found to be of the same order as in the mill wastewaters, and can be considered to be highly concentrated (in the order of $g.L^{-1}$), equal to or greater than many industrially produced phenolic waste streams. In most olive wastewaters the phenolic compounds occurred at concentrations exceeding 700 $mg.L^{-1}$, at which phytotoxic and antimicrobial effects are generally observable (Visioli *et al.*, 1999). In terms of black olive brines, OW-04, -08, and –15 came from the production of *Calamata* olives from the large production facility, and were from different fermentation batches. Values were relatively consistent between these samples, and greater than OW-13, which was

from the small-scale traditional producer. Lower COD and total phenol values in OW-13 resulted from a shorter brining period (only two weeks as opposed to six months), which was followed by regular changes of fresh water.

The simple (low molecular weight) phenolic compounds in the brine samples accounted for a significant percentage of the total phenols, and were in the same range as those reported by other researchers (Owen *et al.*, 2003; de Castro and Brenes, 2001; Brenes and de Castro, 1998). Black olive brines contained the highest concentration, followed by green olive brine and the NaOH wastewater. The green olive brine and NaOH wastewaters, however, had higher percentages of simple phenolics in relation to total phenols (63 and 82% respectively). This is because these wastewaters do not contain higher molecular weight polymerised phenolics and tannins that give black olive brines and OMW their dark colour. Phenolic compounds are discussed in more detail in the following section.

HPLC chromatograms of organic acids in various wastewater samples are shown in Figures 2.1a-d. Figure 2.1a shows the profile of a typical black olive brine. In this sample lactic acid occurred at a concentration of 14.38 $g.L^{-1}$, which compares well with published data of around 16 $g.L^{-1}$ (Duran Quintana *et al.*, 1999; Chorianopoulos *et al.*, 2005). Peak 2 was unidentified, while peak 3 is elenolic acid. It was not possible to quantify the elenolic acid, as it was not available as a pure compound. It was, however, positively identified, as it is the only organic acid component from the hydrolysis of oleuropein for the production of hydroxytyrosol (see section 2.3.4). This hydrolysate was therefore used a reference. Green olive brines gave very similar organic acid profiles to that shown in Figure 2.1a.

Lactic, acetic and elenolic acids are known to be common in black and green olive brines, while malic and citric acids have been reported in lesser quantities (de Castro and Brenes, 2001; Duran Quintana *et al.*, 1999; Brenes and De Castro, 1998). Figure 2.1b shows the organic acid profile of NaOH wastewater used for the debittering pre-treatment of green olives, before they are brined. The pre-treatment is performed to extract oleuropein, which is responsible for the bitterness of green olives (see section 1.3, Figure 1.2). Oleuropein degrades into elenolic acid glucoside and hydroxytyrosol during the pre-treatment. Acidifying this wastewater to pH 2 with HCl results in the profile shown in Figure 2.1c.

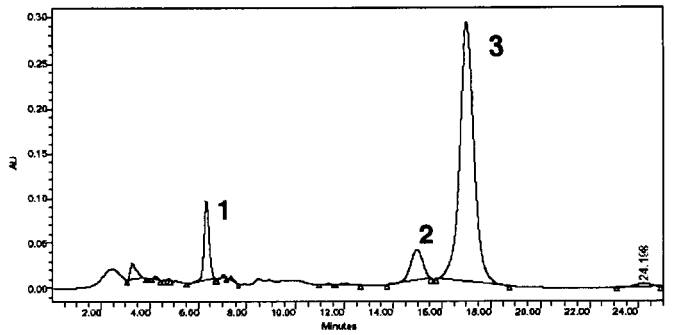


Figure 2.1a: Organic acids in black olive brine OW-08.
1) Lactic acid 2) Unknown 3) Elenolic acid

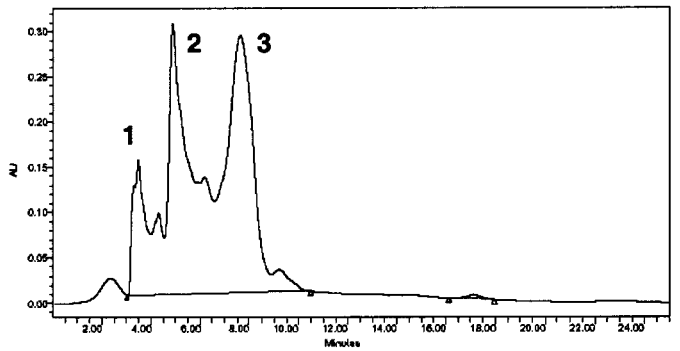


Figure 2.1b: Organic acids in NaOH debittering wastewater OW-09
1) Citric acid 2) Gluconic acid 3) Unknown

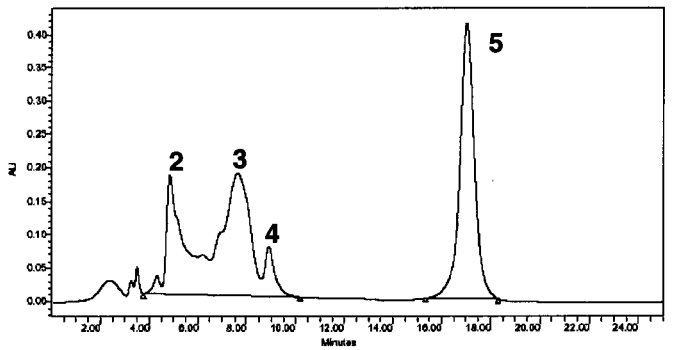


Figure 2.1c: Organic acids in NaOH pretreatment wastewater OW-09 after acid hydrolysis
2, 3) as above 4) Propionic acid 5) Elenolic acid

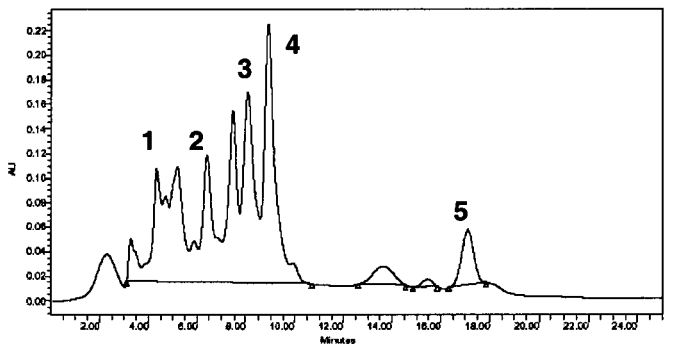


Figure 2.1d: Organic acids in olive mill wastewater sample OW-17
1) Citric acid 2) Lactic acid 3) Acetic acid 4) Propionic acid 5) Elenolic acid

It was evident that a significant amount of elenolic acid was produced through the hydrolysis of the glucoside, although the glucoside itself was not positively identified for lack of a standard. In contrast, when the black and green olive brines were subjected to acid hydrolysis there was minimal change in composition. This indicates that elenolic acid glucoside is hydrolysed during the brining process. Microorganisms then consume the glucose (Brenes and de Castro, 1998; de Castro and Brenes, 2001), while the elenolic acid remains present in the brine solutions.

An example of an organic acid profile from a mill wastewater sample is shown in Figure 2.1d. These wastewaters generally have more organic acid components than occur in the brines. Compounds reported to occur in OMW include: formic acid, succinic acid, palmitic acid, as well as all compounds mentioned above (Pinelli *et al.*, 2003). Whether this is a result of the milling process (in the case of oil), or of microbial activity (in the case of brines), is uncertain. Table 2.3 shows summarised results of organic acids in various wastewaters analysed in this work compared to that of other researchers, where possible.

Table 2.3: Organic acids present in olive wastewaters

	Citric ($g.L^{-1}$)	**Lactic** ($g.L^{-1}$)	**Acetic** ($g.L^{-1}$)	**Propionic** ($g.L^{-1}$)
Black olive brine				
OW-04	n.d.	13.24 ± 0.63	n.d.	n.d.
OW-08	n.d.	14.38 ± 0.85	n.d.	n.d.
Green olive brine				
OW-10	0.23 ± 0.06	16.08 ± 0.37	0.31 ± 0.01	0.07
Brenes & De Castro (1998)	n.d.	10.44 – 13.08	0.73 – 1.88	n.d.
Duran Quintana *et al.* (1999)	0.4 ± 0.04	1.54 ± 0.07	0.48 ± 0.01	
NaOH wastewater				
OW-09	0.35 ± 0.05	0.17 ± 0.02	0.76 ± 0.18	0.14
Brenes *et al.* (2000)	n.d.	3.78	2.03	0.09
Mill wastewater				
OW-17	0.29 ± 0.04	0.11 ± 0.03	8.94 ± 0.09	2.83 ± 0.04
Dionisi *et al.* (2005)	n.d.	n.d.	1.32	0.37

n.d. = not detected

Elenolic acid concentrations in the brined olive wastewater samples were greater than in the mill wastewater samples analysed in this study. Elenolic acid is known to have antioxidant and antimicrobial properties (Briante *et al.*, 2002, 2001; Visioli *et al.*, 1999, 1995; Obied *et*

al., 2005), and is a possible valuable component of the brines that could be recovered for commercial purposes.

2.3.2 Analysis of phenolic compounds in olive wastewaters

As discussed in Section 1.3, the phenolic compounds in olive wastewaters comprise a large range in terms of molecular weight distribution. A typical molecular weight distribution of black olive brine, determined by size exclusion chromatography, is illustrated in Figure 2.2. In size exclusion chromatography the larger compounds elute first, and therefore the peak occurring around fraction 17 represents highly polymerised phenolic compounds such as lignans and humic substances. The sizes of the eluting compounds became progressively smaller until the second major peak around fraction 44, which includes condensed tannins and other pigmented polyphenols. The large peak at fraction 49 consisted of monomeric phenolic compounds, predominantly hydroxytyrosol. This was verified by subjecting the individual fractions to HPLC.

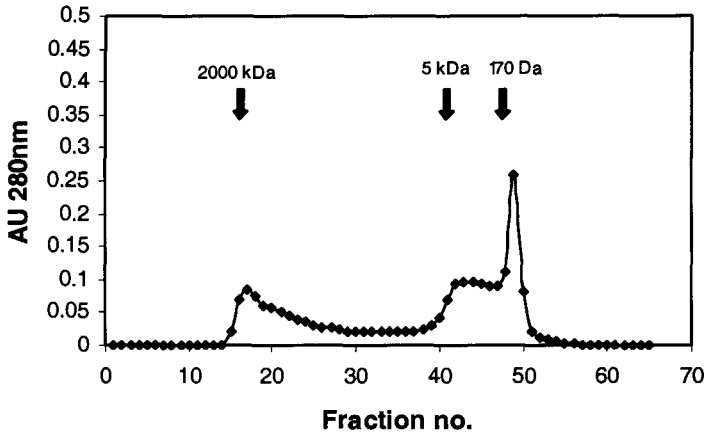


Figure 2.2: Molecular weight distribution of phenolic compounds in black olive brine OW-15

Green olive brines and NaOH pre-treatment wastewater exhibited primarily the last peak, with only small traces of tannins. Mill wastewaters contain similar size fractions, although relative quantities of the different fractions vary (Assas *et al.*, 2002; Ayed and Hamdi, 2003, 2002).

Isocratic elution HPLC chromatograms for the various different wastewaters are shown in Figure 2.3. Hydroxytyrosol is the main component of the all the brine wastewater samples. Excepting (d) OW-13, which is the dilute brine from the small traditional table olive producer, and (f) OW-17, which is a mill wastewater sample, hydroxytyrosol occurs at concentrations in excess of 1 $g.L^{-1}$. It accounts for approximately 70% of the low molecular weight phenolics. Tyrosol and 4-hydroxyphenylacetic acid were also routinely detected in the brines, at concentrations around 0.2 and 0.1 $g.L^{-1}$ respectively. Other compounds identified in trace quantities were gallic acid, 3,4-dihydroxyphenylacetic acid and protocatechuic acid.

It is evident from Figure 2.3 that the brines have simpler profiles exhibiting less compounds than mill wastewaters. Presumably this is because many compounds that are released into the mill wastewaters from crushing the olives are not extracted from the olives during brining. Mill wastewaters are known to contain phenolic glucosides and hydrolysable tannins that are present in the olive fruit, but do not occur in the brines (Mulinacci *et al.*, 2001; Piga *et al.*, 2005).

Acid hydrolysis was therefore performed (with HCl, to pH 2) on the wastewaters to observe possible changes in profiles due to hydrolysis of glucosidic compounds or hydrolysable tannins. Gradient elution was performed in this case as glucosides and tannins are less polar than the simple phenolics, and thus did not elute with the isocratic mobile phase. These chromatograms are shown in Figure 2.4.

Acid hydrolysis had little effect on the black olive brines. Figures 2.4 (a) and (b) show essentially the same chromatographic profiles, with the main and secondary peaks being hydroxytyrosol and tyrosol respectively. Concentrations of these two substances remained unchanged, and there were only minor traces of other simple phenolic compounds. This illustrates that there are no glucosidically bound complex phenolics or hydrolysable tannins in the black olive brines (e.g. anthocyanins). This is in agreement with Piga *et al.* (2005), who demonstrated that anthocyanins do initially occur in black olive brines, but these disappear during fermentation and are replaced by more stable larger pigmented polyphenols (such as condensed tannins).

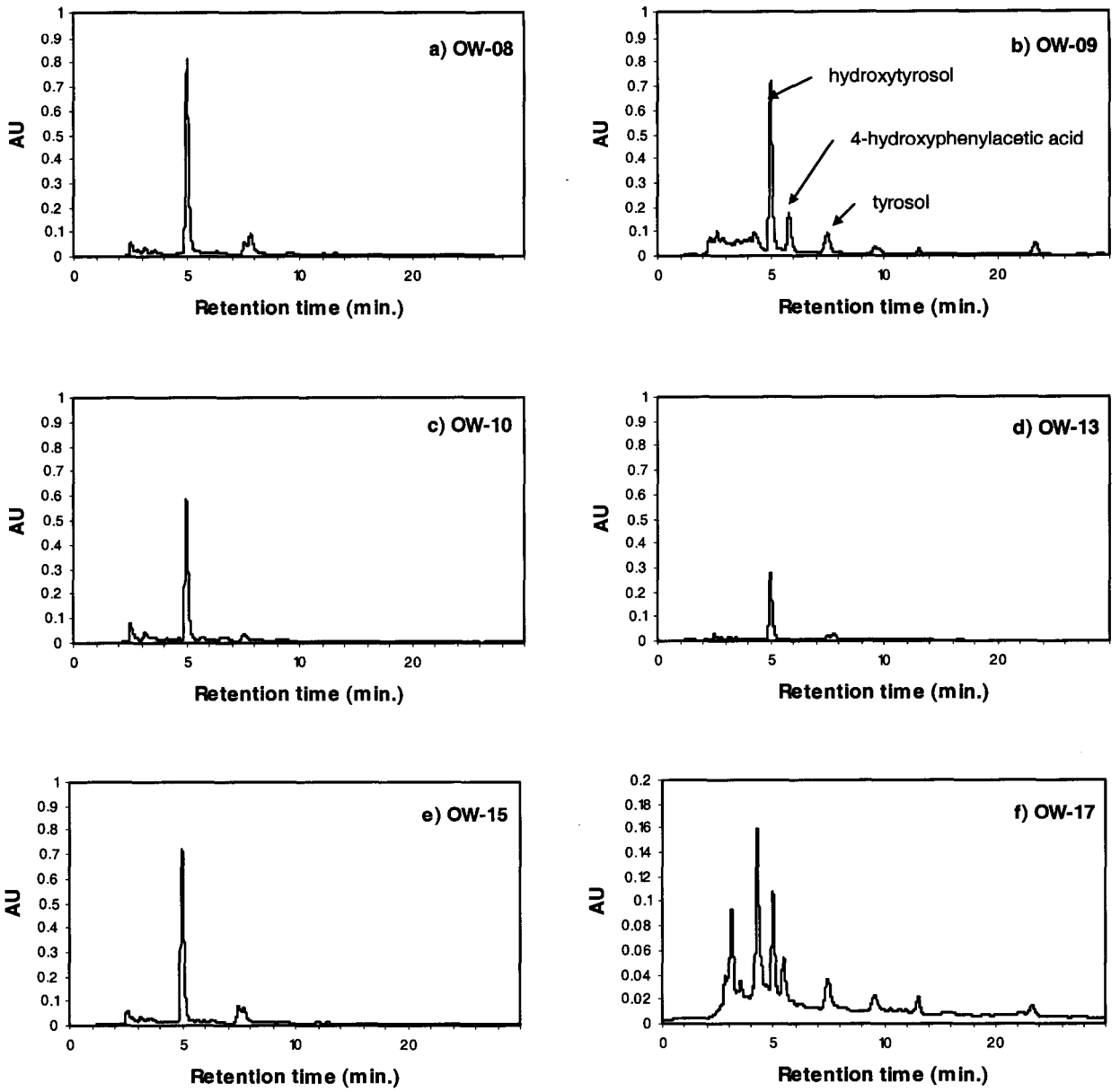


Figure 2.3: HPLC chromatograms of wastewater samples

a) Black olive brine, b) NaOH green olive debittering wastewater, c) Green olive brine, d) Black olive brine, e) Black olive brine, f) Mill wastewater.

In contrast, the mill wastewater samples shown in Figures 2.4 (c) and (d) did undergo slight alteration after hydrolysis. See for example the peaks that elute around 18 and 29 minutes. It was evident again that the mill wastewater samples contained a larger diversity of phenolic compounds than the brines. However, hydroxytyrosol was the most abundant, and its concentration in the mill wastewater increased slightly after hydrolysis. The vertical scales of the brine and mill wastewater chromatograms are not the same: the brines have more than twice the hydroxytyrosol concentration than the mill wastewaters. The sample OW-15 in

Figure 2.3 (e) was diluted 1 in 2 and therefore shows a smaller peak than those shown in Figure 2.4 (a) and (b), which were undiluted.

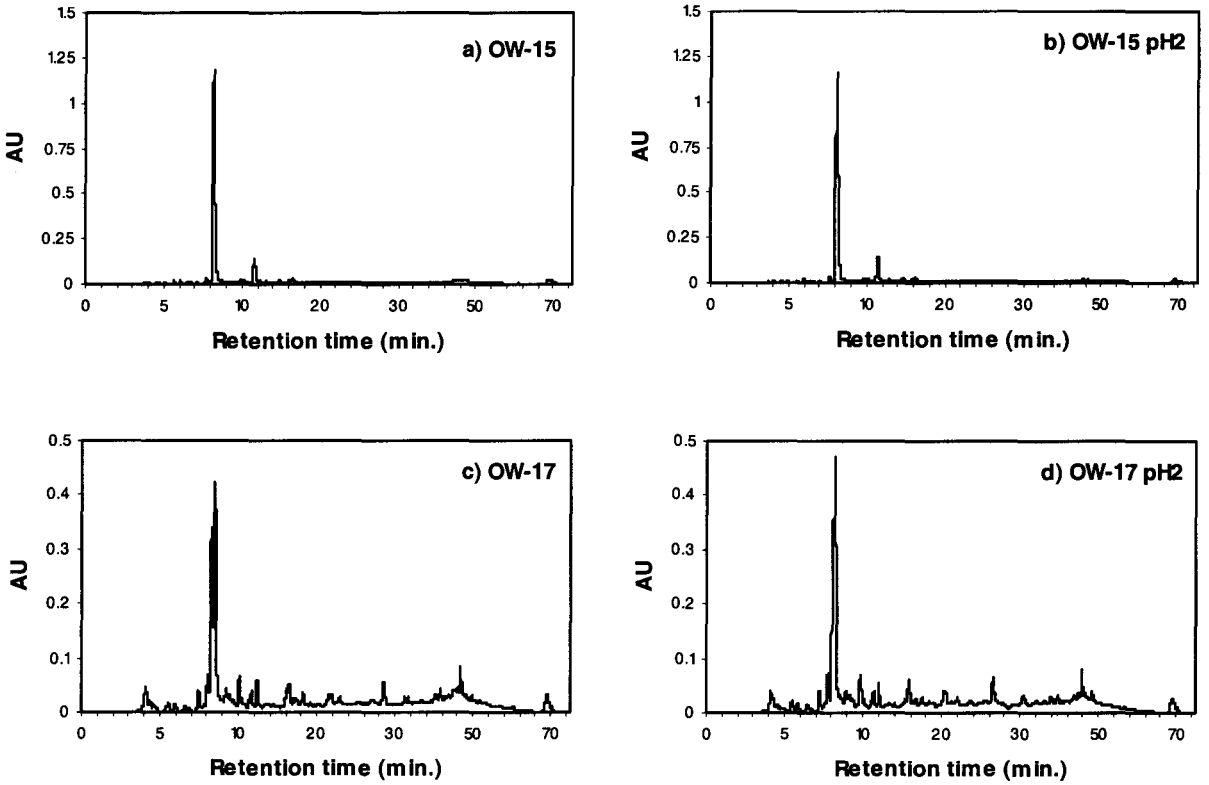


Figure 2.4: Wastewater samples before and after acid hydrolysis

a) black olive brine, b) black olive brine acidified to pH2,
c) mill wastewater, d) mill wastewater acidified to pH2.

Figure 2.5 shows HPLC chromatograms of black olive brine before and after liquid/liquid extraction, using ethyl acetate, for recovery of low molecular weight phenolic compounds. In this sample hydroxytyrosol occurred in the brine at a concentration of 1.21 $g.L^{-1}$, and was extracted at a yield of 0.92 $g.L^{-1}$. Mulinacci *et al.* (2001) report a maximum hydroxytyrosol concentration in mill wastewaters of 0.131 $g.L^{-1}$, while extraction yields have been reported to range from 0.065 - 0.091 $g.L^{-1}$ (Capasso *et al.*, 1999, 1994). Hydroxytyrosol concentrations generally appear to be higher in fermentation brines than mill wastewaters; values ranging from 0.60 - 4.84 $g.L^{-1}$ have been reported to occur in the brines (Owen *et al.*, 2003; de Castro and Brenes, 2001; Brenes and de Castro, 1998).

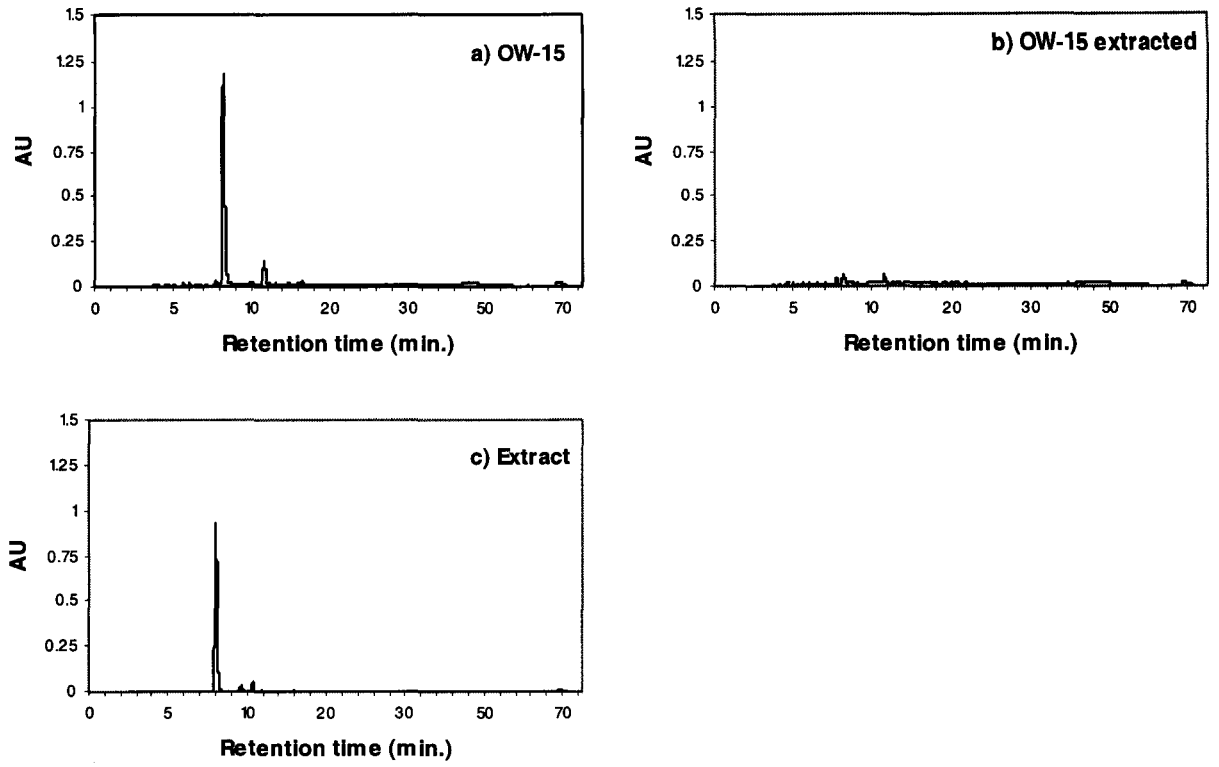


Figure 2.5: Extraction of low molecular phenolic compounds from wastewaters

a) Black olive brine OW-15 before extraction, b) after extraction,
c) extract (diluted back to original wastewater volume).

COD determination was performed on extracts from the different wastewater samples, after they had been resuspended in water equivalent to the original sample volume. This allowed for evaluation of the portion of COD attributable to simple phenolics. Simple phenols were accountable for 65, 48 and 34% of total COD for the green olive brine, NaOH wastewater and black olive brines, respectively. The NaOH wastewaters had significant amounts of sugar, while the black olive brines contained polymerised phenolics and tannins that accounted for most of the remaining COD. In the mill wastewater samples, extractable simple phenolic were responsible only for around 12% of the total COD.

2.3.3 Antioxidant activity of phenolic extracts from olive brines

Black olive brine extract (as in Figure 2.5 (c)) was used for the investigation of antioxidant activity, as it contained the highest quantity of simple phenolics. The extracts also contained elenolic acid, and therefore the antioxidant activity cannot be ascribed completely to the phenolic components, however they are expected to predominate.

Figure 2.6 shows the final DPPH$^{\bullet}$ concentrations after reaction with various increasing ratios of extract and antioxidant standards to DPPH$^{\bullet}$. From this plot it is possible to estimate the amount of antioxidant necessary to reduce the initial DPPH$^{\bullet}$ concentration by half (EC_{50}). Estimates of EC_{50} from Figure 2.6 are 12 $mg.g^{-1}$ for gallic acid, 14 $mg.g^{-1}$ for the extract and 98 $mg.g^{-1}$ for ascorbic acid. Comparatively, Bouzid *et al.* (2005) reported an EC_{50} of approximately 24 $mg.g^{-1}$ for a hydroxytyrosol extract from olive mill waste.

The antioxidant activity of the extract is thus very close to that of gallic acid, which is considered to be a powerful antioxidant (Brand-Williams *et al.*, 1995). This activity is mostly attributable to the hydroxytyrosol content of the extract, as it is known that hydroxytyrosol is a more potent antioxidant than tyrosol and elenolic acid (Tuck and Hayball, 2002). It is notable that the radical scavenging activity of hydroxytyrosol is much higher than commercially used lipidic food preservatives such as butylated hydroxyanisole (BHA, EC_{50}=93 $mg.g^{-1}$; Sanchez Moreno *et al.*, 1998).

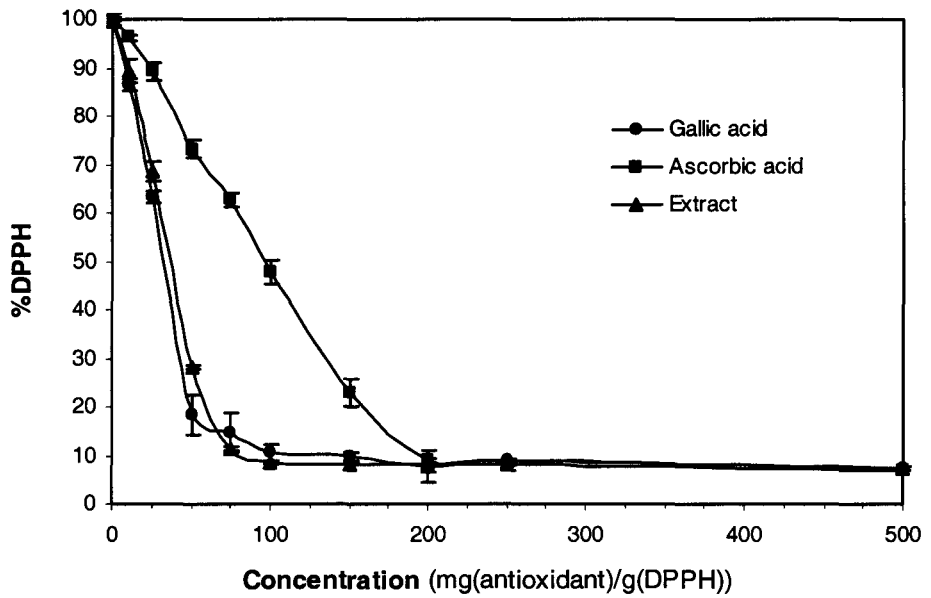


Figure 2.6: Percentage DPPH remaining at steady state after reaction with increasing concentrations of antioxidant

2.4 CONCLUSIONS

A detailed analysis was performed on brined olive and related wastewater samples, and on olive mill wastewater samples for comparison. These wastewaters can be considered to be of high strength, due their high phenolic content and high organic load (COD). As such these wastewaters are unsuitable for discharge into sewers or the environment, particularly because of their antimicrobial activity, which makes them resistant environmental biodegradation.

Although the olive-derived wastewaters are complex effluents containing many organic and inorganic components, there are several important distinctions between the black olive brine wastewaters and the (more extensively studied) mill wastewaters (OMW). Mill wastewaters contain high quantities of suspended solids from the olive pulp, and readily available carbohydrates, which are not present in the brines. The brines, in contrast, contain high quantities of dissolved solids, particularly salt (NaCl) that is used during the brining process. The high salt concentration is recognised to be detrimental to biological degradation processes.

Size exclusion chromatography of phenolic compounds in brined olive wastewater samples illustrated that there was a large molecular weight distribution, including low molecular weight monomeric phenols, tannins, and highly polymerised polyphenolic compounds approaching 2 000 kDa. HPLC analysis of the brined black olive wastewater samples before and after acid hydrolysis indicated that the tannins were condensed, whereas mill wastewater samples contained both condensed and hydrolysable tannins.

The brines were shown to contain considerable concentrations of low molecular weight phenolic compounds (more than occur in mill wastewaters and significantly more than occur in olive oil), particularly the valuable compound hydroxytyrosol. Comparatively, the mill wastewater samples contained lower concentrations of low molecular weight phenolics, but there was a much larger diversity of compounds.

A crude phenolic extract of black olive fermentation brine was shown to have powerful antioxidant activity. These waste streams could thus serve as a source of valuable phenolic antioxidants, subject to the development of an efficient extraction system. Elenolic and lactic acids are other components of the brines that could be recovered for commercial purposes.

The fermentation brines appeared to be more attractive than mill wastewaters for the recovery of phenolic antioxidants. This is mainly because they contain hydroxytyrosol, as the predominant low molecular weight phenolic component, at high concentrations. In addition, brines have low suspended solids concentrations, compared to mill wastewaters. This is advantageous from a processing point of view.

In crude form, such extracts could be used as natural food preservative or as nutraceuticals, or they could be further processed to yield hydroxytyrosol in pure form, which has a high market value. Hydroxytyrosol occurred in locally produced black olive brine in excess of $1 g.L^{-1}$, and since millions of litres of this waste are produced and discarded annually, this waste source could be of considerable potential economic interest.

Simple phenolics account for between 34% and 65% of the total COD of the brines. Extraction of the low molecular weight phenolic components of this waste can thus also be beneficial in subsequent biological treatment, as the organic load of the remaining wastewaters can be significantly reduced.

CHAPTER 3: THE USE OF MEMBRANE-ASSISTED SOLVENT EXTRACTION FOR THE RECOVERY OF PHENOLIC ANTIOXIDANTS FROM OLIVE BRINES

3.1 INTRODUCTION

The low molecular weight phenolic antioxidants that occur in olive wastewaters have considerable potential as value-added products, as discussed in Chapter 2. Hydroxytyrosol, in particular, has high market value as a pure compound (approximately \$US 1 000 g^{-1}). It has also recently been made available as an antioxidant nutraceutical under several different trade names. Various procedures for the recovery of hydroxytyrosol from olive mill wastewaters have been described (Fki *et al.*, 2005; Visioli *et al.*, 1999; Capasso *et al.*,1999, 1994). Hydroxytyrosol has also been recovered from the solid residue that results from the olive milling and oil extraction process, using a hydrothermal treatment (Fernandez-Bolanos *et al.*, 2004, 2002). Two comprehensive reviews about the recovery, analysis, antioxidant activity and commercial use of hydroxytyrosol have recently been published (Allouche *et* al., 2004; Obied *et al.*, 2005). Although the occurrence of hydroxytyrosol in olive fermentation brines and de-bittering wastewaters has previously been reported (Brenes *et al.*, 2004, 2000, 1998, 1995; Aggelis *et al.*, 2001; de Castro and Brenes, 2001; Benitez *et al.*, 2001, 1999; Beltran-Heredia *et al.*, 2000), there is minimal scientific literature concerning its recovery from these sources.

The two most common methods used for the recovery of phenolic compounds from an aqueous liquid are solid phase extraction and liquid/liquid extraction. Combinations of these two processes are also widely employed. Liquid/liquid extraction (as described in Chapter 2) involves the mixing of a solute-containing aqueous phase with an immiscible organic solvent phase. Mass transfer of solute occurs from the aqueous phase into the organic phase by diffusion, due to higher solubility of solute in the organic solvent and the concentration gradient that exists between the two phases. The mixture is then allowed to settle and phase separation is performed. Organic solvents used for liquid/liquid extraction are usually volatile, and thus the solutes can be recovered by subsequent evaporation of the solvent. There exists a solubility distribution coefficient between the aqueous and organic phases; therefore several consecutive extraction steps are required for complete recovery of solute(s).

Liquid/liquid extraction of hydroxytyrosol from mill wastewaters is well documented, and a continuous counter-current extraction process for industrial purposes has been optimised (Fki *et al.*, 2005; Allouche *et al.*, 2004).

The disadvantages of liquid/liquid extraction are that large quantities of solvent are required, and foaming and the formation of stable emulsions may occur during mixing. If so, long time periods are necessary for phase separation of such emulsions. There is also significant complexity involved in automating and controlling the liquid/liquid extraction process, especially for continuous systems. In addition, mixing and extraction systems in general are expensive and energy-intensive. However, liquid/liquid extraction remains the most common method for the recovery of phenolics in industry, as it is applicable to highly concentrated waste streams containing concentrations of > 3000 $mg.L^{-1}$ (Doherty and Malone, 2001).

In solid phase extraction, a liquid stream is passed through a chromatographic column or packed bed containing a selective particulate solid phase. The solid phase bed material has a higher affinity for the phenolic solutes than the aqueous phase, and therefore the solutes adsorb onto the solid surface. After adsorption, the phenolics can be eluted from the solid phase using an organic solvent such as methanol or ethanol. The solvent containing the extracted phenolics can then be removed *in vacuo*, and the extract can be further purified through successive chromatographic steps or fractionation. Many variations of the solid phase extraction process are possible. For example, Visioli *et al.* (1999) describe a process whereby mill wastewaters were first lyophilised (freeze dried) to obtain a solid residue, which was then purified through chromatographic fractionation.

An advantage of solid phase extraction is that considerably smaller quantities of organic solvent are used compared to liquid/liquid extraction, but a major disadvantage is that loading rates for sorbents are relatively low, and therefore this method is generally only applied directly to dilute phenolic waste streams with concentrations less than 1000 $mg.L^{-1}$ (Rodriguez *et al.*, 2000). For industrial-scale operations large quantities of sorbent are required which tends to make the process uneconomical compared to liquid/liquid extraction, as the sorbents are costly (Earhart *et al.*, 1976). Solid phase extraction has, however, been used to recover pectin and phenolic compounds directly from wastewaters arising from the production of apple juice, although the concentrations of phenolic compounds was notably lower than occur in olive mill wastewaters and fermentation brines (Schieber *et al.*, 2003).

High salt concentrations are also known to adversely affect solid phase extraction efficiency of phenolic compounds (Rodriguez *et al.*, 2000), and therefore the application of solid phase extraction for the recovery of phenolic compounds from olive fermentation brines is unlikely to be successful.

In this study, solid phase extraction was investigated in preliminary extraction experiments, using Amberlite XAD-40 adsorbent resin packed into a small laboratory-scale column. As anticipated, the process was found to be unsatisfactory, due to the high concentrations of phenolic components and salt in the wastewater. The column rapidly became overloaded and breakthrough of the low molecular weight phenolic components occurred at less than one bed volume of wastewater. In addition, higher molecular weight pigmented phenolics (tannins etc.) also adsorbed onto the column, and several bed volumes of washing water were not sufficient to remove these. As a result, after adsorption, the organic solvent used for elution contained a mixture of low and high molecular weight phenolics, and therefore a secondary purification step would be required for the separation of these fractions. Further research of solid phase extraction for the recovery of low molecular weight phenolic compounds from the fermentation brines was therefore abandoned.

The focus of work described in this chapter is a membrane-assisted solvent extraction process for the recovery of the low molecular weight phenolic compounds from olive fermentation brines. It is a relatively new technology, and has been made possible by rapid advances in the polymer sciences. Membrane-assisted solvent extraction is based on a similar principle to liquid/liquid extraction, except that the aqueous and (extracting) organic phases are not mixed; they are separated by a hydrophobic (water resistant) membrane, which is non-permeable to liquids at low pressures. The compounds to be extracted diffuse through this membrane from the aqueous phase into the solvent phase in a process called membrane contacting, but the liquid phases do not pass through the membrane because of the hydrophobicity. In terms of mass transfer there are similarities between this process and dialysis. This method has been investigated for several applications such as the separation-concentration of phenol (Urtiaga *et al.*, 1992a, 1992b) and cresol (Ferreira *et al.*, 2005) from aqueous waste streams, recovery of valeric acid (Rodriguez *et al.*, 1997) and the extraction of aroma compounds from fermentation broths (Baudot *et al.*, 2001). As yet, the technique has not been investigated as a method for the extraction of the valuable phenolic antioxidants from olive wastewaters.

The main advantage of membrane-assisted solvent extraction is that since there is no mixing of the aqueous and organic phases, subsequent separation and decanting are not required. This makes the system design very simple. The formation of emulsions is avoided, and in addition, the method is well suited to continuous processing. A further benefit is that no pre-treatment (filtration or settling) of wastewater is required, as compared to conventional liquid/liquid or solid phase extraction. Because the extraction is a diffusion-based process, there are no significant pressure requirements; small pumps are sufficient, and thus energy costs are kept to a minimum. The membrane system used for these applications is usually a module consisting of many long thin hollow fibres. In such modules, there is a large surface area available for mass transfer incorporated into a small volume, and this results good extraction rates, as well as compact plant size (small footprint) compared to liquid/liquid extraction. A disadvantage of membrane-assisted solvent extraction is that the membrane offers additional resistance to mass transfer, but this can be overcome by the use of membrane modules with large surface area to volume ratios.

The objective of the work reported in this section was thus to determine the feasibility of using membrane-assisted solvent extraction for the recovery low molecular weight phenolic antioxidants from olive fermentation brines. After initial proof of concept, the efficiency of the extraction process was evaluated by determination of the overall mass transfer coefficient for a batch extraction process, and this result was compared to mass transfer coefficients determined by other researchers using similar processes for various purposes. Thereafter, conclusions were drawn and recommendations are made concerning the application of this technology to the olive industry.

3.2 MASS TRANSFER THEORY

Calculation of the overall mass transfer coefficient for an extracting solute in a membrane module is a complex mathematical problem due to the variation of concentration driving force along the axial length of the membrane, which is affected by flow rates, concentrations, distribution coefficients and time. Several mathematical models have been proposed and evaluated for determination of the overall mass transfer coefficient. These include models based on fundamental principles (Urtiaga *et al.*, 1992a, 1992b), Wilson-plot methodology (Viegas *et al.*, 1998) and the Olander and Hatta models (Ferreira et al., 2005). In this work mass transfer within the membrane module was modeled according to Gonzalez-Munoz *et al.*

(2003). These authors provide an analytical solution for the mass transfer coefficient based on solute concentrations in the two phases and various mass balances. The complete mathematical derivation appears in Appendix A. The model was developed for bulk aqueous and organic phases contained in separate reservoirs, with both phases continuously recycled through the membrane unit in a co-current manner. It is assumed the initial concentration of solute in the organic phase is zero.

The equation for mass transfer can be described by:

$$J_s = K_a A_m (c_a - c_a^*) \tag{3.1}$$

where J_s = overall solute flux ($g.s^{-1}$)

K_a = overall mass transfer coefficient ($m.s^{-1}$)

A_m = membrane surface area (m^2)

c_a = solute concentration in the aqueous phase at time t ($mg.L^{-1}$)

c_a^* = solute concentration in aqueous phase in equilibrium with organic phase at time t ($mg.L^{-1}$)

The objective is thus to determine the coefficient K_a in equation (3.1). By performing various steady- and unsteady state mass balances, and several integrations, the following expression is obtained for the solute concentration over time in the aqueous phase:

$$c_a(t) = \frac{V c_a(0)}{1+V} + \frac{c_a(0)}{1+V}\exp(-ct) \tag{3.2}$$

where

$$c = \frac{Q_a}{V_a}\frac{(1+V)}{(1+Q)}\left[1-\exp\left[-\frac{A_m K_a}{Q_a}(1+Q)\right]\right] \tag{3.3}$$

$$V = \frac{V_a}{V_o D}$$

$$Q = \frac{Q_a}{Q_o D}$$

and

$c_a(0)$ = initial concentration of solute in the aqueous phase ($mg.L^{-1}$)

V_a, V_o = volume of aqueous and organic phases respectively (m^3)

Q_a, Q_o = flow rate of aqueous and organic phases respectively ($m^3.s^{-1}$)

D = distribution coefficient of solute between the aqueous and organic phase (-).

Equation (3.2) can be expressed in the form:

$$c_a = a + b\exp(-ct) \tag{3.4}$$

where a and b depend only on known parameters, and the value of c can be obtained from the slope of a straight line plot of $\ln(c_a(t) - a)$, *i.e.* the concentration in the aqueous phase feed tank over time minus coefficient a. Equation (3.3) can be re-arranged to solve for K_a:

$$K_a = \frac{-Q_a}{(1+Q)A_m}\ln\left[1 - c\left(\frac{V_a}{Q_a}\right)\left(\frac{1+Q}{1+V}\right)\right] \tag{3.5}$$

and a value for the mass transfer coefficient can thus be obtained.

3.3 MATERIALS AND METHODS

3.3.1 Materials

The wastewater used for the membrane extraction experiments was black olive fermentation brine (OW-08) as discussed previously. The organic solvent was ethyl acetate. A Microdyne polypropylene hollow fibre membrane module (MD020CP2N) was used as contactor, with the following properties:

Membrane specifications	$n = 40$
	ID = 1.8mm, OD = 2.0 mm
Pore size	0.2 μm
Total membrane surface area	$A_m = 0.1\ m^2$
Lumen cross-section area	$A_l = 2.5 \times 10^{-6}\ m^2$
Shell cross-section area	$A_s = 1.8 \times 10^{-4}\ m^2$
Membrane length	$L = 0.5$ m

3.3.2 Membrane extraction experimental system

Figure 3.1 shows a schematic diagram of the membrane extraction system, and Figure 3.2 shows a photograph of the actual system used for extraction experiments. Reservoirs of wastewater and solvent were placed on magnetic stirrers to ensure proper mixing of the bulk phases. The reservoirs were 1 L Schott bottles, and were sealed to prevent evaporative losses. The aqueous and organic phases were recycled through lumen and shell space of the membrane module respectively, using peristaltic pumps, and returned to their respective reservoirs. Gonzalez-Munoz *et al.* (2003) reported that a slight overpressure of the aqueous phase in the membrane lumen is necessary to stabilise the interface between the two phases within the membrane, and therefore a flow-control needle valve was fitted to the aqueous phase lumen outlet.

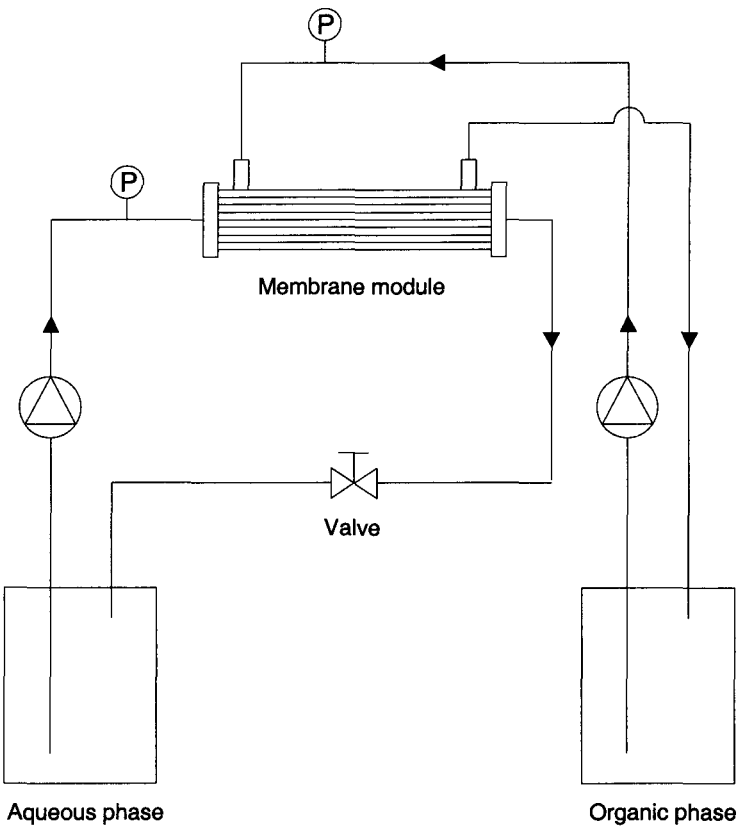


Figure 3.1: Schematic diagram of the system used for membrane-assisted solvent extraction

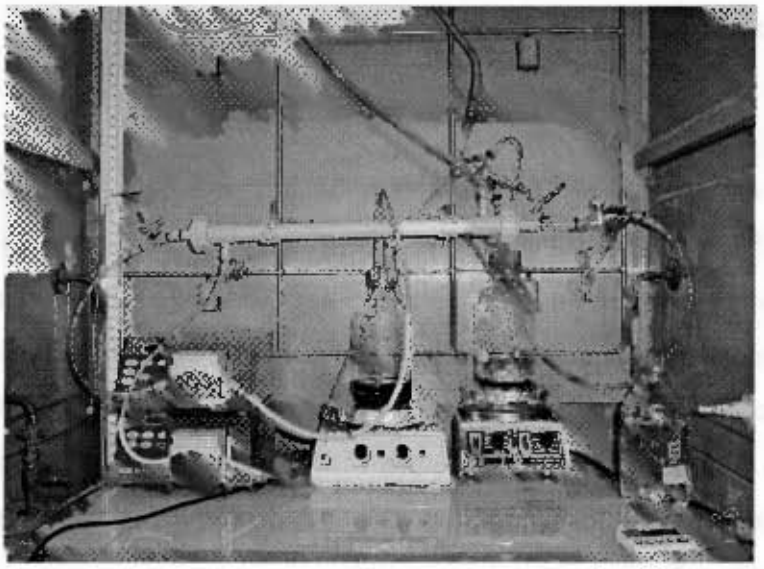


Figure 3.2: Laboratory-scale experimental membrane extraction system

Transmembrane pressure between the aqueous and organic phases was measured using a mercury manometer. Flow rates (and corresponding Reynolds numbers) were set so as to ensure minimal boundary layer resistance to mass transfer in the aqueous and organic phases. Gonzalez-Munoz *et al.* (2003) recommended that the aqueous and organic phase Reynolds numbers are at least 4 and 0.4 respectively. This ensures minimal concentration boundary layer resistance to mass transfer at the membrane surface, and increasing the Reynolds numbers above these values has no appreciable effect upon improving the mass transfer coefficient. All experiments were performed at ambient temperature ($25 \pm 2^{\circ}C$).

3.3.3 Experimental procedure for the determination of the overall mass transfer coefficient

The distribution coefficients of the various phenolic solutes between the aqueous and organic phase were first determined. Six dilutions of wastewater (0 – 100%) were mixed with an equal volume (20 ml) of organic solvent, shaken for 30 minutes and left to settle. This allowed for concentrations of solutes in the two phases to reach equilibrium. After separation, concentrations in the aqueous and corresponding organic phases were measured for the different dilutions, with the resulting ratio so determined being the effective distribution coefficient. Distribution coefficients were measured for hydroxytyrosol,

combined low molecular weight phenols and total phenol concentrations between the two phases.

Batch extraction experiments were performed to determine overall mass transfer coefficients. Equal volumes (500 ml) of wastewater and solvent were placed in the respective reservoirs, and the pumps were switched on. The variation with time of phenolic concentrations in the aqueous wastewater and organic solvent phases were monitored by periodic sampling, until equilibrium concentrations had been reached (corresponding to the distribution coefficient between the two phases). This data, together with the distribution coefficients and physical parameters of the system allowed for the calculation of overall mass transfer coefficients according to equation (3.5).

3.3.4 Analytical methods

HPLC was used to quantify hydroxytyrosol and other low molecular weight phenolic compounds, as described in section 2.2.14. Total phenol concentrations were determined by colorimetric assay as described in section 2.2.10. Concentrations of solutes in the organic phase were determined by first removing the ethyl acetate *in vacuo*, and re-dissolving the residue in an equal volume of water.

3.4 RESULTS AND DISCUSSION

The distribution coefficients of phenolic solutes between the aqueous (wastewater) phase and the organic phase, at equilibrium, are shown in Figure 3.3, for increasing wastewater concentrations. The slope of the straight line fitted to the data (by least squares regression) is the effective distribution coefficient.

The distribution coefficients for hydroxytyrosol and combined low molecular weight phenolic compounds were 1.753 and 1.005 respectively (least square regression coefficients were > 0.98). The distribution coefficient for total phenols was 0.712, meaning that the total phenol content remained higher in the wastewater than in the organic solvent. This is because ethyl acetate is selective towards low molecular weight phenolic compounds

(Allouche *et al.*, 2004), and the higher molecular weight phenolic components thus remain in the aqueous phase.

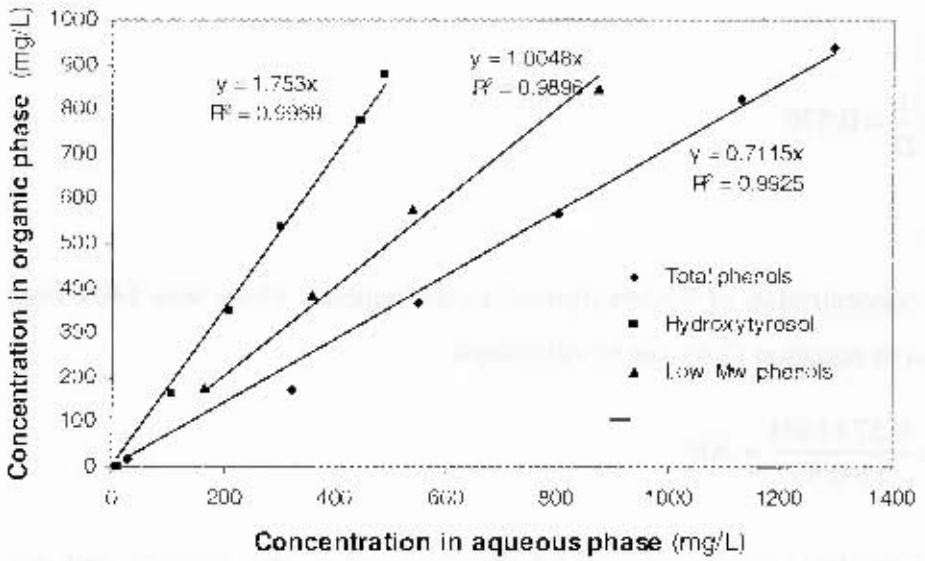


Figure 3.3: Equilibrium distribution coefficients of phenolic components between the aqueous (wastewater) and organic phases

Calculation of the mass transfer coefficient for hydroxytyrosol during a batch contacting experiment is shown below.

Aqueous phase flow rate:

$Q_a = 138$ ml.min^{-1} $= 2.30 \times 10^{-6}$ m^3.s^{-1} (Reynolds no. = 4.32)

Organic phase flow rate:

$Q_o = 127$ ml.min^{-1} $= 2.12 \times 10^{-6}$ m^3.s^{-1} (Reynolds no. = 15.2)

From Figure 3.3, the distribution coefficient for hydroxytyrosol is

$D = 1.753$

Thus

$$Q = \frac{Q_a}{Q_o D} = \frac{138}{127 \cdot 1.753} = 0.620$$

Aqueous and organic phase volume:

$$V_a = V_o = 500 \text{ mL} = 0.5 \text{x} 10^{-3} \text{ m}^3$$

Thus

$$V = \frac{V_o}{V_a D} = \frac{1}{D} = 0.570$$

The initial concentration of hydroxytyrosol in the aqueous phase was 1401 mg.L^{-1}, thus the coefficient a in equation (3.4) can be calculated:

$$a = \frac{Vc_{a,0}}{1 + V} = \frac{0.57 * 1401}{1 + 0.57} = 509$$

The time-dependent concentrations of hydroxytyrosol in the aqueous and organic phases during a batch extraction experiment are shown in Figure 3.4. The sum of these two components is also shown; the mass balance closed to within ±5%.

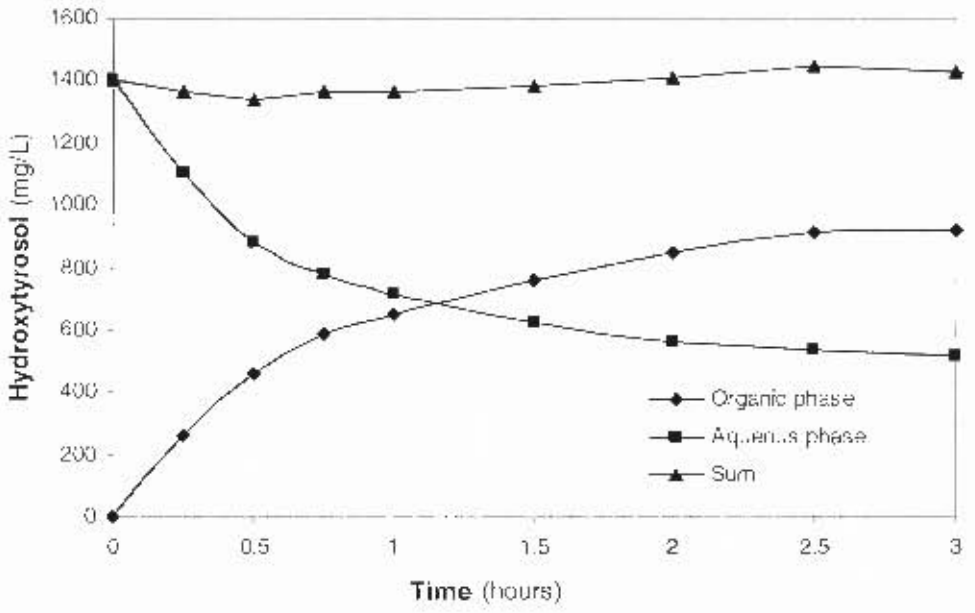


Figure 3.4: Concentrations of hydroxytyrosol in the aqueous and organic phases over time during membrane extraction

Taking the concentration of hydroxytyrosol in the aqueous phase, and plotting it as the natural log of concentration (less coefficient *a*) against time results in the graph of Figure 3.5, where the slope is the coefficient *c* in equations (3.4) and (3.5). The relevant values can then be substituted in equation (3.5) to solve for K_a.

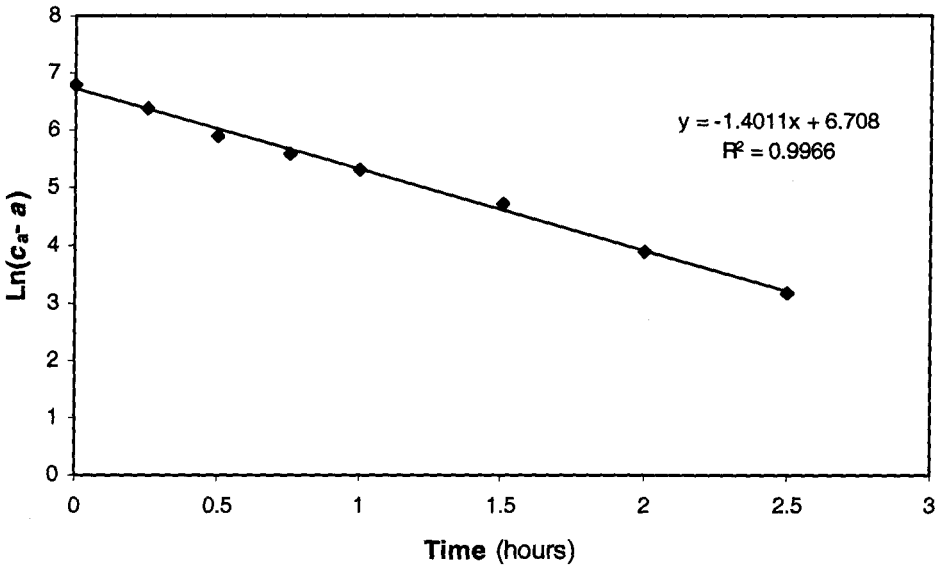


Figure 3.5: Linearised plot of hydroxytyrosol concentration over time during membrane extraction, for the purpose of calculating the mass transfer coefficient

From Figure 3.5 the value of coefficient *c* was found to be –1.401. Referring to equation (3.5):

$$K_a = \frac{-Q_a}{A_m(1+Q)} \ln\left[1 - c\left(\frac{V_a}{Q_a}\right)\left(\frac{1+Q}{1+V}\right)\right] = \frac{-2.30\times10^{-6}}{0.1(1+0.62)} \ln\left[1+1.401\left(\frac{0.5\times10^{-3}}{2.30\times10^{-6}}\right)\left(\frac{1+0.62}{1+0.57}\right)\right]$$

Thus for hydroxytyrosol the mass transfer coefficient was found to be $K_a = 8.17\text{x}10^{-5}$ m.s^{-1} (the negative symbol is irrelevant as it merely indicates the direction of mass transfer). Similarly, for simple phenols $K_a = 6.18\text{x}10^{-5}$ m.s^{-1}, and for total phenols $K_a = 5.15\text{x}10^{-5}$ m.s^{-1}. The mass transfer coefficients of the three phenolic components are a function of the distribution coefficient, and therefore decrease in the same order.

Hydroxytyrosol was successfully and selectively extracted from the olive fermentation brine, but a problem of the aqueous phase breaking through into the organic phase was encountered during some experiments. This can be ascribed to the high porosity of the membranes (~70%) and large pore size (0.2μm), compared to modules used by other researchers. This also resulted in large mass transfer coefficients, an order of magnitude larger than those reported in the literature. Gonzalez-Munoz *et al.* (2003) report a mass transfer coefficient of $6x10^{-6}$ $m.s^{-1}$ for the extraction of phenol from a synthetic wastewater, using a similar system and a Liqui-Cel® membrane module. The membrane pore size in these modules is 0.03 μm, which is most likely the reason for their smaller mass transfer coefficients. However, the Liqui-Cel® modules contain a large number (10176) of much thinner membranes (0.3 mm diameter), which results in a large membrane surface area (1.4 m^2) compared to the module used in this work (0.1 m^2), even though the modules are of similar size. Therefore, extraction rates (as can be determined from equation (3.1)) can be expected to be better in the Liqui-Cel modules, despite the smaller mass transfer coefficient.

Figure 3.6 shows HPLC profiles of the aqueous and organic phases before and after the extraction experiments. Hydroxytyrosol (the major peak at a retention time of 5 minutes) was initially present in the wastewater at a concentration of 1.40 $g.L^{-1}$, and was extracted into the organic phase at a concentration of 0.92 $g.L^{-1}$, *i.e.* 64% extraction yield efficiency.

Residual (unextracted) hydroxytyrosol in the aqueous phase is a function of the distribution coefficient, but this is inconsequential, since vast quantities of wastewater are available for extraction. In terms of process design, the (energy-intensive) organic solvent removal by vacuum distillation for the recovery of solutes from the extract is much more likely to be a rate-limiting factor, compared to the extraction process.

For mill wastewaters with a similar hydroxytyrosol concentration to that used in this work (1.43 $g.L^{-1}$), Allouche *et al.* (2004) report an extraction yield of 85%, using a 3-stage counter-current liquid/liquid extraction unit. However, these authors used a solvent to wastewater ratio of 2:1, and therefore their results are not directly comparable to this work.

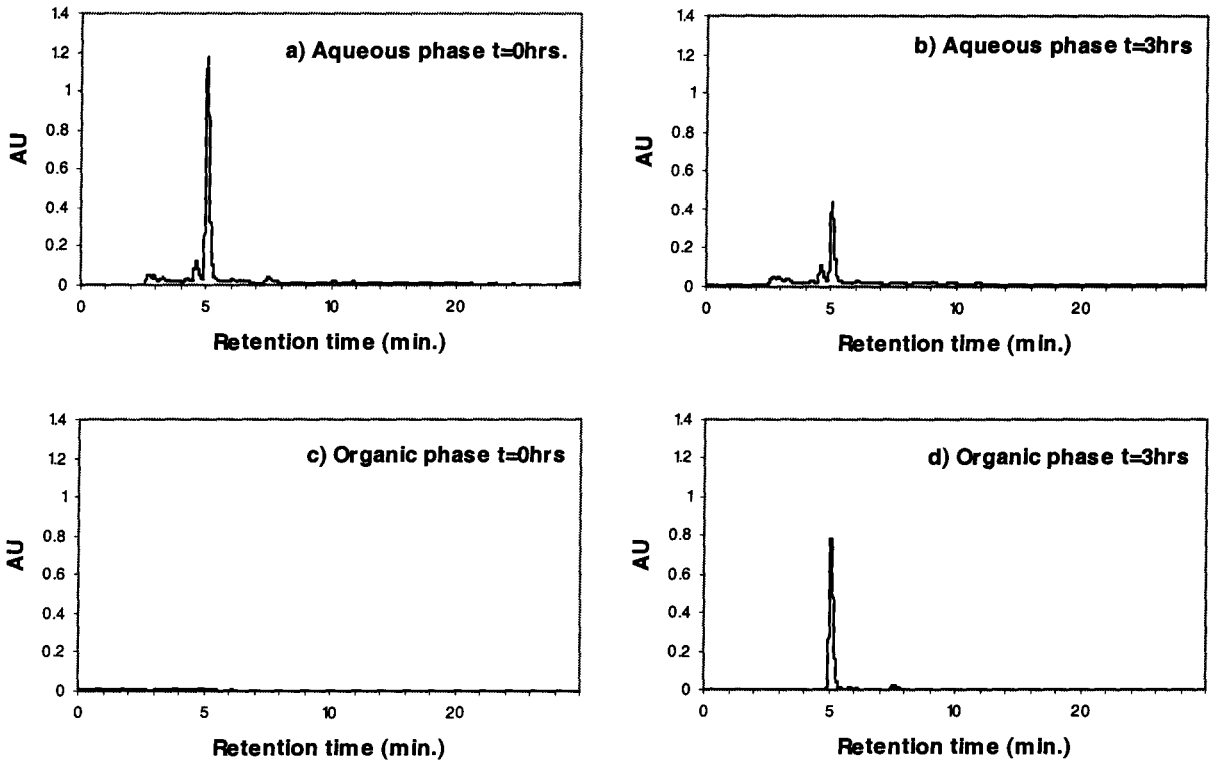


Figure 3.6: HPLC profiles showing hydroxytyrosol concentration in aqueous and organic phases before and after extraction

3.5 CONCLUSIONS

Valuable low molecular weight phenolic compounds, predominantly hydroxytyrosol, were successfully extracted from black olive fermentation brine using a membrane-assisted solvent extraction process. The simplicity of the membrane extraction process, compared to conventional liquid/liquid extraction, makes this approach particularly appealing and merits further research and development. It is feasible that such membrane-based extraction systems could be installed on-site at olive producing facilities, because, in addition to their simplicity, they are expected to cost less than comparable conventional liquid/liquid extraction systems, and are also expected to be easier to operate and automate.

The overall mass transfer coefficient of hydroxytyrosol for the extraction process was determined to be 8.17×10^{-5} $m.s^{-1}$. The relevance of the mass transfer coefficient (once it has been determined for a particular system and solute), is that it can be used for design and determination of required membrane surface area, based on wastewater flow rates, volumes

and concentrations, and the desired extraction rate. In this study the mass transfer coefficient was found to be an order of magnitude larger than that reported by other researchers for a similar process, but this is ascribed to the use of different membrane modules. The Microdyne module used in this work is in fact not completely appropriate for the membrane extraction process, as it is too porous, and thus breakthrough and mixing of phases occurs too easily. These modules are designed for filtration purposes, unlike the Liqui-Cel membrane modules, which are purpose-designed for contacting applications.

The olive fermentation brines appear to be particularly well suited to the membrane extraction process. These wastewaters were shown to have high concentrations of hydroxytyrosol as the principal phenolic component (Chapter 2). In addition, they contain only minimal amounts of suspended solids (compared to mill wastewaters), which means they can be processed directly without any pre-treatment such as filtration or settling.

Further research and optimisation is required before the membrane-based extraction system can realistically be used in industry. In particular, the main objective would not be to maximise the mass transfer coefficient, but rather to maximise the extracted solute concentration in the solvent, as subsequent processing thereof (to recover the solute) is much more likely to be a rate-limiting step than the extraction process itself. More appropriate membrane modules also need to be investigated.

Although not falling within the scope of this work, downstream processing of the organic solvent extract will also need to be addressed. There are two main aspects to be considered: firstly, *in vacuo* solvent removal (and recovery for recycling), and secondly, subsequent purification of the crude extract, which will most likely be a chromatographic process.

Future possible applications of the membrane-assisted solvent extraction technology described in this chapter include its use for recovery of phenolic antioxidants from olive mill wastewaters, as well as from similar wastewater sources, such as in the fruit juice and wine industries. Furthermore, phenol and related compounds in waste streams are a common problem arising from many industrial processes *e.g.* production of plastics, dyes, drugs, pesticides, paper, petrochemicals, resins, leather, paint and steel. Due to their toxicity (or inherent value), there is a need to remove and recover these compounds before discharging waste streams, to prevent adverse environmental effects. Although still in its infancy,

membrane-assisted solvent extraction technology could provide an efficacious alternative to conventional processes.

CHAPTER 4: BIOLOGICAL DEGRADATION OF OLIVE WASTEWATERS

4.1 INTRODUCTION

The objective of work reported in this chapter was to investigate the microbial degradation of black olive brines. The black olive brines are the least well-studied of the various olive-derived wastewaters, and there are few reports on the microbial degradation of them. The production of black table olives accounts for a large proportion of the local olive harvest, and thus, large quantities of the associated brine and rinsing wastewaters are produced locally. Currently, these wastewaters are generally disposed of in evaporation ponds, but this is not perceived to be an optimal solution for various reasons discussed in Chapter 1. Therefore, microbial degradation was investigated as a method of treatment for this wastewater.

A broad comparison of the degradative ability of various microbial species was performed, in terms of their ability to metabolise phenolic compounds and reduce the organic load of the wastewater. The microbial species investigated included pure and mixed cultures of wild-type strains isolated from an appropriate source, and various other species available from local or international culture collections. The wild-type isolates were predominantly bacteria; these were compared to some microbial strains that were chosen on the basis of having been reported in the literature as being capable of degradation of olive mill wastewaters. This work was limited to aerobic degradation processes, as the wastewaters are unlikely to be suitable for direct anaerobic treatment, due to their high salinity and phenol content. If anaerobic treatment is to be investigated, it is likely that an aerobic pretreatment will in any case be necessary, as is generally performed with olive mill wastewaters.

Amongst the bacteria, *Lactobacillus* species (that are predominantly responsible for the olive fermentation process) have been reported to be capable of the degradation of olive wastewaters, particularly the green olive de-bittering wastewater and green olive fermentation brines (Ayed and Hamdi, 2003; Brenes *et al.*, 2004; Marsilio and Lanza, 1998). These wastewaters have high COD and phenol concentrations, but they are not darkly coloured as in the black olive brines and olive mill wastewaters, due to a lower concentration of tannins and other high molecular weight polyphenolics. The natural occurrence of

Lactobacillus species in fermentation brines suggests that they are tolerant of high phenol and salt concentrations, and they were therefore investigated for the degradation of black olive brines.

Yeasts, including *Candida* and *Saccharomyces* species, are also known to occur in fermentation brines and have thus been used for the degradation of olive wastewaters (Ettayebi *et al.*, 2003; Fialova *et al.*, 2004; Yan *et al.*, 2005; Giannoutsou *et al.*, 2004; Borja *et al.*, 1995, 1992; Garcia Garcia *et al.*, 2000, 1997; Fadil *et al.*, 2003; Assas *et al.*, 2002). These species have been used for the degradation of green olive brines and de-bittering wastewaters, as well as the (darkly coloured) mill wastewaters, and have been reported to be able to decolourise this waste through the depolymerisation of high molecular weight phenolic compounds.

Filamentous molds of the genus *Aspergillus* have been widely employed for the degradation of mill wastewaters, with resultant reduction of phenol content and COD, and in some cases also resulting in decolourisation of the wastes (Borja *et al.*, 1995; Hamdi *et al.*, 1991; Martinez Nieto *et al.*, 1993; Hamdi 1991; Garcia Garcia *et al.*, 2000, 1997). In addition, they have been used for the treatment of green olive wastewaters in conjunction with chemical oxidation processes, resulting in high quality effluents (Kotsou *et al.*, 2004; Kyriacou *et al.*, 2005). *A. niger* has also been used for treatment of solid wastes (pulp) from oil production, resulting in the release of large quantities of hydroxytyrosol through the action of cinnamoyl esterases (Bouzid *et al.*, 2004). There are, however, no reports of the use of *Aspergillus* species for the treatment of black olive brines.

Many different species of white-rot fungi have been investigated for the treatment of mill wastewaters. These include: *Pycnoporous coccineus* (Jaouani *et al.*, 2005, 2003), *Lentinula edodes* (D'Annibale *et al.*, 2000, 1999, Vinciguerra *et al.*, 1995, 1993), *Panus tigrinus* (D'Annibale *et al.*, 2004; Fenice *et al.*, 2003), *Pleurotus ostreatus* (Setti *et al.*, 1998; Martirani *et al.*, 1996; Aggelis *et al.*, 2003; Flouri *et al.*, 1996; Fountoulakis *et al.*, 2002) and *Phanerochaete chrysosporium* (Sayadi and Ellouz 1993; Dias *et al.*, 2004; Blanquez *et al.*, 2002; Sayadi *et al.*, 2000). The specific interest in white-rot fungi is due to the production of extracellular lignolytic enzymes, which are capable of attacking high molecular weight polyphenolics and hence have a good ability to decolourise the wastewaters. However, none of these fungi have been investigated for the degradation of fermentation brines. White-rot

fungi of the genus *Trametes* have not been investigated for treatment of any olive-derived wastes. This species is known to be a particularly good producer of the lignolytic enzyme laccase (Galhaup *et al.*, 2002).

Despite the potentially beneficial effects of the above organisms for the treatment of olive wastewaters, they require aseptic techniques and necessary sterility associated with the cultivation of monocultures. This is a definite disadvantage in terms of wastewater treatment, and increases the cost and complexity of a bioprocess. Mixed microbial cultures, on the other hand, do not require aseptic conditions. A stable consortium of microorganisms develops over time, with the organisms most suited to the metabolism of the substrate predominating. However, mixed culture treatment of olive-derived wastewaters generally results in polycondensation and humification, *i.e.* decolourisation is not likely to be achieved using this approach. Aerobic mixed culture fermentation is usually performed as a pre-treatment for anaerobic fermentation (Brenes *et al.*, 2000; Aggelis *et al.*, 2001; Benitez *et al.*, 1997; Hafidi *et al.*, 2005). The only reports in the literature to date concerning the aerobic degradation of black olives brine have been performed using mixed cultures (Benitez *et al.*, 1999; Beltran-Heredia *et al.*, 2000).

Cultures of the above microbial species, mixed cultures and wild-type isolates were cultivated in shake flasks using black olive brine as substrate, in order to identify organism(s) that would be best suited for biological treatment of this wastewater. The fate of the phenolic compounds after microbial metabolism was also investigated. The work presented here was essentially a preliminary screening process, and was performed in collaboration with C. Werner (2005) and A. van Schalkwyk (2005), who performed in-depth microbiological and biochemical investigations of the metabolism of the different species in the black olive brines.

4.2 MATERIALS AND METHODS

4.2.1 Environmental sampling and isolation of wild-type microbial strains

Phenol-tolerant wild-type microbial species were obtained by taking water and sludge samples from olive wastewater evaporation ponds. These were isolated by conventional spread-plating techniques. Selected single colonies were streaked onto fresh agar plates, and

were subcultured 3 times to ensure pure cultures. Isolated pure cultures were identified by morphological, biochemical and 16s rRNA analysis. This work was performed in collaboration with A.van Schalkwyk (2005). Mixed microbial cultures were also obtained directly from the raw wastewater and sludge samples.

4.2.2 Maintenance of microorganisms

Wild-type isolates were cultivated on Petri plates containing 25% (v/v) black olive brine, 5 $g.L^{-1}$ yeast extract and 16 $g.L^{-1}$ agar. *L. plantarum* (ATCC 14917) was obtained from the Agricultural Research Council (ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa), and was cultivated on 30 $g.L^{-1}$ malt extract agar plates, with 2% (v/v) black olive brine. *Candida maltosa* (UOFS Y-1298) was obtained from the University of the Orange Free State culture collection, and was cultivated on agar plates with the following composition: 2% (v/v) black olive brine, glucose 10 $g.L^{-1}$, peptone 5 $g.L^{-1}$, yeast extract 3 $g.L^{-1}$, malt extract 3 $g.L^{-1}$ and agar 16 $g.L^{-1}$. A wild-type strain of *Aspergillus niger* NRRL-3 was obtained from the Council for Scientific and Industrial Research (CSIR), and was cultivated on plates with the following composition: 2% (v/v) black olive brine, malt extract 20 $g.L^{-1}$, glucose 20 $g.L^{-1}$, peptone 1 $g.L^{-1}$ and agar 16 $g.L^{-1}$. *Trametes pubescens* (CBS 694.94) was obtained from the Institute of Applied Microbiology, Universitat fur Bodenkultur, Wien., and was cultivated on 30 $g.L^{-1}$ malt extract agar with 2% (v/v) black olive brine.

Black olive brine (2% v/v) was added to the agar plates in order to acclimatise the different microbial strains to the presence of olive wastewater phenolics. All microbial cultures were routinely sub-cultured every two months to ensure strain vitality. Plates were incubated at 25°C until fully colonised, then stored at 4°C.

Mixed cultures were maintained in liquid culture; these were acclimatised and enriched through shake flask culture by successive inoculation into fresh media once a week. The media contained 25% (v/v) black olive brine and 5 $g.L^{-1}$ yeast extract; flasks were shaken at 170 rpm and 25°C.

4.2.3 Shake flask degradation experiments

All experiments were performed at 25°C, in triplicate, in cotton wool stoppered Erlenmeyer flasks (500 ml) containing 200 ml of media. These were orbitally shaken at 170 rpm. Media contained 25% (v/v) black olive brine and was supplemented with 5 $g.L^{-1}$ yeast extract to promote the growth of microorganisms, as it contains readily available nitrogen sources and vitamin B. Although yeast extract contains no fermentable carbohydrates (such as reducing sugars), at a concentration of 5 $g.L^{-1}$ it adds approximately 4.4 $g.L^{-1}$ of oxidizable material as determined by the COD test. The pH of the media was uncorrected and had an initial value between 4 and 5. Initial total phenol content of the media was 900 ± 100 $mg.L^{-1}$, and initial COD was 16 ± 2 $g.L^{-1}$. The variation in these values was because of the use of different batches of wastewater over the duration of the work.

Black olive brine from the fermentation of *Calamata* olives was used in all cases (OW-04, OW-08, OW-15 and OW-18, see Table 2.1). The black olive brines were routinely diluted to 25% (v/v) in order to bring the total phenol content down to less than 1 $g.L^{-1}$. Preliminary experiments indicated that this dilution did not impede the growth of microorganisms, whereas higher phenol (and salt) concentrations in 50% dilutions appeared to have an inhibitory effect. During the table olive production process the brines are diluted with rinsing and factory washing wastewaters to at least this value prior to discharge, and therefore it was considered valid to dilute the raw brine wastewaters to a certain extent.

Sterilised flasks were (aseptically) inoculated with 10% (v/v) of pre-grown liquid starter culture. With the exception of *T. pubescens*, starter cultures were prepared with the same composition as the agar plates used for maintenance, excluding the agar. Starter cultures were inoculated from agar plates using an inoculation loop. These were cultivated for 1 to 2 days as required, depending on the organism. *T. pubescens* starter culture contained glucose 10 $g.L^{-1}$, peptone 10 $g.L^{-1}$ and KH_2PO_4 1 $g.L^{-1}$ and 2% black olive brine. The starter culture was inoculated by homogenisation of squares cut from agar plates in starter culture media; this was then transferred to the shake flasks. All flasks and media were sterilised by autoclaving at 120°C for 20 minutes before use. The experiments involving *Bacillus megaterium* (a wild-type isolate) and *Trametes pubescens* were performed in collaboration with C. Werner (2005).

Sampling of flasks was performed aseptically in a UV sterilised hood with flaming. The total sample volume per flask was limited to 10%. Except for the mixed cultures, samples were routinely analysed microscopically to check for contamination. Samples were taken every 24 hours, and were subjected to analysis as described below. The degradative ability of the different cultures was evaluated on the basis of their maximum specific degradation rates during the exponential growth phase, *i.e.* grams of substrate (total phenol or COD) removed per litre of wastewater per day ($g.L^{-1}.day^{-1}$), and the total amount of substrate removed over the course of the experiment (%).

Control flasks were carried for all experiments to determine abiotic effects; these were prepared in the same manner as the experimental flasks, except these were inoculated with sterile ddH_2O instead of starter culture.

4.2.4 Analytical techniques

All analyses were performed as described in Chapter 2. Routine analyses of samples included pH, conductivity, total phenol concentration and COD. HPLC was performed on certain samples to investigate the effects microbial metabolism on low molecular weight phenolics, while size exclusion chromatography was performed to determine changes in the molecular weight distribution of phenolic compounds. The colour intensity of the wastewater media was measured as absorbance at 500 nm against a distilled water blank. Biomass concentrations were measured as suspended solids (dry weight).

Laccase activity was assayed using the procedure of Roy-Arcand and Archibald (1991). The reaction mixture contained 2.5 ml 0.1 M sodium acetate buffer (pH 5), 0.33 ml 5 mM ABTS (2,2'-azinobis 3-ethylbenzthiazoline-6-sulfonic acid) and 0.17 ml enzyme sample. Oxidation of ABTS was measured by determining the increase in absorbance of the mixture at 420 nm (ε = 36 000 M^{-1}). One unit (U) of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmol of ABTS per minute, and enzyme concentrations are reported as $U.ml^{-1}$.

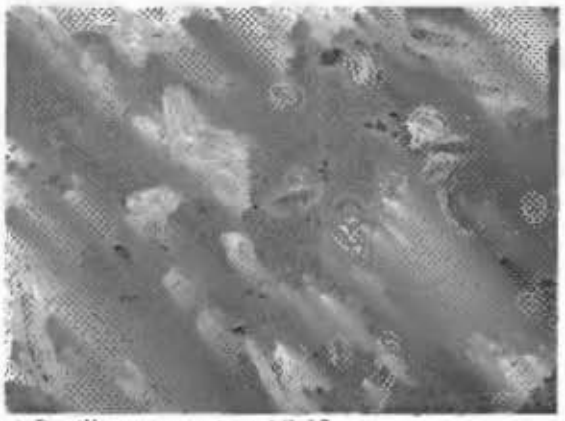
4.3 RESULTS AND DISCUSSION

4.3.1 General discussion

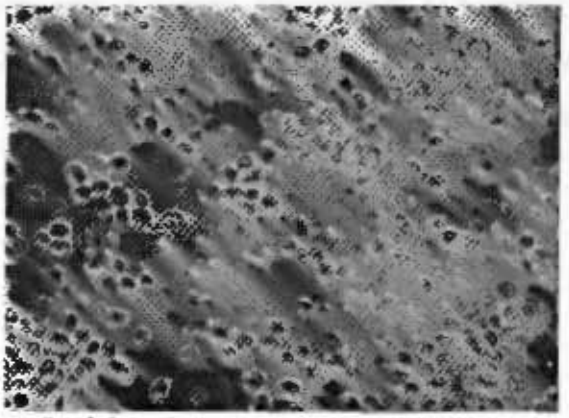
In collaborative work on this research project, thirty-five morphologically different wild-type strains were isolated from the evaporation pond samples. These were initially screened for degradative ability on agar plates containing black olive brine wastewater. Five candidate strains were chosen on the basis of growth. These were transferred to liquid culture and evaluated in terms of total phenol degradation. Of the five isolates, one was chosen for further analysis and comparison to pure cultures obtained from various sources. This microorganism was identified as having 98% homology with the gram-positive bacterium *Bacillus megaterium* (van Schalkwyk, 2005), and was labelled isolate number AS-13.

Figure 4.1 shows microscope images of the different microbial species used for the wastewater degradation experiments. With the exception of *Lactobacillus plantarum*, all the species investigated in this work were able to remove significant quantities of the total phenol content and COD of the wastewater. Table 4.1 shows the summarised results of the degradation experiments. These results were calculated from the data presented in Figure 4.2. Of the monocultures, *Trametes pubescens* removed the highest amount of total phenol content from the black olive brine, followed by *Candida maltosa* and *Bacillus megaterium*. *C. maltosa*, however, showed the fastest growth and had the highest rate of removal of total phenol content, and also showed the best results in terms of overall COD removal. Results from experiments with *Aspergillus niger* were the poorest and were also inconsistent, whereas *L. plantarum* exhibited very little growth and had hardly any effect on the total phenol concentration or COD of the wastewater.

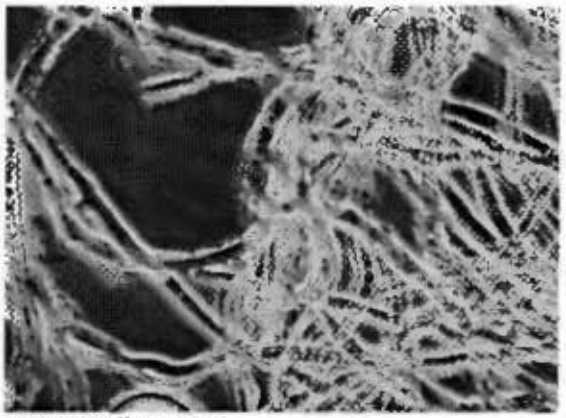
The mixed microbial culture used in this work was also capable of removing phenol content, albeit at a slower rate than the other microbial species, less than half that observed with *C. maltosa*. However, in terms of COD removal, the mixed culture produced the best results (78% total removal). Figure 4.2 shows total phenol and COD concentrations over time during the degradation experiments for the different cultures. These are discussed in turn in the following sections. In general, abiotic effects measured in control flasks accounted for less than 5% change from original values of total phenol and COD concentrations.



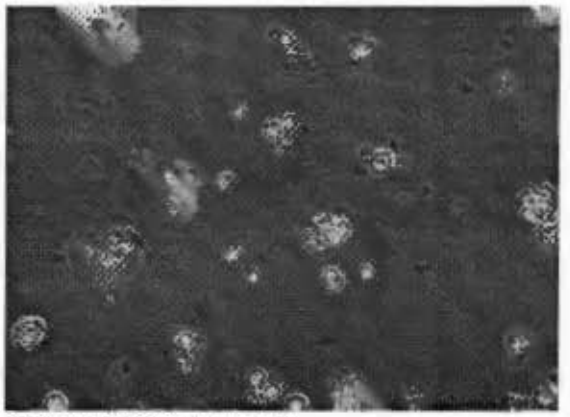
a) *Bacillus megaterium* AS-13



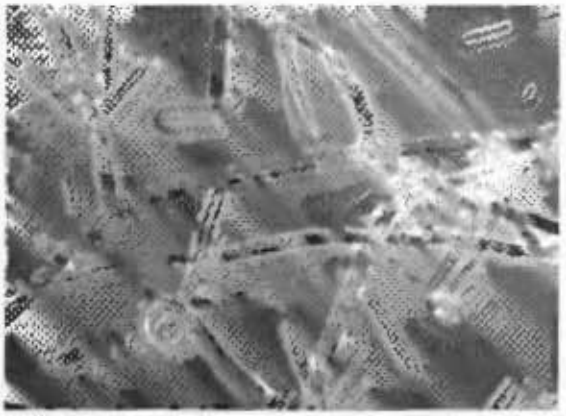
b) *Candida maltosa*



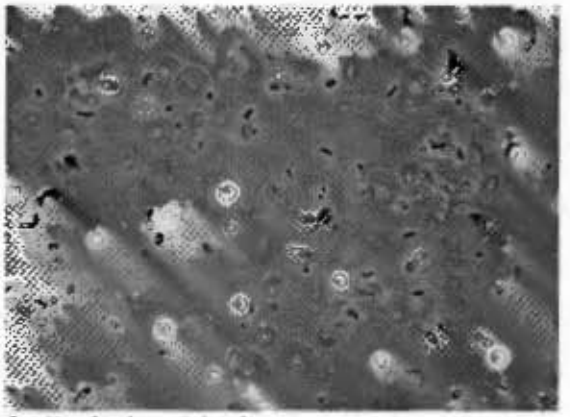
c) *Aspergillus niger*



d) *Lactobacillus plantarum*



e) *Trametes pubescens*



f) Mixed microbial culture

Figure 4.1: Microscope images of the different microbial species used for degradation of black olive brine, taken from shake-flask degradation experiments (100x magnification).

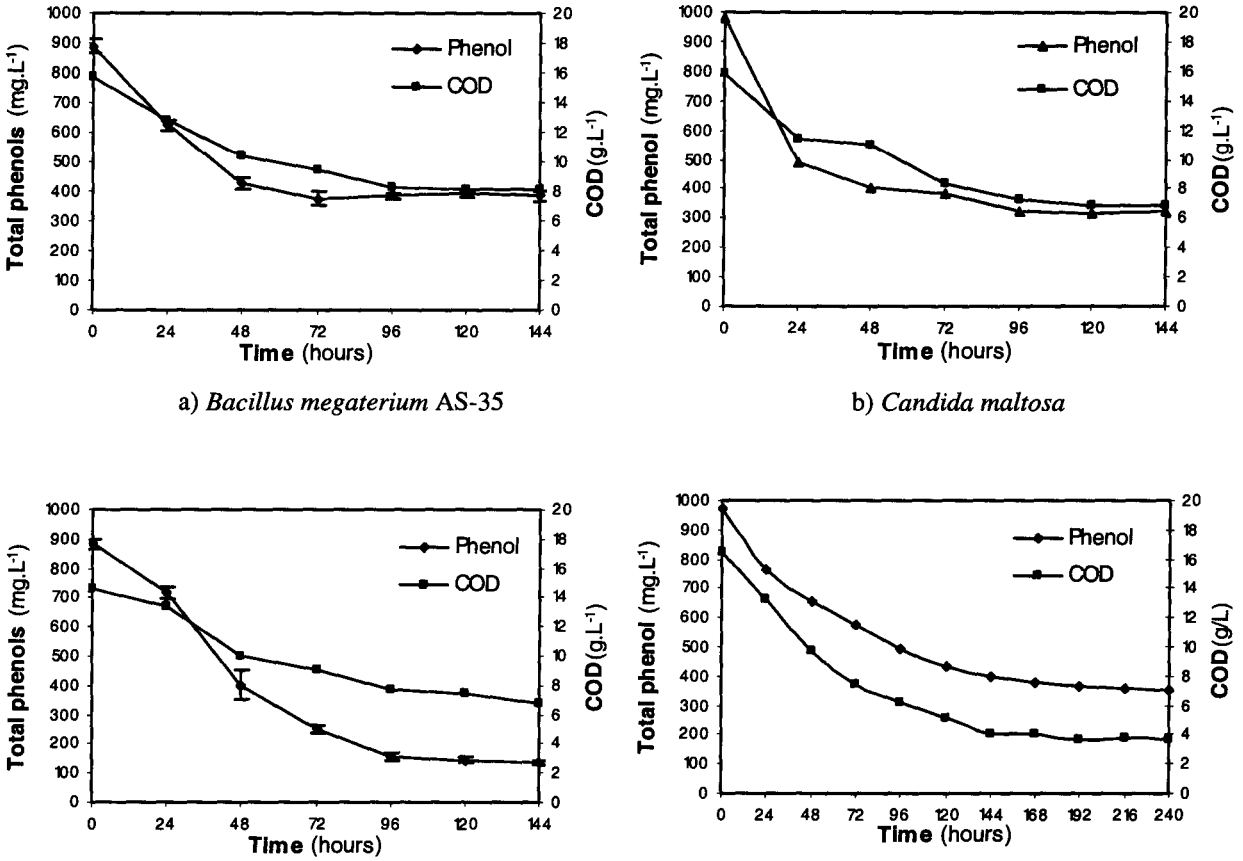
Table 4.1: Percentage removal and maximum degradation rates of total phenolic content and COD from black olive brines by different microbial species in shake flask culture

Organism	X_{TP} (%)	Max. X_{TP} ($mg.L^{-1}.day^{-1}$)	X_{COD} (%)	Max. X_{COD} ($g.L^{-1}.day^{-1}$)
Bacillus megaterium	57	265	49	3.0
Candida maltosa	67	490	57	4.4
Aspergillus niger	18 - 45	-	22 - 36	-
Trametes pubescens	85	318	54	3.3
Mixed cultures				
This work	64	212	78	3.6
Benitez *et al.* (1999)	46	-	83	13.4
Beltran-Heredia *et al.* (2000)	48	-	75	3.2

X_{TP} = percentage removal of total phenolic content; Max. X_{TP} = maximum degradation rate of total phenolic content; X_{COD} = percentage removal of COD; Max. X_{COD} = maximum degradation rate of COD.

4.3.2 *Bacillus megaterium* AS-35 and *Candida maltosa*

B. megaterium AS-35 and *C. maltosa* showed similar growth and degradation patterns, and are therefore discussed together. For *C. maltosa* (the better of the two), total phenols were reduced by 67% from an initial value of $980mg.L^{-1}$. The COD was reduced by 57% from an initial value of $15.8g.L^{-1}$. The maximum degradation rates were $490mg.L^{-1}.day^{-1}$ and $4.4g.L^{-1}.day^{-1}$ for total phenols and COD respectively. For both *B. megaterium* AS-35 and *C. maltosa*, there was no significant further reduction in either variable after 96 hours. This corresponded to the end of exponential growth and the stationary phase of these organisms. Biomass concentrations at this stage had reached a maximum of 5.62 and 6.44 $g.L^{-1}$ respectively. The wastewater had turned a distinctly darker colour, indicating the formation of high molecular weight (recalcitrant) humic substances, which were responsible for the residual total phenol content and COD. The colour intensity of the wastewater increased from 1.6 AU to approximately 2.5 AU at the end of the experiments. In both cases the pH had risen from an initial value of 4.5 to approximately 9.



a) *Bacillus megaterium* AS-35

b) *Candida maltosa*

c) *Trametes pubescens*

d) Mixed microbial culture

Figure 4.2: Time course of removal of total phenol content and COD from black olive brine wastewater by different microbial species

In comparison, Fadil *et al.* (2003) used *Candida tropicalis* for the degradation of olive mill wastewater. They reported 47% and 53% reduction in COD and total phenols, from initial values of 30 $g.L^{-1}$ and 2000 $mg.L^{-1}$ respectively. Their initial concentrations for these parameters were almost double those in this work, but their mineral solids (salts) concentrations were significantly lower than in the brines: approximately 1.5 $g.L^{-1}$ compared to 30 $g.L^{-1}$ in this work. Contrary to this work, they reported decolourisation of around 30%, indicating the depolymerisation of the larger phenolic compounds. It would thus appear that the high salt concentrations in the black olive brines inhibit decolourisation of the wastewater.

Figure 4.3 shows the molecular weight distribution of phenolic compounds in the brine wastewater initially, and after 48 hours of degradation by *C. maltosa*. After degradation there

was a significant accumulation of highly polymerised humic substances (around fraction 20), and a corresponding decline in low molecular weight compounds (fraction 40), which resulted in the increased colour of the wastewater after degradation. The different fractions have different UV absorbance coefficients, and therefore peak sizes across fractions cannot be compared quantitatively in terms of total phenol content. Condensed tannins of intermediate molecular weight (~5 kDa, fraction 35) also increased slightly after degradation. It is thus evident that the low molecular weight compounds were polymerised into higher molecular weight compounds.

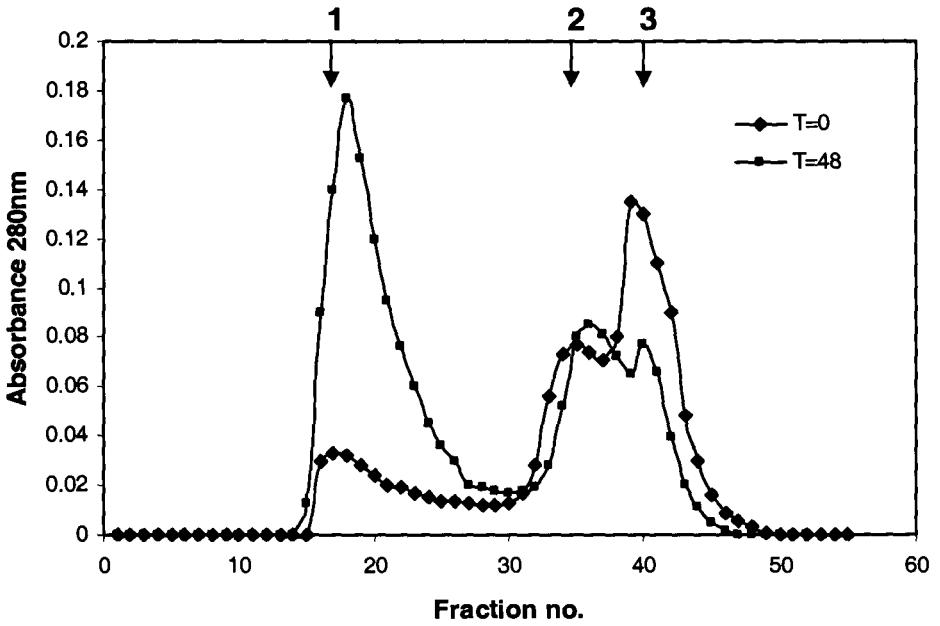


Figure 4.3: Change in molecular weight distribution of phenolic compounds in black olive brine wastewater as a result of degradation by *C. maltosa*

(1) 2 000 kDa (2) 5 kDa (3) 0.2 kDa

The humification observed in this work is in contrast to results reported by Ettayebi *et al.* (2003), who reported a reduction in all phenolic fractions, and hence decolourisation, when *Candida tropicalis* was used for the degradation of mill wastewater. It is likely that the differences in composition between the mill wastewaters and brines (in particular the high salt concentration in the brines), was responsible for these contrary results. However, Hafidi *et al.* (2004), using the yeast *Saccharomyces cerevisiae* for degradation of mill wastewaters, reported the polycondensation and humification of phenolic compounds in certain instances (*i.e.* increase in colour), and concluded that the mechanisms of phenol degradation are highly influenced by media composition. Toscano *et al.* (2003) report the oxidative polymerisation

of phenols, by phenol oxidase enzymes. Oxidation products were reported to be quinones, which then spontaneously underwent non-enzymatic polymerisation, resulting in the formation of humic of dark colour. Humification is not, however, perceived to be necessarily detrimental, as it represents the stabilisation of organic matter and preservation of nitrogen in humic form (Hafidi *et al.*, 2005). In addition, humification of phenolic compounds results in a decrease in total phenol concentration and organic load, as the polymerisation results in the loss of carbon atoms (to biomass or CO_2) as aromatic rings combine.

The disappearance of low molecular phenolic compounds in the brine wastewater during degradation experiments was verified by HPLC, as illustrated in Figure 4.4. As before, the main peak at a retention time of 5 minutes was hydroxytyrosol. It was evident that the hydroxytyrosol was completely metabolised by the end of the degradation experiment, but there remained a residual mixture of (unidentified) compounds.

Based on HPLC peak areas, the low molecular weight phenolics were reduced by 76%. As discussed in section 1.4.3, it is likely that these compounds were oxidised to quinones, and these then reacted to form polyphenols and humic substances of higher molecular weight and darker colour (Ayed and Hamdi, 2003). It is also possible that some of the low molecular weight phenols were mineralised through ring cleavage. However, no extracellular enzymes were detected in the wastewater media during these degradation experiments, and therefore it is not possible to state with certainty the exact path followed in the conversion of these compounds. The lack of detection of enzymes was possibly a result of the protein binding capacity of polyphenols and tannins in the wastewater. In related work, Fiavola *et al.* (2004) used *C. maltosa* for degradation of pure phenol as sole carbon and energy source. In their work *C. maltosa* produced the enzyme phenol hydroxylase that effected phenol degradation, and it is possible that the same or a similar enzymes were responsible for the degradation results achieved in this work.

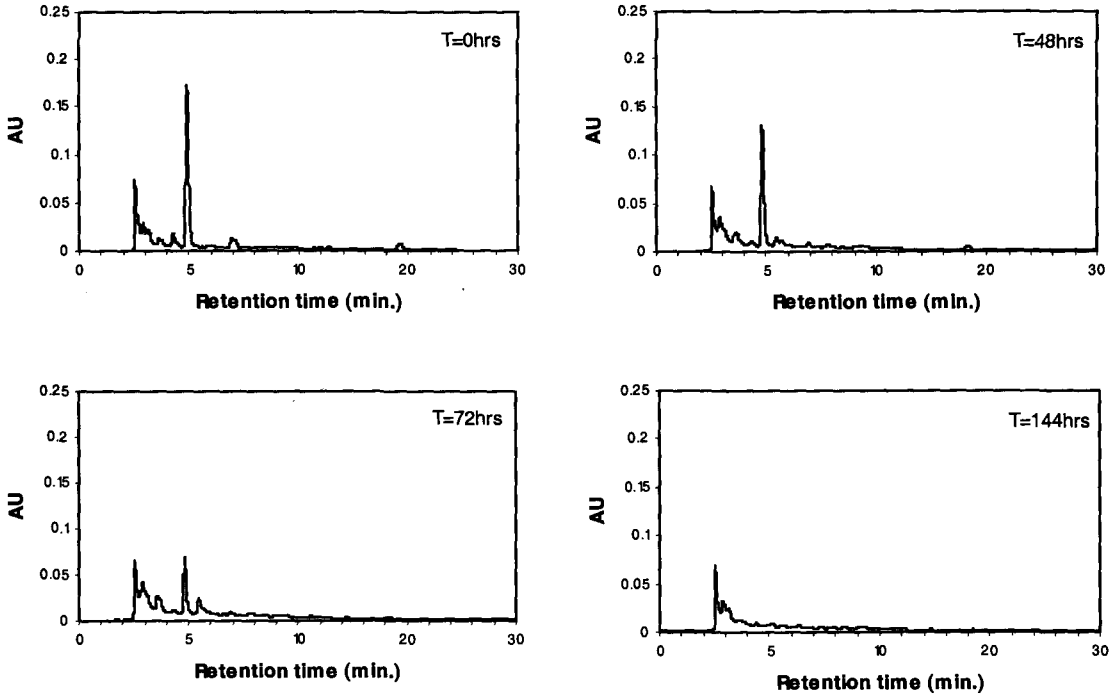


Figure 4.4: HPLC chromatograms showing the successive decrease of low molecular weight phenolic compounds in black olive brine wastewater during degradation effected by *C. maltosa*

4.3.3 *Trametes pubescens*

T. pubescens showed a longer lag phase than the other cultures, but subsequent removal of total phenol content was comparatively the highest (see Figure 4.2). The rate of degradation was not as high as that achieved with *C. maltosa*, but the total amount of phenol metabolised was the greatest of all the cultures investigated (85%). Unlike the other cultures, the wastewater did not go significantly darker in colour, and an acidic pH was maintained (4.4 to 5.6) throughout the course of the degradation experiment. The COD was reduced by 54% from an initial value of 14.7 $g.L^{-1}$, while the maximum degradation rate for COD was 3.3 $g.L^{-1}.day^{-1}$. The maximum biomass concentration was 5.5 $g.L^{-1}$. It would thus appear that at an acidic pH, oxidation of phenolic compounds results in less polymerisation than occurs when the pH is high.

The white-rot fungi have not previously been investigated for the degradation of black olive brines, but they have been extensively investigated for the degradation of mill wastewaters.

As a comparison, Garcia Garcia *et al.* (2000) used *Phanerochaete chrysosporium* for the degradation of mill wastewaters with initial total phenols and COD concentrations of 1 200 $mg.L^{-1}$ and 82 $g.L^{-1}$ respectively. 90% of the total phenols and 75% of the COD were removed from these mill wastewaters by *P. chrysosporium*, and significant decolourisation was evident. These results appear better than those achieved with *T. pubescens* and the black olive brines used in this study, but the difference in composition between the two types of wastewater, and the use of different organisms, does not allow for direct comparison of results.

T. pubescens is known to be a good producer of the extracellular enzyme laccase (Galhaup *et al.*, 2002), and this was the only experiment in which any enzyme was detected in the wastewater during degradation experiments. Total phenol and extracellular laccase concentrations over time are shown in Figure 4.5. The laccase concentration did not correspond to phenol degradation; rather, it started to accumulate only once the total phenol concentration had been significantly reduced (after 48 hours). Whether this is a response of nutrient starvation, or is a consequence of the protein binding capacity of polyphenols and tannins, is uncertain. Dias *et al.* (2004) reported the same phenomenon when using laccase-producing wild-type basidiomycete for the treatment of olive mill wastewaters. These authors also report high phenol removal: 90% from an initial concentration of 800 $mg.L^{-1}$.

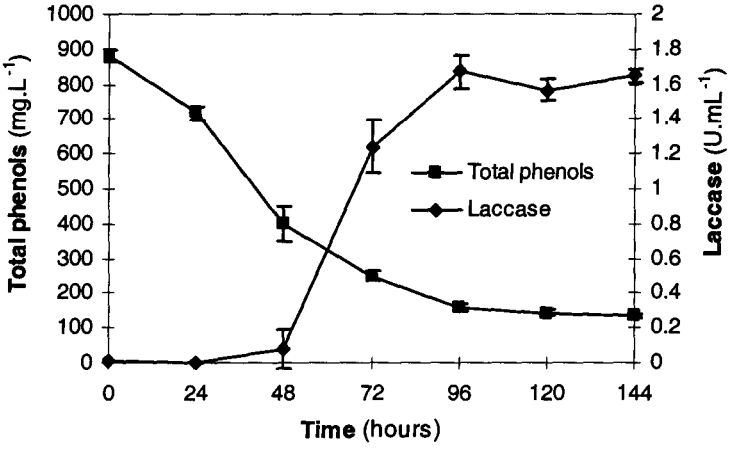


Figure 4.5: Total phenol removal and extracellular laccase production in black olive brine wastewater by *Trametes pubescens*

The effect of laccase from *T. pubescens* on the molecular weight distribution of the phenolic components in the wastewater is shown in Figure 4.6. After degradation, there was a significant reduction of intermediate- and low molecular weight compounds (fractions 30 to 50), compared to the reduction in these compounds by *C. maltosa* (Figure 4.3). There was also an accumulation of high molecular weight polyphenols (fraction 15), but this was not as large as that observed with *C. maltosa*.

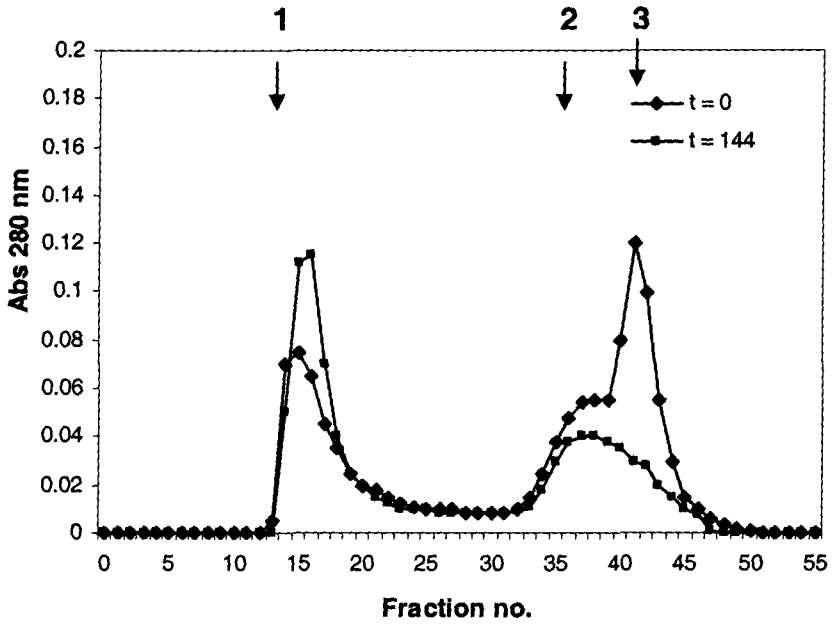


Figure 4.6: Molecular weight distribution of phenolic compounds in black olive brine wastewater before and after degradation by *Trametes pubescens*

(1) 2 000 kDa (2) 5 kDa (3) 0.2 kDa

In this case the removal of colour due to reduction of intermediate weight polyphenols (condensed tannins) must have been closely balanced by the increase in colour caused by the accumulation of high molecular weight polyphenols (humic substances), and therefore there was no net increase in colour of the wastewater. HPLC analysis of the low molecular weight phenolic fraction indicated that these had been reduced by 89%.

There are several reports in the literature detailing the reduction in quantity of all three phenolic fractions in mill wastewaters, through the action of laccase and related enzymes produced by white rot fungi (Sayadi *et al.*, 2000; Sayadi and Ellouz, 1993, 1992) The different metabolic path that resulted in the increase of the high molecular weight fraction observed in this work is most like due to the differences in composition between mill

wastewaters and the fermentation brines, the most fundamental of which is the high salt concentrations in the brines, although the mill wastewaters also contain lipids and sugars that do not occur in the brines.

4.3.4 *Aspergillus niger* and *Lactobacillus plantarum*

In this work, degradation results observed with *A. niger* and *L. plantarum* were unsatisfactory compared to the other microbial cultures investigated, and are therefore not presented in any detail. *A. niger* was able to degrade the black olive brine wastewater, but results were highly variable and not comparable to the other species investigated. The growth of *L. plantarum* was very poor, and total phenol and COD concentrations remained essentially unaffected.

Although *A. niger*, and to a lesser extent *A. terreus*, have been widely used for degradation of green olive brines and de-bittering wastewaters, and mill wastewaters, results published in the literature are contradictory. For green olive wastewaters, some authors claim that the growth of *A. niger* hardly affected polyphenolic compounds (Aggelis *et al.*, 2001), while others claim that phenolics were reduced by between 41 and 85% (Kotsou *et al.*, 2004; Kyriacou *et al.*, 2005).

For mill wastewaters the results are also contradictory. It seems to be the general case that *Aspergillus* species degrade low molecular weight aromatic compounds (simple phenolics) and hydrolysable tannins to a greater extent than higher molecular weight phenolics such as condensed tannins and humic substances (Sayadi and Ellouz, 1993; Hamdi, 1991; Hamdi *et al.*, 1991). *A. niger* is known to be able to degrade hydrolysable tannins through the production of the enzyme tannase (Bouzid *et al.*, 2004; Belmares *et al.*, 2004). It is possible that Sayadi and Ellouz (1993) refer to the degradation of hydrolysable tannins (that occur in mill wastewaters), while other authors refer to condensed tannins that are not degraded. As discussed in section 2.3.5, the black olive brines do not contain hydrolysable tannins, only condensed ones, and this could have attributed to the poor results obtained. It is also possible that the wastewater composition (especially the high salt concentration) of the black olive brines had a detrimental effect on the growth and metabolic processes of *A. niger.*

In contrast to degradation studies on mill wastewaters and green olive wastewaters, *L. plantarum* appeared to be unsuitable for the degradation of black olive brines. In this case it

is unlikely that it was the high salt concentration in the brines that was responsible for poor degradation, because *Lactobacillus* species occur naturally in the fermentation brines. *L. plantarum* is known to produce tannase (Ayed and Hamdi, 2002), as well as β-glucosidase and esterase enzymes (Marsilio and Lanza, 1998). Ayed and Hamdi (2003) report the decolourisation of undiluted mill wastewaters through reductive depolymerisation by *L. plantarum*, when grown anaerobically (*L. plantarum* is a facultative anaerobe). These authors report the depolymerisation of phenolic compounds of high molecular weight (including condensed tannins) into smaller compounds. In the present study, similar (anaerobic) experiments with black olive brines also had little or no effect. It is possible that fermentable carbohydrates are important for the growth of *L. plantarum* and subsequent degradation of phenolic compounds. The mill wastewaters contain significant quantities fermentable carbohydrates (sugars) compared to the fermentation brines, and this could possibly be a reason for the poor results obtained in this work.

4.3.5 Mixed microbial cultures

The mixed microbial culture exhibited the same general degradation trends as *B. megaterium* AS-13 and *C. maltosa*, that is, darkening of the wastewater and increase in pH. Degradation occurred at a slower rate (see Figure 4.2), but was sustained for longer, and stationary phase biomass concentration (at approximately 144hrs) was higher at 10.4 $g.L^{-1}$. The COD was also reduced to the greatest extent, indicating that the mixed cultures are better equipped to remove and convert organic substrates from the residual phenolic fraction than are the monocultures. HPLC analysis showed that the organic acids detectable in the original wastewater were completely consumed by the mixed culture, whereas this was not the case for the monocultures. This could account for the lower final COD achieved by the mixed culture, despite the fact that residual total phenol concentrations were approximately the same as results achieved with *B. megaterium* and *C. maltosa*.

The increase of pH and concomitant oxidation of phenolic compounds to form darkly coloured compounds of high molecular weight has been previously reported for mixed culture aerobic olive mill wastewater treatment (Hafidi *et al.*, 2004). The degradation results obtained in this work compared favourably with the only other published data for black olive brines and mixed cultures (see Table 4.1). Benitez *et al.* (1999) report a maximum COD

degradation rate of 13.4 $g.L^{-1}.day^{-1}$, which is about four times higher than that achieved in this work, but their initial concentrations were significantly lower than those used in this work, at 126 $mg.L^{-1}$ and 7 $g.L^{-1}$ for total phenols and COD respectively. At these concentrations they achieved 46 and 83% removal for total phenols and COD, respectively. Beltran-Heredia *et al.* (2000) reported a degradation rate of 3.2 $g.L^{-1}.day^{-1}$ for COD, 48% and 75% removal from initial (low) concentrations of and 102 $mg.L^{-1}$ and 3.6 $g.L^{-1}$ for total phenols and COD, respectively. These authors do not report any data for colour or molecular weights of residual phenolic compounds. In addition, their salt concentrations varied from 4.4 to 12.9 $g.L^{-1}$, compared to an average of around 30 $g.L^{-1}$ in this study. During the degradation of black olive brines by mixed cultures used in this study, the total phenols were reduced by 64% from an initial value of 976 $mg.L^{-1}$, while the COD was reduced by 78% from 16.5 $g.L^{-1}$. Maximum degradation rates were 212 $mg.L^{-1}.day^{-1}$ and 3.6 $g.L^{-1}.day^{-1}$ for phenol and COD respectively.

Despite the slower degradation rates observed (compared to the monocultures), the mixed cultures are particularly attractive for the biological treatment of olive wastewaters, due to the convenience of not having to follow sterile techniques. The mixed cultures were therefore subjected to further experiments. It was decided to investigate the degradation of the black olive brine wastewater, using mixed aerobic culture, after the (valuable) low molecular weight phenolic antioxidants had been extracted (as described in Chapters 2 and 3). Figure 4.7 shows the removal of total phenols and COD from the extracted wastewater, compared to degradation in the un-extracted wastewater.

In this case, the total phenols were reduced by 53% from an initial value of 488 $mg.L^{-1}$, while the COD was reduced by 80% from an initial value of 17 $g.L^{-1}$. The maximum degradation rates were 93 $mg.L^{-1}.day^{-1}$ and 4.3 $g.L^{-1}.day^{-1}$ for phenol and COD respectively. The rate of degradation of phenolics in the extracted waste was less than half that observed for the unextracted waste, which indicates the recalcitrance of these compounds to degradation. The high molecular weight polyphenols are known to be more resistant to biodegradation than the low molecular weight aromatic compounds (Sayadi *et al.*, 2000).

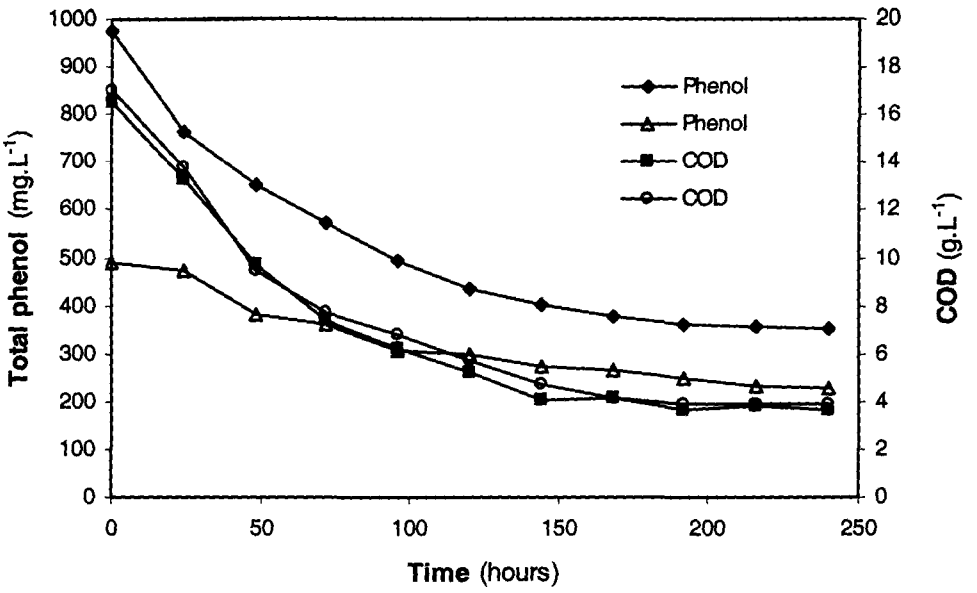


Figure 4.7: Total phenol and COD reduction by mixed microbial culture of black olive brine wastewater (solid data points), compared to degradation of wastewater from which the low molecular weight phenolics had been previously extracted (open data points)

Nevertheless, there was still appreciable reduction in total phenol content, again through the process of polymerisation and humification, resulting in a darkly coloured final effluent. The rate of COD degradation was unaffected, although it will be noticed that the initial concentration seems high considering that the low molecular weight phenolics had been extracted. This was attributed to residual ethyl acetate dissolved in the wastewater after the extraction process (liquid/liquid extraction was performed in this case), as was evident from the smell of the wastewater after extraction.

Benitez *et al.* (1999) and Beltran-Heredia *et al.* (2000), who used mixed aerobic cultures for the degradation of black olive brine wastewater, did not add yeast extract or other supplements to their wastewater media. The effect of yeast extract supplementation was therefore investigated in a brief experiment. Flasks were prepared with and without $5g.L^{-1}$ yeast extract, but the wastewater was diluted to 10% in this case. The results for this experiment are shown in Figure 4.8. Removal of total phenol concentration was initially slightly slower for the flasks that lacked nitrogen, but the final concentrations measured were essentially identical. The samples containing yeast extract initially had a higher COD due to the addition of oxidizable components in the yeast extract, but this was reduced to a value

comparable to the samples that were not supplemented. Other observations such as increased colour and pH were again observed.

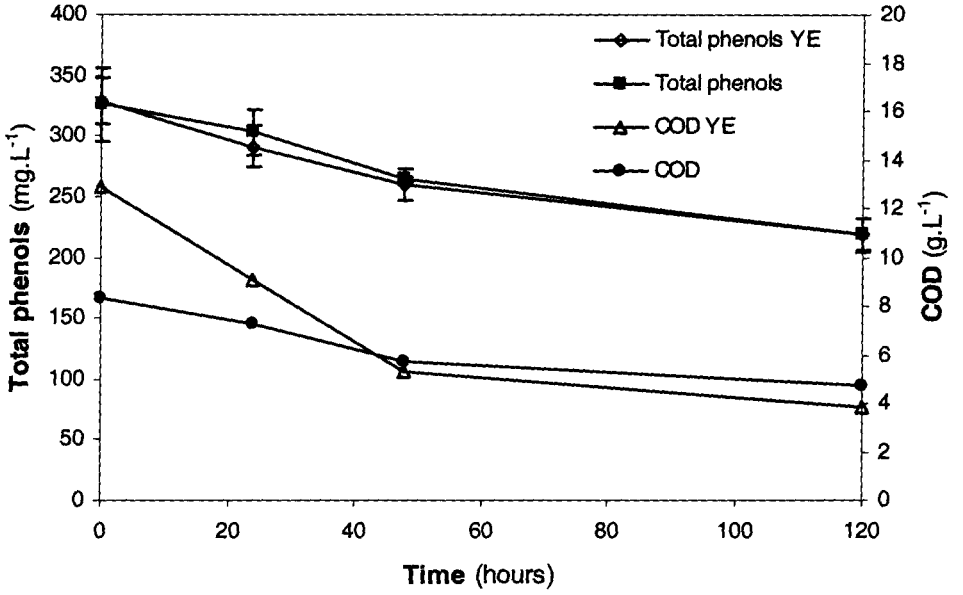


Figure 4.8: Mixed culture degradation of black olive brine in wastewater media supplemented with yeast extract (open data points) and without supplementation (solid data points)

It was evident that the addition of yeast extract did not have a significant effect on the degradation process, indicating that for mixed cultures the addition of nitrogen substrate was not necessary. This is contrary to results observed during preliminary experiments with monocultures, where addition of nitrogen was found to enhance the growth and degradation process. This is another advantage of using mixed microbial cultures as opposed to pure cultures for wastewater degradation purposes.

4.4 CONCLUSIONS

In work discussed in this chapter, black olive brine wastewaters were successfully degraded using both pure microbial species and mixed microbial consortia. Degradation was evident through the reduction of total phenol content and COD, and essentially involved the conversion of soluble organic compounds into biomass (and off gasses), thereby reducing the organic load of the residual wastewater effluent.

Degradation of the black olive brine wastewater, generally, involved polymerisation and polycondensation of phenolic compounds into high molecular weight humic-like substances. This has been previously observed with aerobic degradation of some olive-derived wastewaters. However, the decolourisation of mill wastewaters (as a result of depolymerisation of high molecular weight phenolic compounds), reported by many researchers, was not achieved with the black olive brines. The high salt concentrations in the brines and lack of readily fermentable sugars and lipids are the main distinctions between brine wastewaters and mill wastewaters, and are suspected to be the main reason for the humification process as opposed to decolourisation, but the mechanism for this is not known.

The process of humification can be considered to be beneficial, as humic substances represent a stabilised form of organic matter that incorporate organic nitrogen, and are thus a suitable feedstock for subsequent anaerobic digestion, depending on the salt concentration. In addition, the aerobic degradation and humification process resulted in the removal of low molecular weight aromatic phenolic compounds, and hence reduced toxicity of these wastewaters.

Of the pure cultures investigated in this work, *Candida maltosa* exhibited the best degradation rates. It was able to degrade phenolic compounds that occur in the black olive brines at a rate of 490 $mg.L^{-1}$ in 24 hours. However, *Trametes pubescens* showed the highest percentage degradation of total phenolic compounds (85%), although this took longer. Results using *Aspergillus niger* were inconsistent and marginally successful, while no degradation was achieved with *Lactobacillus plantarum*.

The degradation rates observed using mixed microbial cultures were not as good as those observed for the pure microbial cultures, but the percentage removal of total phenolic compounds was of the same order (64%). Mixed cultures are more feasible for such wastewater treatment applications, as it is difficult to maintain pure microbial cultures for extended periods of time. In addition, wastewaters need to be sterilised before treatment if pure cultures are to be used, and this is not feasible at large scale due to the costs incurred. For mixed microbial cultures this is not the case. Mixed cultures also appeared to be more capable of removing non-phenolic substrates from the wastewaters, as was evident from their superior removal of COD compared to pure cultures.

The ability of the mixed cultures to degrade wastewater that had been previously extracted (to recover the low molecular weight phenolic antioxidants) is of some interest. The extraction process resulted in the removal of approximately 50% of total phenol content, while subsequent degradation then resulted in a removal of a further 25%, and hence the total amount of phenol removed (approximately 75%) was greater than that removed from the unextracted wastewater by degradation alone (approximately 65%), even though degradation rates were slower. The combination of extraction and subsequent degradation processes is thus very appealing, as a valuable by-product is so obtained, and the organic load of the wastewater is reduced to a greater extent than is achieved using biological degradation alone.

Future work to be considered should involve the appropriate modelling of the degradation process, in order to determine kinetic rate constants (specific growth rates, substrate and biomass yield etc.) and inhibition coefficients. Such knowledge would be useful for the optimisation of the degradation process, and for the design and scaling of biological processes or bioreactors for this purpose. The influence of the different non-phenolic wastewater components (sugars, lipids and salt), as well as pH, upon the metabolic pathways of phenol degradation, needs to be further investigated.

CHAPTER 5: DEVELOPMENT OF A SMALL SCALE BIOREACTOR SYSTEM FOR TREATMENT OF HIGH-STRENGTH WASTEWATERS

5.1 INTRODUCTION

The objective of the work reported here was to design and evaluate a small-scale bioreactor for the treatment of high strength wastewaters, using the black olive brines as a model agro-industrial effluent. There is a need for such small-scale, end-of-pipe treatment systems for the numerous olive and olive oil producers, who are often isolated and do not have access to appropriate treatment facilities. Such a system should be capable of treating wastewaters by reducing COD and total phenol content, thereby reducing the organic load and environmental burden of the wastewater before discharge or appropriate reuse. A submerged membrane bioreactor (sMBR) was identified as a likely treatment system, as discussed in section 1.5. A laboratory scale sMBR was, therefore, designed, constructed and evaluated. Because of the impracticality of transporting and storing large quantities of wastewater, the working volume of the reactor was limited to 1 L for initial studies, but this was later scaled-up 4 L for further evaluation.

Evaluation of the bioreactor involved appropriate hydrodynamic modeling and characterization, followed by degradation studies that could be compared to the literature and to the results of the work reported in the previous chapter. Hydrodynamics within an sMBR have an important effect, not only in terms of mixing and mass transfer, but also on membrane performance, as the gas-induced liquid velocity past the membrane surface directly affects concentration boundary layers and thus membrane fouling. High crossflow velocities are desirable as they reduce the phenomenon of concentration polarization, which occurs as a consequence of deposition of solid material onto the membrane surface. This results in cake layer formation and subsequent reduction in membrane permeate flux, increased transmembrane pressures and, therefore, increased operating cost. Hydrodynamics, aeration effects and other parameters such as solids concentration, hydraulic residence time and suction pressure all affect operational performance of an sMBR. These have been extensively studied and modeled (Fane *et al.*, 2002; Sofia *et al.*, 2004; Liu *et al.*, 2000, 2003; Ueda *et al.*, 1997; Gui *et al.*, 2002; Le-Clech *et al.*, 2003).

Hydrodynamics in the reactor were modelled according to the work of Chisti and Moo-Young (1993). These authors provide an empirical model that predicts the gas-induced liquid circulation velocity based on aeration intensity, friction coefficients, reactor geometry and liquid column height. It has been found to be applicable for a wide range of draught tube airlift reactor designs and operating conditions, including draught tube and annulus sparged internal loop reactors, split cylinder reactors and external loop reactors, of both round and rectangular cross-sectional areas. The effects of hydrodynamics on mixing and mass transfer were also determined by measuring the oxygen mass transfer coefficient.

The molecular weight cut-off (MWCO) of the membranes used in an sMBR depends on the application. For high quality treated domestic wastewater effluents where water reuse is required MWCO's of 0.2μm are common, *i.e.* the membrane serves a filtration function and assists in the purification of the effluent. For high strength wastewater where a single biological unit operation is unlikely to be capable of complete treatment (as is the case for olive wastewaters), larger MWCO's can be used, as the main purpose of the membrane is then to retain microbial cells within the reactor. This improves permeate flux and thus reduces required membrane surface area and cost. A ceramic membrane was chosen for use in the bioreactor. Ceramic membranes are more robust than their polymeric counterparts and have better chemical resistance, which allows for facile sterilisation and membrane cleaning (using strong acids and bases, for example).

The effects of hydrodynamic factors in the reactor on membrane performance were evaluated by performing critical flux experiments. Critical flux can be defined as the rate of flow through a membrane (per unit area) above which the rate of deposition of solid (or colloidal) material onto the membrane surface exceeds the rate of removal due to hydrodynamic factors. Therefore, when critical flux is exceeded rapid fouling of the membrane occurs; transmembrane membrane permeate flux declines and transmembrane pressure (driving force) increases (Sofia *et al.*, 2004; Fane *et al.*, 2002; Wu *et al.*, 1999; Bouhabila *et al.*, 2001; Stephenson *et al.*, 2000). Below the critical value flux is directly proportional to transmembrane pressure. The critical flux is significantly enhanced with increased liquid crossflow velocity past the membrane surface. It is desirable to operate membrane bioreactors below the critical flux as this extends operational time, and minimizes energy and cleaning requirements.

Critical flux is generally determined by performing a flux-step experiment. Here, flux is increased in a step-wise fashion until transmembrane starts to increase exponentially, which then corresponds to the critical value. The aim of these experiments was to investigate the effect of draught tube-reactor geometry on critical flux, using a model colloidal system. A narrow draught tube increases liquid velocity past membrane (as well as constraining air bubbles to enhance the scouring effect), which is expected to improve the critical flux.

To evaluate the bioreactor in terms of wastewater treatment, batch degradation studies were first performed using several of the microbial species investigated in Chapter 4. Parameters of interest were substrate degradation rates (in terms of COD and total phenols), % substrate removal and membrane performance, well as the versatility of the bioreactor in terms of growing different types microorganisms (bacteria, fungi, yeast and mixed cultures). Although it is of interest to combine a degradation process with an upstream extraction process, the membrane-assisted extraction system (Chapter 3) was not yet suitably developed to produce the large quantities of extracted wastewater required for degradation experiments in the reactors. Un-extracted (raw) wastewater was thus used for degradation experiments in the reactors.

Fed-batch and continuous feed degradation experiments were then performed, in order to evaluate the ability of the reactor system to treat the wastewater in a sustained manner, as would be required in industry. The effects of wastewater concentration were first investigated in fed-batch experiments, by successively increasing the concentration of feed. Next, the influence of organic loading rate (OLR) was investigated in continuously-fed experiments, by increasing the feed flow rate. These experiments resulted in an approximate estimate of operating conditions for industrial purposes.

5.2 MATERIALS AND METHODS

5.2.1 Design, construction and operation of a submerged membrane airlift bioreactor system

A 1 L laboratory bench-top reactor was designed as a standard bubble column with a centrally located single tubular ceramic membrane. One of two different sized draught tubes could be inserted into the reactor, creating a rising (and recirculating) liquid flow regime past

the membrane, due to the rising passage of air bubbles. This allowed for investigation of hydrodynamic effects with different riser-to-downcomer cross-sectional area ratios (A_R:A_d), and also for comparison of draught tube-to-bubble column configurations. The reactor was constructed from clear Perspex to allow for visual observation. Figure 5.1 shows a schematic cross-section of the reactor with a draught tube in place (see Appendix B for full design details).

After the 1 L reactor had been successfully tested, modelled and used for initial degradation studies, it was scaled up to 4 L, and two of these reactors were constructed. The larger reactor size allowed for larger and more numerous samples. The reactor design was essentially the same as for the smaller reactor, except for dimension changes. The 4 L reactor design is shown in Appendix (B). Table 5.1 shows the details of the two reactors.

The oxygen sparger beneath the membrane was made from sintered steel and can be considered to be a fine bubble diffuser. All other tubing and fittings were made from stainless steel. The membrane was potted and sealed into the end fittings using epoxy glue

Airflow to the reactor was supplied by a compressor. The inlet air was filtered (Millipore 0.45 μm) and then directed through a sterile water-filled flask for humidification prior to entry into the reactor. Pre-humidification of the air was performed in order to minimize evaporation (*i.e.* loss of working fluid volume during operation). Airflow rate was controlled using a needle valve, and measured using a calibrated rotameter. Exhaust air was directed through a water trap to prevent escape of microbes to the environment.

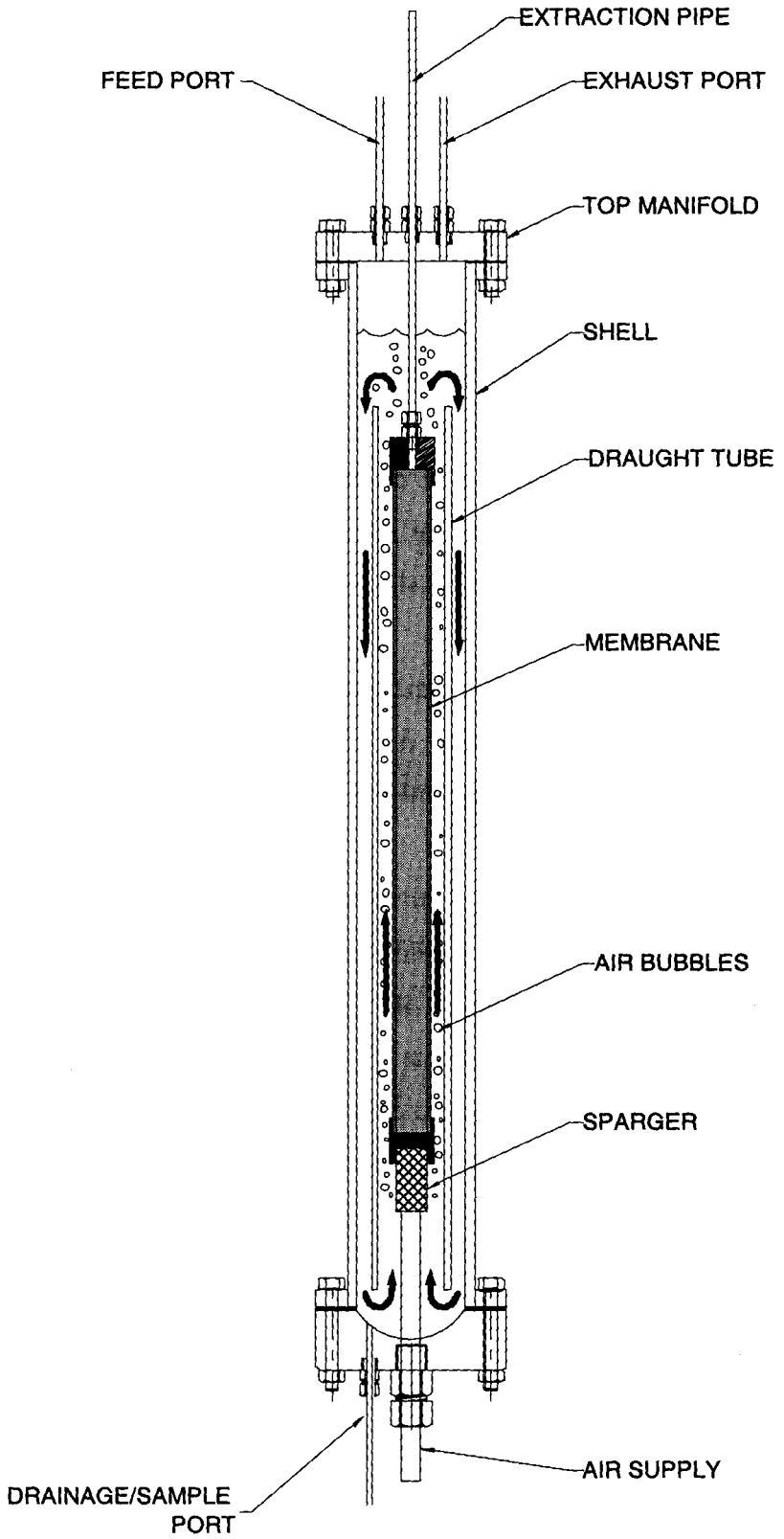


Figure 5.1: Schematic of 1 L submerged membrane airlift reactor

Table 5.1: Details of submerged membrane airlift reactors used in this work

Parameter	1 L Reactor		4 L Reactor
Total height	500 mm		536 mm
External diameter	60 mm		110 mm
Internal diameter	54 mm		100 mm
Total volume	1.3 L		4 L
Working volume	1 L		3 L
Draught tube	**1**	**2**	
External diameter	30 mm	34 mm	50 mm
Internal diameter	26 mm	40 mm	40 mm
Riser cross-sectional area (A_r)	4.18×10^{-4} m^2	7.95×10^{-4} m^2	1.14×10^{-3} m^2
Downcomer cross-sectional area (A_d)	1.58×10^{-3} m^2	1.03×10^{-3} m^2	5.89×10^{-3} m^2
Riser to downcomer ratio (A_r : A_d)	1 : 3.8	1 : 1.2	1 : 5.2
Sectional area below draught tube (A_b)	2.20×10^{-3} m^2	2.98×10^{-3} m^2	4.24×10^{-3} m^2
Membrane			
Active length	195 mm		195 mm
Diameter	12 mm		12 mm
Surface area	7.54×10^{-3} m^2		7.54×10^{-3} m^2
Nominal pore size	1μm		1μm

A peristaltic pump was used for withdrawal of permeate through the membrane. A mercury manometer was connected to the suction line to monitor transmembrane pressure (TMP). Permeate could be recycled back into the reactor to maintain working volume during hydrodynamic studies. Pure water flux (PWF) through the membrane was routinely measured to check for membrane fouling.

Figure 5.2 shows a schematic diagram of the entire reactor system.

5.2.2 Hydrodynamic modelling of liquid recirculation in the reactor

Modelling of the liquid velocity past the membrane surface was performed by filling the reactor with water and then measuring the liquid velocity at different air flow rates, using black rubber pellets as tracers. The passage of the pellets past the membrane was filmed using a digital camera (Logitec Quickcam®, California , USA). Frame-by-frame analysis

allowed for the measurement of distance travelled during a specific time period, allowing for calculation of average velocity in the riser. Frames were captured at 25 per second, and were analysed using open source Virtualdub software (www.sourceforge.com). The measured liquid velocity in the draught tube riser was then compared to theoretical values for the different airflow rates. Theoretical values were calculated as described in section 5.3.1.

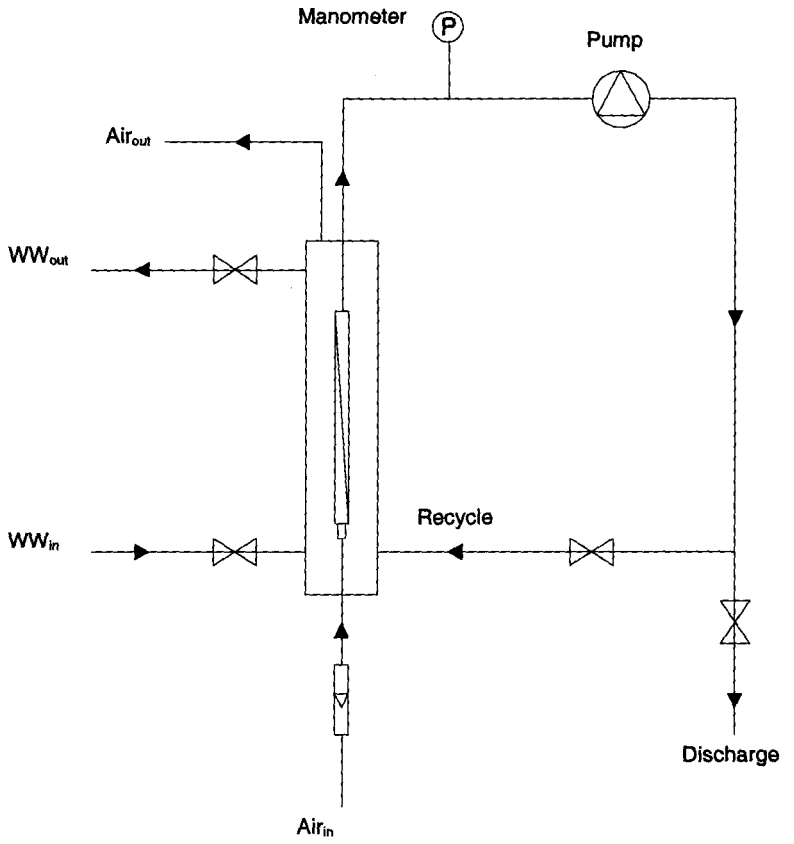


Figure 5.2: Schematic diagram of bioreactor system

5.2.3 Measurement of the oxygen mass transfer coefficient

The 4 L bioreactor was used for evaluation of the oxygen mass transfer coefficient, as the available dissolved oxygen (DO_2) probes were too large to be incorporated into the 1L reactor. The procedure was as follows: firstly the DO_2 probe (YSI 5739 field probe, Yellow springs, Ohio, USA) was calibrated in a deoxygenated saturated Na_2SO_3 solution as a zero point, and then in an air-saturated pure water solution (DO_2 = 8.68 mg.L^{-1} at 21°C). The probe was then inserted into the top reactor above the downcomer zone, and the reactor was

filled with ddH_2O. The reactor was deoxygenated by sparging with nitrogen for 30 minutes. Once the DO_2 concentration had reached near zero, the nitrogen stream was replaced with air at a specified flow rate, and the DO_2 concentration was logged using a PC. Experiments were performed in triplicate, at an airflow rate of 3 $L.min^{-1}$, which was the aeration intensity used for degradation experiments.

5.2.4 Determination of the critical flux through the membrane

The critical flux experiments were performed in the 1 L reactor. For a model colloidal solution, *Candida maltosa* cell broth was prepared by performing a batch degradation experiment in shake flasks, using black olive brine wastewater as substrate (as described in section 4.2.3). After 96 hours this was sterilised by autoclaving. Sterilisation removed biological factors (such as variation in viscosity and cell density) from the experiment. The viscosity of the broth was measured using a Physica Rheolab MC 1 viscosity meter, and was found to be approximately Newtonian, with a value of 0.95 mPa.s. The suspended solids concentration of broth was 7.32 $g.L^{-1}$.

Permeate was withdrawn continuously through the membrane by suction using a peristaltic pump. Permeate flow rate and membrane flux are directly related through membrane surface area, therefore a step increase in flux was obtained by increasing the suction pump flow rate. After a flux-step increase the system was monitored until steady state was reached (~30 minutes), after which the next flux-step increase was made.

Transmembrane pressure (TMP) was measured with mercury manometer. Flow rates were measured using a volumetric flask and a stopwatch. Air was supplied at 1 $L.min^{-1}$ in all experiments. Permeate withdrawn from the membrane was returned to the reactor to maintain a constant volume.

The flux step experiment was repeated for the three different configurations (bubble column, narrow and wide draught tube). Pure water flux measurements were made between experiments to monitor long-term irreversible fouling. After each experiment the membrane was soaked overnight in a 5N NaOH solution to clean the membrane.

5.2.5 Batch degradation experiments

Batch degradation experiments were performed in a similar manner as described in Chapter 4, except that the reactor system was used instead of shake flasks. The wastewater used for batch degradation experiments was black olive brine, and was supplemented with yeast extract as required. Wastewaters were sterilised by autoclaving before use.

The experimental system was set up as illustrated in Figure 5.2. Before operation the reactor system was sterilised using a 10% bleach solution, after which the reactor was rinsed with sterile tap water. After inoculation, the supply air was switched on and set to a flow rate of $1L.min^{-1}$ (1vvm). Air was filter-sterilised and humidified before introduction into the reactor. The suction pump was then switched on, and permeate withdrawal through the membrane was set to approximately $0.75ml.min^{-1}$, which was well below the critical flux previously determined. Samples were taken (daily) from the permeate withdrawn from the reactor, while the remainder of the permeate was returned to the reactor so as to maintain working volume and consistent hydrodynamic conditions. Membrane fouling was monitored throughout experiments by measuring suction pressure and permeate flux as described above. Some experiments were repeated under identical conditions for different reactor configurations (bubble column or draught tube), in order to investigate the effects of different hydrodynamic conditions on degradation rates and membrane performance.

The organisms used for the batch degradation experiments were *B. megaterium, C. maltosa, T. pubescens* and mixed microbial culture as described in Chapter 4. A 10% (v/v) inoculum was used in all cases. Initial and steady state biomass concentrations were measured as suspended solids, while wastewater analyses were performed as described in Chapter 4. These experiments were performed in the 1 L reactor.

5.2.6 Fed-batch and continuous degradation experiments

The 4 L reactor system was modified slightly from the system shown in Figure 5.2 in order to make the fed batch and continuous experiments possible. Figure 5.3 shows a schematic diagram of the modified system, while a photograph of the actual reactor and system is shown in Figure 5.4.

Peristaltic pumps were used to supply the feed stream and withdraw permeate from the reactor, and transmembrane pressure was monitored with a mercury manometer as before. Flow rates were calculated volumetrically.

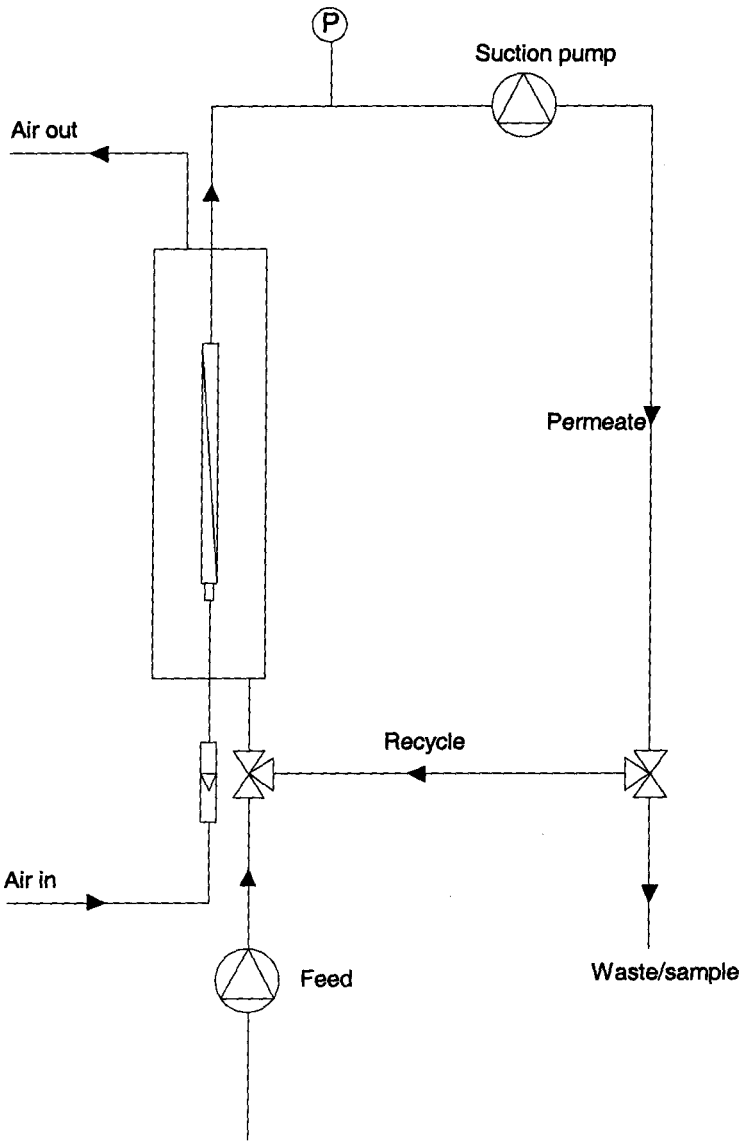


Figure 5.3: Schematic diagram of reactor system for fed batch and continuous degradation experiments

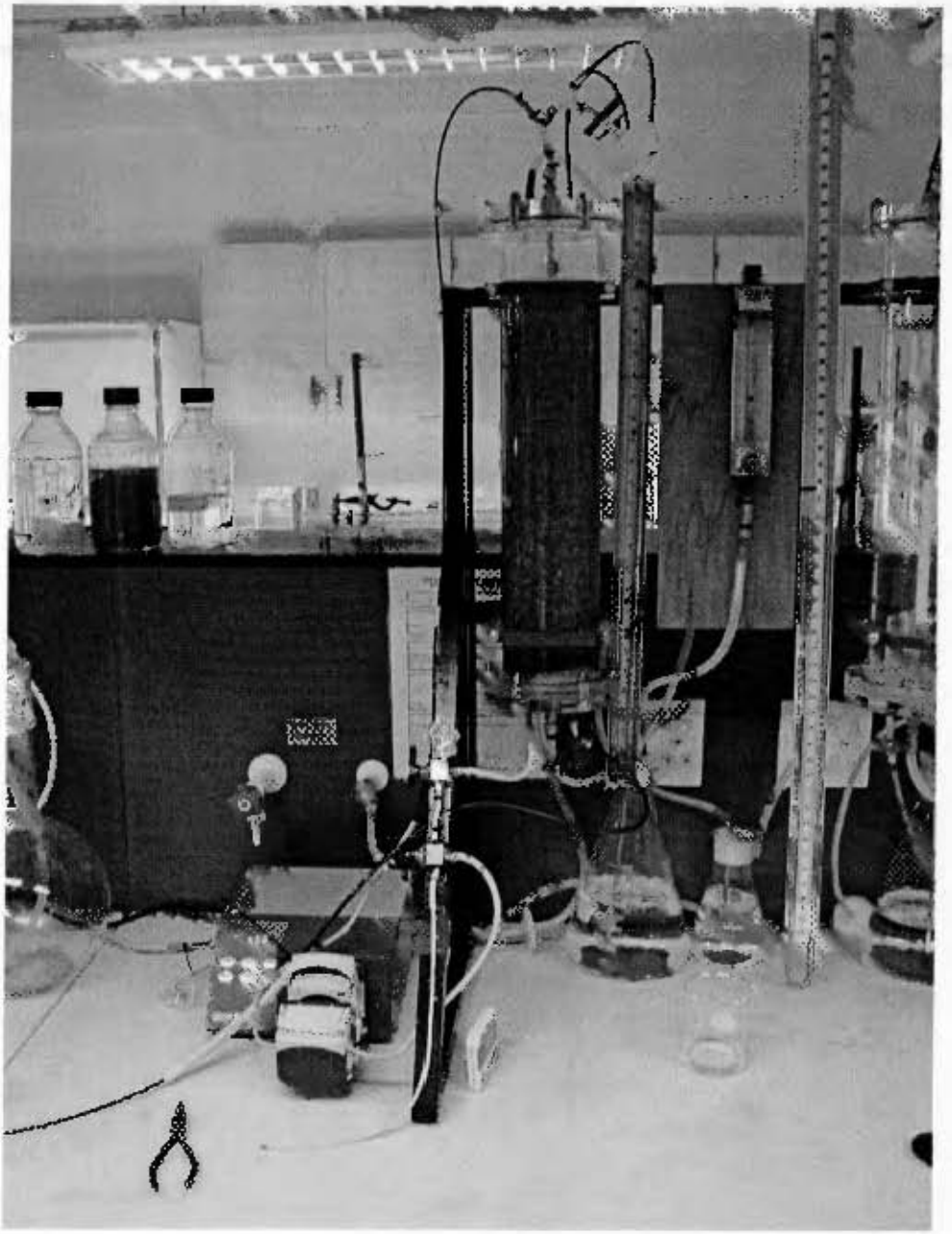


Figure 5.4: Photograph of the 4L bioreactor system

Fed batch experiments were performed by repeatedly supplying fresh doses of wastewater to the reactor, and then monitoring subsequent degradation. Initial batch fed studies were performed by dosing the reactor every 48 hours with a consistent volume (300 mL or 10% reactor volume) and concentration (25%) of wastewater, which was equivalent to adding approximately 300 $mg.L^{-1}$ to the total phenol concentration in the reactor at that time. Permeate was continuously recycled as in batch degradation experiments, except during draining prior to the addition of a new dose, when an equivalent dose volume was discharged

to waste. Membrane fouling was monitored through permeate suction pressure, using a mercury manometer as before. No cleaning or backwashing was performed during experiments.

Subsequent fed batch experiments were then performed to investigate the effect of increasing wastewater concentrations on degradation in the reactor. In this case the batch volume of wastewater supplied to the reactor was also 300 ml, but the concentration of the doses was successively increased. Degradation rates after dosing were then monitored as before.

For continuous feed experiments, wastewater was introduced into the reactor using a step-wise increase in flow rate, corresponding to an increase in organic loading rate (OLR) and decrease in hydraulic residence time (HRT). In this manner it was possible to determine the point at which organic overload occurred, and hence to estimate the maximum degradative capability of the reactor system.

Based on the results of the fed batch experiments, it was decided to use a total phenol concentration in the wastewater feed of approximately 800 $mg.L^{-1}$ for continuous degradation experiments. Due to the problem of eventual contamination of pure microbial cultures, a mixed culture was used for continuous degradation experiments. In order to maintain a consistent wastewater volume in the reactor, effluent was continuously withdrawn from the reactor (through the membrane) at the same volumetric flow rate as the feed influent.

The reactor was filled with wastewater at the appropriate dilution, inoculated, and operated for 3 days in recycle mode (to allow for biomass growth and acclimatization) before continuous feeding of wastewater was commenced. The organic loading rate was then increased by increasing the feed flow rate every 24 hours, until such a time that the effluent concentration from the reactor started to increase, indicating organic overload.

5.3 THEORY

5.3.1 Hydrodynamic modelling

In a draught-tube airlift reactor, the main frictional loss occurs as the recirculating liquid passes underneath the draught tube, because flow is constrained here. It is much larger than

the frictional loss at the membrane walls or at the top (open channel) of the reactor, and these can both be neglected. From Chisti and Moo-Young (1993), the empirical equation for (superficial) velocity of the liquid in the riser of an internal loop is given by:

$$U_{Lr} = \left[\frac{2gh_D(\varepsilon_r - \varepsilon_d)}{K_B \left(\frac{A_r}{A_d} \right)^2 \frac{1}{(1-\varepsilon_d)^2}} \right]^{0.5} \qquad (5.1)$$

Where U_L = liquid velocity in the riser ($m.s^{-1}$)

g = gravitational acceleration = 9.81 $m.s^{-2}$

h_D = gas-liquid dispersion height (m)

ε_r , ε_d = fractional gas holdup in riser and downcomer respectively

K_B = frictional loss coefficient for the bottom of the reactor

A_r , A_d = cross-sectional area of riser and downcomer respectively (m^2)

Solving this equation is an iterative process, as the parameters are all mutually dependent. One such iteration is shown below for the 1 L reactor with draught tube 1 inserted, at an airflow rate of 1.2 $L.min^{-1}$. Firstly, a value is assumed for the liquid riser velocity, and then the corresponding fractional gas hold-up in the riser is calculated:

$$\varepsilon_r = \frac{U_{Gr}}{0.24 + 1.35(U_{Gr} + U_{Lr})^{0.91}} \qquad (5.2)$$

where U_{Gr} = superficial gas velocity in the riser ($m.s^{-1}$).

Equation (5.2) was developed by Hills (1976) for independently controlled flow of air and water ($U_L + U_G$) of less than 1.3$m.s^{-1}$.

$$U_{Gr} = \frac{\phi_G}{A_r} \qquad (5.3)$$

where ϕ_G = gas flow rate = 1.2×10^{-3} $m^3.min^{-1}$ = 2×10^{-5} $m^3.s^{-1}$

A_r = riser cross-sectional area = 4.36×10^{-4} m^2

therefore $U_{Gr} = 0.0459$ m.s^{-1}

and $U_{Lr} = 2$ m.s^{-1} (assumed value)

The above values are substituted into equation (5.2), resulting in

$$\varepsilon_r = 0.016$$

For annulus-sparged draught tube reactors without gas-liquid separators, there is the following empirical relationship between riser and downcomer for gas hold-up (Chisti, 1989):

$$\varepsilon_d = 0.9\varepsilon_r \tag{5.4}$$

therefore $\varepsilon_d = 0.014$

The overall gas hold-up is then calculated by:

$$\varepsilon = \frac{\varepsilon_r A_r + \varepsilon_d A_d}{A_r + A_d} \tag{5.5}$$

therefore $\varepsilon = 0.014$

Next, the gas-liquid dispersion height is calculated:

$$h_D = \frac{h_L}{1-\varepsilon} \tag{5.6}$$

where h_D = dispersed height (m)

h_L = ungassed liquid height = 0.43m

therefore $h_D = 0.436$

The frictional loss coefficient for the bottom of the reactor is given by:

$$K_B = 11.402\left(\frac{A_d}{A_r}\right)^{0.789} \tag{5.7}$$

Due to the difficulty in determining gas bubble interfacial area a_L the mass transfer coefficient is usually expressed as the product $k_L a_L$ (s^{-1}). Equation (5.8) can be integrated to obtain:

$$\ln\left(1-\frac{C_L}{C^*}\right)=-k_L a_L t \qquad (5.9)$$

Thus, if the dissolved oxygen concentration is measured over time for an initially de-oxygenated liquid, it is possible to calculate the mass transfer coefficient by plotting the left hand side of equation (5.9) against time, with $k_L a_L$ being the slope of the straight line so obtained.

5.3.3 Critical flux

Flux of a fluid through a membrane can be described by the D'Arcy equation:

$$J = K\Delta P \qquad (5.10)$$

where J = permeate flux ($L.m^{-2}.hr^{-1}$)

ΔP = transmembrane pressure (kPa)

and $$K = \frac{A_m k_m}{\mu}$$

where A_m = membrane area (m^2)

k_m = intrinsic membrane permeability (m^{-1})

μ = liquid viscosity (mPa.s)

The system permeability K ($L.m^{-2}.hr^{-1}.kPa^{-1}$ or $m^2.s.kg^{-1}$) is a dynamic coefficient that includes permeating liquid properties, whereas k_m is a function only of membrane properties (*e.g.* pore size, pore tortuosity, membrane material, hydrophobicity). K is the inverse of total resistance to flow of liquid through the membrane for the system, and as such can be used as a measure of fouling occurring on or within the membrane, as the viscosity in this case remains constant. Through measurement of flux and transmembrane pressure it was possible to calculate the value of K (as a function of time) for given hydrodynamic conditions. At some stage during the flux-step increase there comes a point at which the resistance to flow

starts to increase exponentially: this is the critical flux value. In this case it was measured as a corresponding exponential decline of the membrane permeability *K*.

5.4 RESULTS AND DISCUSSION

5.4.1 Hydrodynamic modelling

The empirical model for liquid recirculation velocity according to Chisti and Moo-Young (1993) is shown in Figure 5.5 for the 1 L reactor with the two different size draught tubes, in comparison to measured values (a spreadsheet of calculations is shown in Appendix C).

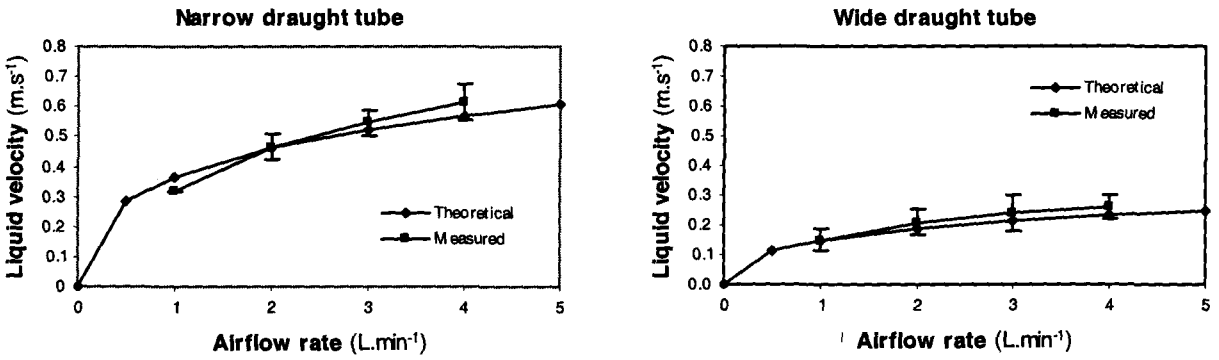


Figure 5.5: Theoretical and measured liquid velocity in the reactor draught tube riser as a function of airflow rate

The effect of the draught tube dimensions is clearly illustrated from the above figure: for the same airflow rate the liquid velocity in the narrow draught tube riser is more than double the velocity in the wide draught tube. The model provided a relatively good approximation to the measured values in both cases, but the fit was marginally better for the wide draught tube. In the case of the narrow draught tube, below an airflow rate of 2 $L.min^{-1}$ the model over-predicted the measured values, while above this airflow rate the converse was true. Possible sources of error between the modelled and measured values are incorrect and unconsidered friction coefficients (due to small scale of the reactor). The frictional losses around the membrane end fittings, in particular, were not taken into consideration, and these are likely to be significant because there the riser channel width changes abruptly. This would be exacerbated in the case of the narrow draught tube, and is a possible reason for the slightly poorer fit of the measured and theoretical values compared to the wide draught tube.

The model can be considered to be robust, as an initial assumption of riser velocity of U_{Lr} = 2 m.s^{-1} (see section 5.3.1) is somewhat unrealistic considering that the superficial gas velocity (U_{Gr}) at the corresponding aeration intensity was only 0.046 m.s^{-1}, nevertheless the iterations still converged rapidly to approximately the measured value.

Since the model provided a good approximation to the measured values for the 1 L reactor, it was used as an estimate for the design of reactor geometry for the scaled up 4 L reactor. This reactor was designed such that the riser to downcomer ratio resulted a liquid velocity in the riser of 0.35 m.s^{-1} at an aeration intensity of 3 L.min^{-1} (See Appendix C for details). This was the aeration intensity used for subsequent degradation experiments, while the desired liquid velocity in the riser was determined from the critical flux experiments (see section 5.4.3).

5.4.2 Measurement of the oxygen mass transfer coefficient

Figure 5.6 shows a plot of the dissolved oxygen concentration over time in the 4L reactor as given by equation (5.9). The slope of the straight line fitted to the data is equivalent to the measured oxygen mass transfer coefficient. The experiment was repeated three times at an airflow at a rate of 3 L.min^{-1}, equivalent to an aeration intensity of 1 vvm. The average mass transfer coefficient at this airflow rate was measured to be 0.0092 ± 0.0008 s^{-1}. This result is consistent with values published in the literature for airlift reactors (Lu *et al.*, 2000; Chisti, 1989; Chisti and Moo-Young, 1993), and is indicative of good mixing and mass transfer in the reactor.

As aeration intensity is the predominant energy input into an airlift reactor, it is common practice to operate airlift reactors at a value of around 1 vvm. Above this value the benefits of increased oxygen mass transfer rate are negated by a sharply increasing energy cost.

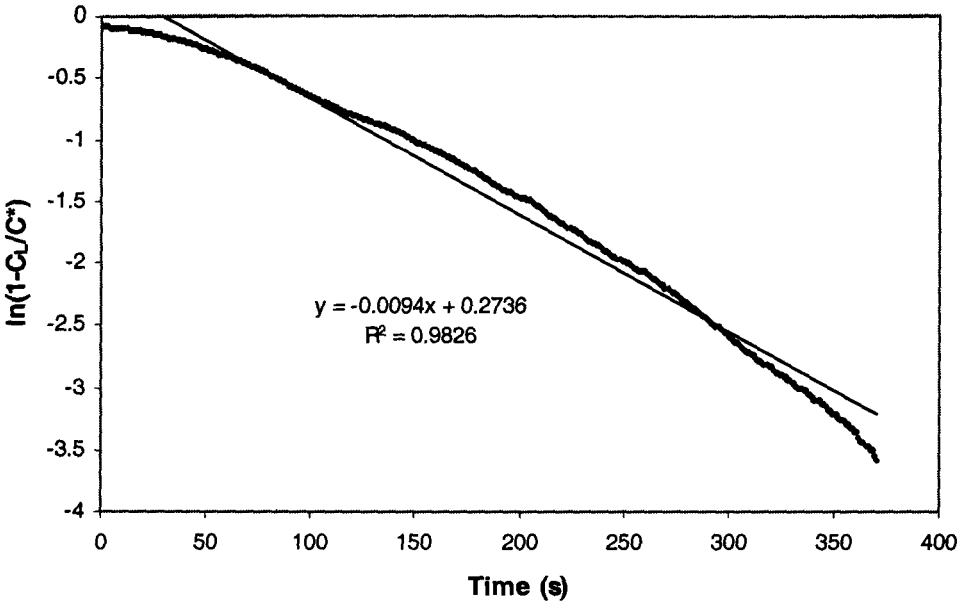


Figure 5.6: Plot of dissolved oxygen concentration over time used to evaluate the mass transfer coefficient

5.4.3 Critical flux experiments

Figure 5.7 shows the effect of increasing membrane flux (flow rate) on the membrane permeability. For the bubble column and wide draught tube (36/40 DT) the membrane permeability started to decline as soon as the permeate flow rate was increased above a value of 1.4 ml.min^{-1}, indicating that critical flux in these conditions had been exceeded.

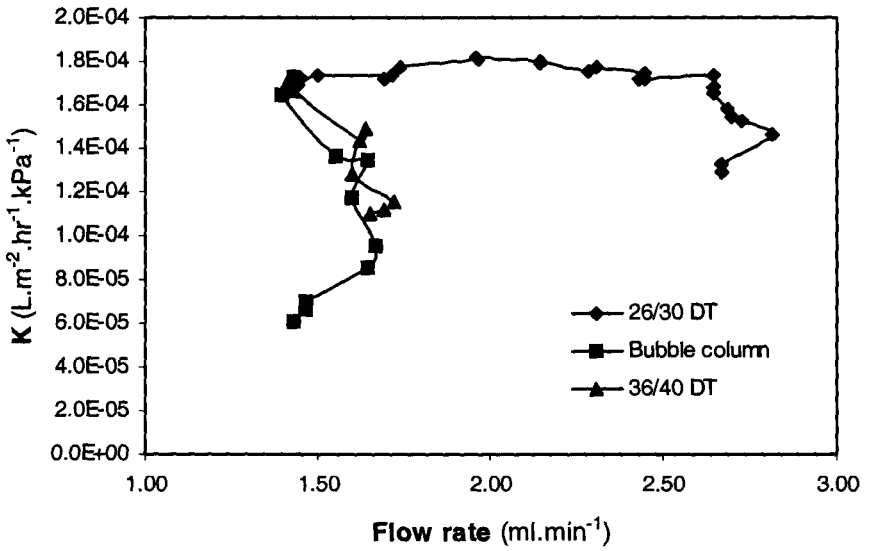


Figure 5.7: Membrane permeability in the airlift reactor as a function of permeate flow rate, for different reactor configurations

For the narrow draught tube (26/30 DT) the situation was much improved, and here the critical flux was only reached at a permeate flow rate of 2.6 $ml.min^{-1}$, or almost double the flux compared to the other two configurations. Thereafter membrane permeability decreased rapidly and drastically, resulting in a declining permeate flow rate and a corresponding increase in transmembrane pressure.

Figure 5.8 shows the membrane permeability as a function of time for the same experiment, illustrating the same trend: rapidly declining permeability for the bubble column and wide draught tube, compared to the relatively stable permeability of the narrow draught tube. The data shown in these two graphs occurs in groups of 3 measurements for each permeate flow rate, so as to ensure that steady-state had been reached before the next flux-step increase was made.

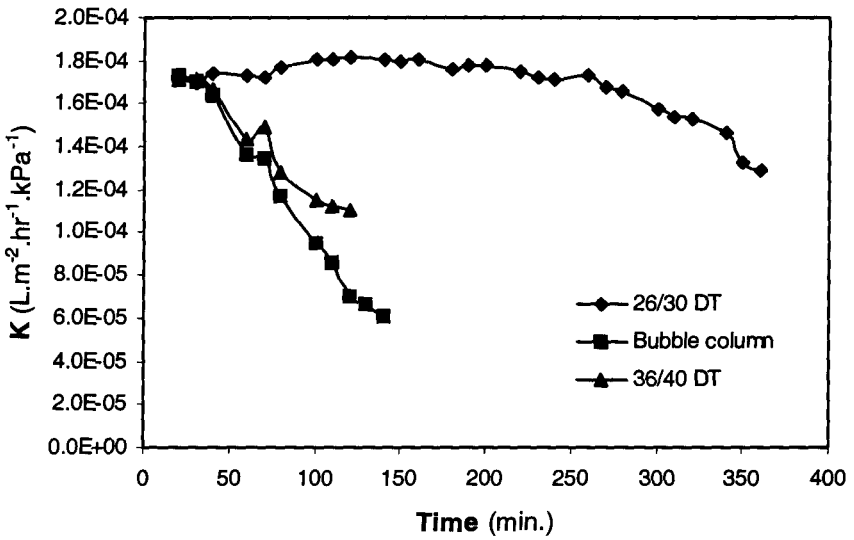


Figure 5.8: Membrane permeability in the airlift reactor as a function of time, for different reactor configurations

The critical flux was clearly improved at higher crossflow velocity, which was achieved by using a narrow draught tube in the reactor while keeping all other parameters constant. The theoretical riser velocity for the narrow draught tube at the given conditions was $0.34 m.s^{-1}$, while for the wider draught tube it was $0.19\ m.s^{-1}$. For the bubble column it was not possible to state a crossflow velocity, as the liquid in the reactor was turbulently mixed and there was no clearly defined flow pattern. These results are in good agreement with those reported by Liu *et al.* (2003). These authors used an sMBR for the treatment of municipal wastewater,

and observed a "critical crossflow velocity" of 0.3 $m.s^{-1}$. Below this value rapid membrane fouling occurred, irrespective of suspended solids concentration or sludge loading rates.

The implications of the above experiment are that membrane bioreactors should be carefully designed (in a geometric sense) to maximise the hydraulic situation. Cross flow velocity past the membrane has a fundamental effect on membrane fouling, and thus also on operational time, cleaning costs, and downtime. In this regard, the scaled up 4 L sMBR was designed with a riser–to-downcomer ratio such that at a nominal aeration intensity of 1 vvm, the theoretical liquid velocity in the riser was 0.35 $m.s^{-1}$.

5.4.4 Batch degradation studies in the 1 L reactor

In order to evaluate the biological efficiency of the reactor, batch degradation studies were performed, so that results could be compared to degradation rates observed in the shake flask experiments of Chapter 4. In addition, hydrodynamic effects were investigated with different reactor configurations. Initial batch degradation experiments using fungal (*T. pubescens*), bacterial (*B. megaterium* AS-35), yeast (*C. maltosa*) and mixed microbial cultures were all successfully performed in the 1 L sMBR, demonstrating the versatility of the system. Except for *T. pubescens*, the degradation rates in the reactor were generally found to be higher than in shake flask cultures, as shown in Table 5.2. The better degradation results were most likely due to improved oxygen mass transfer in the reactor. In degradation experiments with *T. pubescens*, results were much the same in the reactor compared to the shake flasks. It is possible in this case, that despite better oxygen mass transfer, there was a negative effect of turbulence and shear forces on the fungal mycelia.

Detailed results are presented for two batch degradation experiments in the sMBR, using *B. megaterium* AS-35, for the purpose of demonstrating hydrodynamic effects in the reactor with different configurations. The only difference between the two experiments was the presence or absence of a draught tube in the reactor, *i.e.* bubble column or (narrow) draught tube configuration.

Table 5.2: Comparison of degradation rates for different microbial species in shake flask culture and in the 1 L submerged membrane bioreactor

Organism	Shake flask				Bioreactor			
	X_{TP} (%)	Max. X_{TP} ($mg.L^{-1}.day^{-1}$)	X_{COD} (%)	Max. X_{COD} ($g.L^{-1}.day^{-1}$)	X_{TP} (%)	Max. X_{TP} ($mg.L^{-1}.day^{-1}$)	X_{COD} (%)	Max. X_{COD} ($g.L^{-1}.day^{-1}$)
B. megaterium	57	265	49	3.0	67	283	57	3.2
C. maltosa	67	490	57	4.4	68	502	61	4.7
T. pubescens	85	318	54	3.3	83	322	55	3.2
Mixed culture	64	212	78	3.6	71	218	82	3.7

X_{TP} = percentage removal of total phenolic content; Max. X_{TP} = maximum degradation rate of total phenolic content; X_{COD} = percentage removal of COD; Max. X_{COD} = maximum degradation rate of COD.

Figure 5.9 shows reduction in the total phenol content and COD over time by *B. megaterium* AS-35, for the two different reactor configurations. There was not much difference between the two experiments in terms of degradation results, but there was a significant improvement of in terms of membrane performance for the draught tube reactor. The total phenol removal was around 67% from initial concentrations of approximately 960 $mg.L^{-1}$, COD reduction was 57%, from initial concentrations of approximately 16 $g.L^{-1}$. The maximum degradation rates for phenol and COD were 480 $mg.L^{-1}.day^{-1}$ and 4.3 $g.L^{-1}day^{-1}$ respectively.

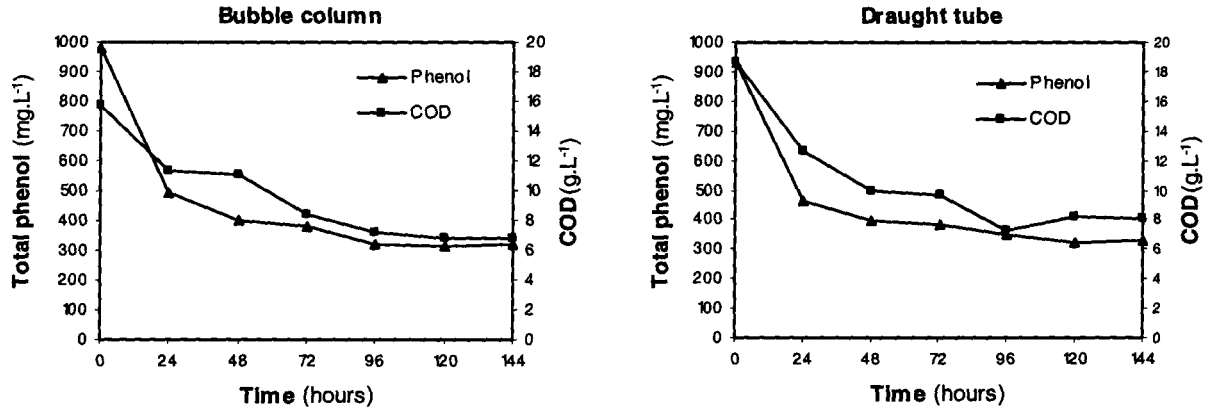


Figure 5.9: Phenol and COD degradation rates of 25% OW by *B. megaterium* AS-35 for different reactor configurations

These degradation rates were a significant improvement compared to results obtained in shake flask cultures. Degradation rates were improved by factors of 1.8 and 1.4 for phenol

and COD respectively, and percentage total removals were also improved by a factor of ~1.2. This was ascribed to better oxygen mass transfer in the reactor compared to shake the flasks.

The final biomass concentration in both of the reactor configurations was approximately 8.5 $g.L^{-1}$, which was 3 $g.L^{-1}$ higher than was reached with *B. megaterium* AS-35 in the shake flask cultures. This is also probably a consequence of improved oxygen mass transfer. However, similar trends of degradation were observed in the reactor as in the shake flasks, that is, increase in colour and pH. HPLC and size exclusion chromatography showed the disappearance of monomeric aromatic compounds and the accumulation of tannins and humic substances as discussed in section 4.3.1.

Figure 5.10 shows membrane permeability over time during the degradation experiments with *B. megaterium* AS-35, for the two different configurations. In both cases, permeate withdrawal was set at 0.75 $ml.min^{-1}$, well below the critical flux determined in section 5.4.3 for the draught tube reactor. Permeate withdrawal was continuous, with no backwashing or membrane cleaning during the course of the experiment. 0.75$ml.min^{-1}$ equates to a theoretical residence time of 22hrs for wastewater in the reactor.

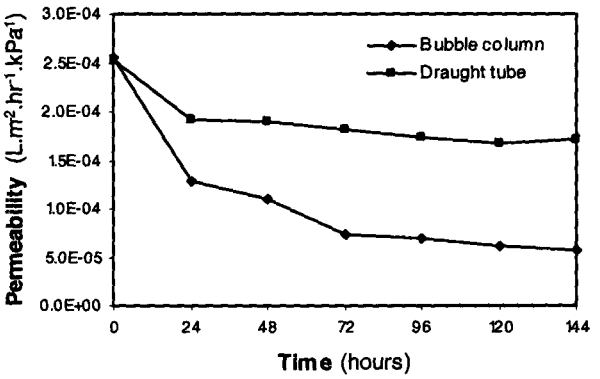


Figure 5.10: Membrane permeability during degradation experiments with *B. megaterium* AS-35, for different reactor configurations

Despite operation below critical flux, gradual fouling of the membrane was observed in both cases, as might be expected. However, the fouling in the bubble column configuration was significantly more severe than that which occurred in the draught tube reactor. This demonstrates the importance of the gas-induced cross flow velocity past the membrane surface, in terms of reducing fouling.

The implication of the above result is that the membrane performance in an sMBR can be significantly improved by careful design of a draught tube located around the membrane unit. The draught tube serves two purposes: firstly, it confines the air bubbles tightly around the membrane which improves the bubble scouring effect that removes deposited material from the membrane surface, and secondly, it induces a recirculating cross flow liquid velocity past the membrane which also reduces membrane fouling. Less membrane fouling means that operational time between cleaning or backwashing can be extended, and because of reduced transmembrane pressure there is also reduced operating cost.

5.4.5 Fed-batch degradation experiments in the scaled-up 4 L reactor

The objective of the fed-batch experiments was to investigate the ability of the reactor to degrade successive wastewater doses, and to investigate the effects of wastewater concentration of the successive doses. Preliminary fed-batch degradation experiments in the 4 L reactor showed that the various microbial species used in section 5.4.4 were all able to repeatedly degrade wastewater doses, at rates initially similar to those shown in Table 5.2 for the (single) batch degradation experiments. However, the number of repeated doses that could be degraded varied somewhat for the different microbial species. In general, contamination rather than other factors caused experiments to be terminated (except for the mixed culture experiments). The experiments showed a gradual increase in residual total phenol in the reactor after degradation of a fed-batch dose (results not shown), corresponding to an accumulation of high molecular weight polyphenolics and humic substances. This was accompanied by a gradual decrease in degradation rates.

The results of a fed-batch degradation experiment using *C. maltosa* in the 4 L reactor are illustrated in Figure 5.11. In this experiment, the wastewater concentration of the fed batch dose was successively increased, and the subsequent phenol degradation was closely monitored.

After the initial lag and growth phase (0 – 48 hours), it was evident that the degradation rates (negative slope after dosing) decreased with increasing dose, particularly after the concentration of total phenols exceeded a value of approximately 1000 $mg.L^{-1}$. During the initial growth phase the maximum degradation rate was 294 $mg.L^{-1}.day^{-1}$. After the first and second doses (at 50 and 98 hours), the degradation rates were 422 and 340 $mg.L^{-1}.day^{-1}$

respectively. After the third dose (146 hours) the degradation rate was significantly impeded (276 $mg.L^{-1}.day^{-1}$), resulting in a slight lag phase, indicating inhibition of metabolism at this wastewater concentration.

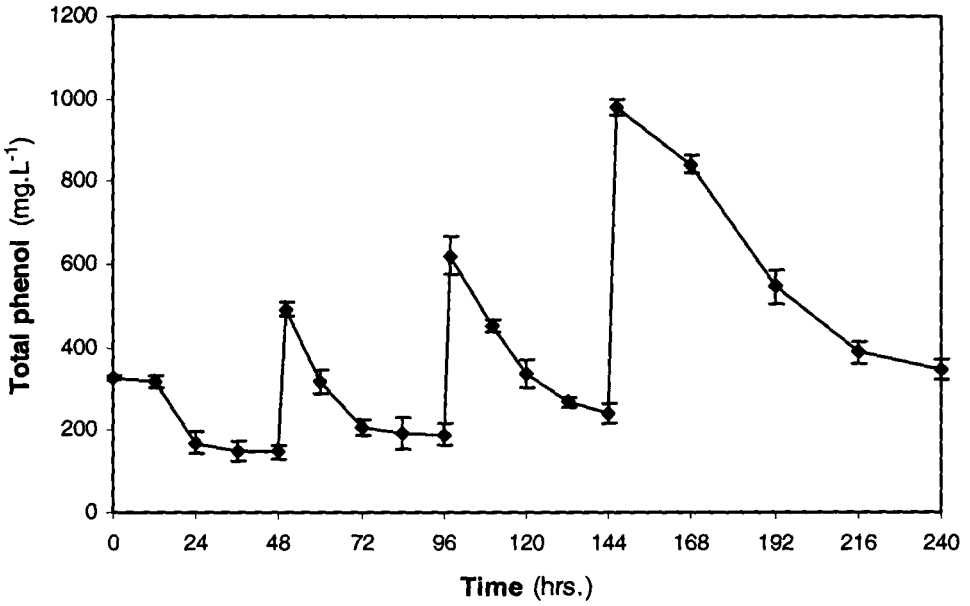


Figure 5.11: Total phenol degradation by *C. maltosa* in the 4 L reactor during a fed-batch experiment, with successively increasing dose concentration

The increase of residual total phenol concentration after degradation of successive fed-batch doses is also evident from Figure 5.11. This was determined to be due to the accumulation of high molecular weight (recalcitrant) polyphenols, by size exclusion chromatography. However, HPLC analysis showed that the majority of the low molecular weight phenolic fraction was metabolized. COD degradation closely followed the phenol degradation pattern. After the last dose (at 146 hours) the COD reached a level of 18.3 $g.L^{-1}$, and this was reduced to 8.2 $g.L^{-1}$ by the end of the experiment (55% reduction).

It was generally evident that inhibition of degradation and growth occurred at total phenol concentrations of around 1000 $mg.L^{-1}$. Increasing concentrations above this value resulted in severe inhibition, apparent through significantly poorer degradation rates and decline in biomass. Total phenol concentrations in the range 500-800 $mg.L^{-1}$ appeared to be the optimal initial concentration in terms of degradation; in this range degradation rates were similar to those obtained during batch degradation experiments performed previously with the different

species. Approximately 48 hours were required to adequately metabolise a wastewater dose in this range, while the average degradation efficiency was around 220 $mg.L^{-1}.day^{-1}$.

5.4.6 Continuous degradation experiments in the 4 L reactor

Continuous wastewater degradation experiments were performed with mixed microbial cultures, due to the contamination problems encountered with monocultures after extended time periods. The objective of these experiments was to determine the maximum organic loading rate (OLR) capable of being treated by the reactor, for a given influent concentration. The organic loading rate was increased by increasing the flow rate of the wastewater influent. Initially the reactor was filled with wastewater at a total phenol concentration of 800 $mg.L^{-1}$, and then operated for three days in recycle mode, to allow for biomass growth and acclimatization. Thereafter continuous feeding was commenced. The wastewater influent total phenol concentration was also 800 $mg.L^{-1}$; this value was decided upon from the fed-batch degradation experiments. Effluent was withdrawn from the reactor through the membrane at the same rate at which the influent was supplied, and the total phenol concentration of the effluent was monitored.

Figure 5.12 shows the results of the experiment. The total phenol concentration in the reactor was reduced to 362 $mg.L^{-1}$ (55%) over the first 72 hours. The average rate of degradation up to this point was 148 $mg.L^{-1}.day^{-1}$. At 72 hours continuous feeding was commenced, at an organic loading rate of 60 $mg.L^{-1}.day^{-1}$. This was increased daily in increments of approximately 60 $mg.L^{-1}.day^{-1}$. Effluent concentration remained relatively constant at approximately 330 $mg.L^{-1}$, until the organic loading rate reached a level of 420 $mg.L^{-1}.day^{-1}$ (at 216 hours). Thereafter, the effluent concentration started to increase, indicating conditions of organic overload (and possibly substrate inhibition).

While the effluent concentration remained relatively constant (72 to 216 hours), the degradation rate was essentially equivalent to the organic loading rate, and therefore, a maximum degradation rate of 420 $mg.L^{-1}.day^{-1}$ was achieved in this experiment, while the degradation efficiency at this rate was 57%. This was an improvement compared to the maximum degradation rate using mixed cultures in section 5.4.4, which was found to be 212 $mg.L^{-1}.day^{-1}$.

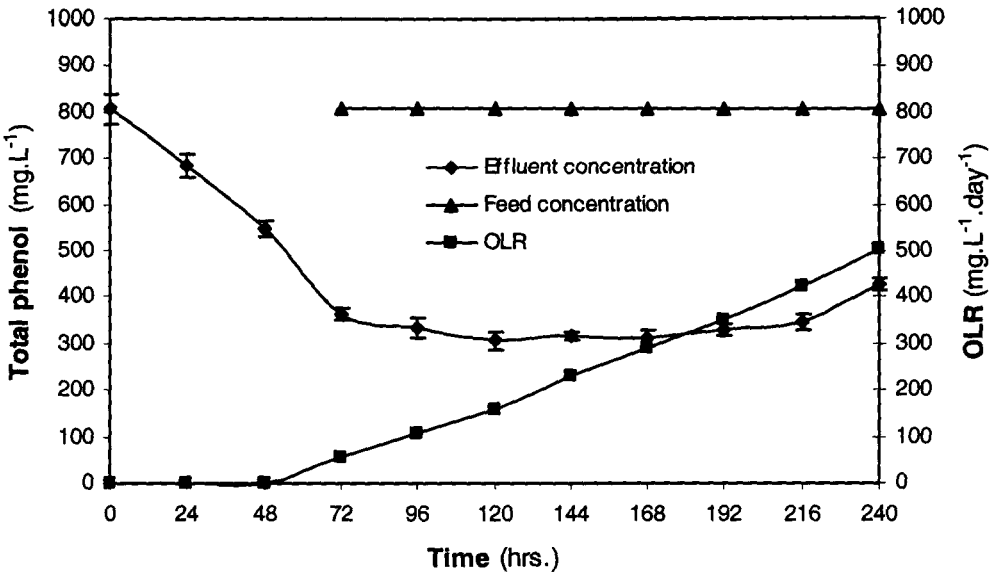


Figure 5.12: Total phenol reduction by mixed microbial culture in the submerged membrane airlift reactor during a continuously fed degradation experiment.

Due to the continual discharge of effluent, there was no gradual accumulation of high molecular weight humic substances in the reactor, as was observed in the batch degradation experiments. From a microbial metabolism point of view, continuous feeding appeared to be a preferable mode of operation (compared to batch-fed operation), because the high molecular weight humic substances (that are the product of metabolism) were continuously discharged, and hence did not accumulate in the reactor. In the batch-fed mode of operation these substances accumulated, and were possibly detrimental to the degradation process; hence the better degradation rates achieved with continuous feed.

The COD of the effluent was approximately proportional to the effluent total phenol concentration. The maximum COD degradation rate in this experiment (corresponding to the phenol degradation rate at 216 hours) was 6.4 $g.L^{-1}.day^{-1}$. These results are in good agreement with results reported by Beltran-Heredia *et al.* (2000) and Benitez *et al.* (1999), who investigated aerobic mixed culture treatment of black olive brines in batch degradation experiments. These authors reported COD degradation rates ranging between 3.2 and 13.4$g.L^{-1}.day^{-1}$, depending on wastewater parameters. The COD of their wastewater was approximately equivalent to the wastewater used in this work, but their total phenol concentrations were significantly lower at values ranging between 103 and 180 $mg.L^{-1}$. In addition, their dissolved solids concentrations were considerably lower than in this work. For

aerobic mixed culture degradation of green olive debittering wastewater, organic loading rates of 0.9 to 3.3 $g.L^{-1}.day^{-1}$ have been reported (Aggelis *et al.*, 2001).

The final biomass concentration reached in the continuous degradation experiment was 13.8 $g.L^{-1}$, and this is the most likely reason for the better degradation rates achieved compared to the shake flask experiments presented in Chapter 4, where the final biomass concentration was 10.4 $g.L^{-1}$.

Another factor that possibly contributed to the better degradation rates achieved in the continuously-fed experiments was the pH. The effluent pH was consistently lower (around 7.5) than in the batch degradation experiments, due to the acidity (pH 4.5) of the wastewater influent.

In this experiment, the permeate flux was dictated by the hydraulic retention time (which is the inverse of the dilution rate), and membrane fouling was monitored by transmembrane pressure, as discussed previously. Even with continuous permeate withdrawal and no backflushing, no significant membrane fouling was observed during any of the experiments. There was, generally, a slight initial decline in membrane permeability (due to biomass accumulation and corresponding increase in broth viscosity), thereafter the membrane permeability stabilized and declined only very gradually. Transmembrane pressure was, in effect, proportional to permeate flux.

Considering that the maximum organic loading rate in the continuous degradation experiment was approximately 500 $mg.L^{-1}.day^{-1}$, and the influent concentration was 800 $mg.L^{-1}$, the corresponding wastewater dilution rate in the reactor was 0.625 day^{-1}. Given that the reactor working volume was 3 L, the corresponding influent flow rate (and thus effluent flow rate through the membrane) can be calculated to be 1.3 $ml.min^{-1}$ (this was also the actual measured value). This value is significantly less than 2.6 $ml.min^{-1}$, which was determined to be the critical flux in section 5.4.3 for this particular membrane and similar hydrodynamic conditions, hence no severe fouling was observed. This illustrates the importance of sub-critical flux operation. In effect, if the organic loading rate, concentration and volume of wastewater to be treated by a submerged membrane bioreactor system are known, and the critical flux for the membrane has been measured in the appropriate conditions, then it is

possible to calculate the membrane surface area required to ensure sub-critical flux operation, and hence minimize membrane fouling.

5.5 CONCLUSIONS

In this chapter a small-scale submerged membrane airlift reactor was designed, constructed and tested for the treatment of black olive fermentation brines. Such small-scale systems are of potential use for isolated olive and oil producers, for whom the produced wastewaters are problematic because of their high strength and organic load. The reactor was able to significantly reduce (by at least 50%) the organic load of the wastewater studied, in terms of both total phenol content and chemical oxygen demand.

The reactor and system were of simple design and construction, exploiting the advantages of both submerged membrane and airlift reactor concepts. Utilisation of a membrane results in the recycle of biomass for degradation purposes, as well as providing a biomass-free effluent *i.e.* settling tanks are not required for post-treatment. This is advantageous if further purification of the wastewater is to be performed. Compared to conventional stirred tank reactors, the airlift design is advantageous in that there are no moving parts, while good mixing and mass transfer are achieved at a low energy cost. This is beneficial for wastewater treatment purposes, where no particularly valuable by-products are produced.

A disadvantage of membrane bioreactors is that the membranes are subject to fouling, which increases operating cost and necessitates periodic cleaning. In this regard draught tubes were investigated, as these create a recirculating hydrodynamic flow regime past the membrane, and in this manner help to minimize the effects of membrane fouling. The effect of different draught tube diameters was investigated and appropriately modeled, and in critical flux experiments it was found that a narrow draught tube around the membrane was the most beneficial. The narrow draught tube served to create a high crossflow velocity past the membrane surface, and also constrained air bubbles, which is thought to enhance the scouring effect that keeps the membrane surface free of deposited material.

Batch degradation experiments were performed with various microbial species, including bacteria, yeasts, fungi and mixed microbial cultures. No backflushing or membrane cleaning was performed during the batch degradation experiments, while permeate was continually extracted through the membrane and recycled into the reactor. Membrane fouling was insignificant when a narrow draught tube configuration was used. Degradation rates for the various microbial species were improved in the reactor compared to shake flask experiments, most likely due to improved oxygen mass transfer.

After proof of the submerged membrane airlift bioreactor concept, the reactor was scaled-up for further testing and degradation experiments. Dimensions of the scaled-up reactor were determined according to the hydrodynamic model, to ensure a critical fluid crossflow velocity past the membrane of 0.35 $m.s^{-1}$, at the desired aeration intensity of 1 vvm. The scaled-up reactor was then used for fed-batch and continuous feed degradation experiments. Batch-fed degradation experiments allowed for the determination of maximum influent wastewater concentrations, which was approximately 800 $mg.L^{-1}$ total phenol concentration. Above this concentration microbial growth and degradation appeared to be inhibited. An increase in residual total phenol concentration was evident after the degradation of successive batch doses, corresponding to the gradual increase of high molecular weight polyphenols.

The continuous feed experiments allowed for the determination of maximum organic loading rates. The maximum organic loading rate for the degradation of black olive brines by mixed cultures was determined to be 420 $mg.L^{-1}.day^{-1}$, for influent total phenol concentration of 800 $mg.L^{-1}.day^{-1}$. These results can be used as an initial approximation of operating conditions for future long-term degradation studies, as well as for estimating the required reactor size for specific wastewater volumes and production rates. During the continuous feed experiments, there was no accumulation of high molecular weight polyphenols (as was observed in the batch fed experiments), as these degradation products were continually discharged. This possibly led to better degradation results than were observed in the fed-batch degradation experiments.

Mixed microbial cultures appeared to be the best option for the degradation of the black olive wastewater, as this approach negates the need for sterility during operation. Contamination of pure microbial cultures was often evident after extended operation periods, while sterilization increases cost and complexity, and as such is undesirable for wastewater treatment purposes.

The mixed microbial cultures were shown to be able to degrade wastewater that had been extracted for the recovery of low molecular weight phenolic antioxidants in Chapter 4, therefore it is reasonable to assume that degradation of extracted wastewater would be possible in the reactor, at similar (or even possibly better) rates. It would thus be of interest in the future to combine the extraction and degradation processes. The small-scale of these systems makes such integration both possible and appealing.

CHAPTER 6: FINAL CONCLUSIONS AND RECOMMENDATIONS

This research project involved the first detailed investigation of olive-derived wastewaters produced in South Africa. The overall objectives of the project have been met, in that, firstly, the local olive wastewaters were analysed and characterised. Secondly, value-added products were identified, and one was recovered from the wastewaters. Thirdly, biological degradation of the wastewater was investigated and successfully performed, and finally, a small-scale biological treatment system was developed and characterised for treatment of the wastewater.

In the investigation and characterisation of olive-derived wastewaters, there are four distinct primary sources were identified: mill wastewaters from the production of olive oil, black table olive brine wastewaters, green table olive brine wastewaters, and alkaline green table olive pre-treatment wastewaters. The wastewaters are accompanied by rinsing and other factory washing wastewaters, but these were not considered in this work as they are essentially dilutions of the primary sources. The raw wastewaters can be considered to be of high strength, as they have a high organic load, and in addition, they contain high concentrations of phenolic compounds, which makes them unsuitable for discharge into the environment or into conventional (municipal) wastewater treatment systems. In this work emphasis was given to the black olive brine wastewaters, as (proportionately) large quantities of this wastewater are produced locally, and they are the least well studied of the olive-derived wastewaters.

The different wastewaters were found to contain many common components, but there were several important distinctions. Apart from the phenolic components, the mill wastewaters were found to contain high quantities of suspended solids (cellular debris) as a result of cell disruption during the oil extraction process, and they also contained sugars, lipids and other organic compounds such as volatile fatty acids. The brines, on the other hand, had low suspended solids concentrations, but had high dissolved solids concentrations, as a result of the addition of salt during the fermentation process. Compared to the mill wastewaters, the brines contained few carbohydrates, as these are fermented by the microorganisms that occur in the brining process.

The olive-derived wastewaters contain a wide diversity of phenolic compounds, including low molecular weight aromatic compounds, higher molecular weight condensed and hydrolysable tannins, and highly polymerised lignin-like compounds and humic substances, with molecular weights approaching 2 000 kDa. The low molecular weight phenolic compounds are of interest because of their powerful antioxidant activity, while the higher molecular weight compounds are responsible for the dark colour of the black olive brines and the mill wastewaters. The low molecular weight phenolic compounds are toxic in the environment, but biodegradable, whereas the higher molecular weight compounds are benign but more resistant to biodegradation.

The low molecular weight phenolic compounds were identified as having the most potential as value-added products, as they can be commercially used as antioxidants. There is significant potential market value for such naturally derived antioxidants, particularly those from olives, because of their potency and additional pharmacological effects. These antioxidants could be used for the preservation of foodstuffs, preservation and/or enhancement of cosmetics, or could be formulated into nutraceutical products (prophylactics), or they could be refined for pharmacological and other research.

The black and green olive brines, and green olive pre-treatment wastewaters were found to contain high quantities of the valuable compound hydroxytyrosol, in excess of 1 $g.L^{-1}$, as the main low molecular weight phenolic component. In contrast, the olive mill wastewaters contained lesser quantities of hydroxytyrosol, and contained many other low molecular weight phenolic compounds, resulting in a complex phenolic profile. The wastewaters from the production of table olives therefore appeared to be a more appealing source for the recovery of antioxidants than the olive mill wastewaters. In addition, the low suspended solids and lipids concentrations in the table olive wastewaters make for easier processing, when compared with the olive mill wastewaters.

A crude (unpurified) extract, containing hydroxytyrosol as the predominant phenolic compound, and also containing elenolic acid, was recovered from the black olive brine. Values approaching 1 g (measured as total phenols) could be extracted from 1 L of black olive brine wastewater. The extract was evaluated for antioxidant activity, and was shown to be approximately six times more active (in terms of DPPH free radical scavenging) than a typical commercially used antioxidant (BHT). A novel extraction process, incorporating the

use of a membrane unit, was therefore investigated for the recovery of antioxidants from the black olive brines.

Membrane-assisted solvent extraction was shown to be a feasible process for the selective recovery of low molecular weight phenolic antioxidants from the olive brines, and has several potential advantages over conventional extraction processes such as liquid/liquid extraction and chromatographic processes. The membrane-assisted extraction process was mathematically modelled in order to determine the overall mass transfer coefficient, which is pertinent to the design, scaling and operation of such systems.

Next, the biological degradation of black olive brines was investigated. Despite the toxicity of the wastewaters, there are numerous microbial species capable of utilising phenolic compounds as a source of carbon and energy. Biological treatment is perceived to be an environmentally friendly and cost-effective method of reducing the organic load of the wastewaters. A selection of different microbial species were thus subjected to a preliminary investigation, in order to determine which would be suitable for the biological treatment of the black olive brines. Microbial species investigated for degradation purposes included a wild-type bacterium *Bacillus megaterium* AS-35, and *Lactobacillus plantarum*, the yeast *Candida maltosa*, the filamentous mold *Aspergillus niger*, the basidiomycete *Trametes pubescens*, and a mixed microbial consortium obtained from olive wastewater evaporation ponds.

Preliminary biological degradation experiments in shake flasks resulted in the removal of approximately 50 to 80% of the organic load and total phenol content of the black olive brines. Unlike reports on the biological degradation of olive mill wastewaters, decolourisation (through the mineralization of high molecular weight phenolic compounds) was not observed in the case of the black olive brines. Rather, degradation occurred through a polycondensation and humification process. This was ascribed to differences in composition between the brines and mill wastewaters. It is thought that the high salinity in the brines significantly influences the biological degradation process, as it affects the metabolic pathways followed.

The pure microbial cultures investigated were able to degrade the black olive brine at a faster rate than a mixed and acclimatised microbial consortium, but there are significant process

constraints involved in the use of a microbial monoculture. In particular, aseptic conditions are difficult to maintain for extended time periods, and wastewater needs to be sterilised before treatment, which significantly affects the cost of biological treatment. A mixed microbial treatment process therefore would be the most feasible, as sterile conditions are not required. Mixed cultures were able to degrade both raw (but diluted) wastewater, and wastewater that had been previously extracted to recover the valuable low molecular weight antioxidant hydroxytyrosol. In addition, wastewater did not need to be supplemented with a nitrogen source for degradation by mixed culture, as was the case for the pure microbial cultures.

A small-scale submerged membrane airlift bioreactor (initially 1 L, then scaled-up to 4 L) was then designed to perform the biological degradation process. Submerged membrane bioreactors are becoming increasingly popular for wastewater treatment applications, as they have numerous advantages over conventional biological treatment processes. Several process steps are incorporated into a single unit operation, which results in high efficiency and a small footprint. Biomass is retained and recycled within the reactor, which results in high cell density, and good degradation rates, while the reactor effluent is purified through membrane filtration and is biomass-free. Down-stream processes such as settling tanks are thus not required after the biological degradation process; this is advantageous if subsequent purification (or polishing) steps are to be performed. In addition, submerged membrane bioreactors are energy efficient, as there are no moving parts and the main energy input into the system is for the supply of compressed air.

However, a problem with submerged membrane bioreactors is that the membranes are prone to fouling, and therefore the hydrodynamics in the reactor were investigated and modelled. Reactor geometry was investigated so as to create a high liquid velocity past the membrane surface, which helps to remove deposited material, thereby minimising fouling effects. The operation of the reactor was then investigated using a model colloidal system, in order to determine the critical membrane flux, below which membrane fouling is gradual, and above which rapid fouling occurs. Design of the draught tube riser-to-downcomer ratio such that the liquid velocity in the riser is at least 0.3 $m.s^{-1}$ significantly improved the critical flux. The hydrodynamics and corresponding critical flux, in conjunction with wastewater and system parameters, determine the required membrane surface area for a given volumetric flow rate (or hydraulic residence time).

Biological degradation of the black olive brine in the submerged membrane bioreactors was investigated using the same microbial cultures as used for the preliminary experiments. These were first performed in batch degradation mode to compare degradation rates with the preliminary shake flask degradation experiments, and then in fed-batch and continuous degradation experiments, to determine approximate influent concentrations and organic loading rates. In this work, optimal conditions for the treatment of the black olive brine wastewater by mixed microbial culture were determined to be approximately 800mg.L^{-1} for influent total phenol concentration, at an organic loading rate of 420 mg.L^{-1}.day^{-1}. The influent concentration and maximum organic loading rate determine the size of a required bioreactor system for industrial purposes, based on wastewater production volumes. Submerged membrane bioreactors have not yet been extensively utilised for the treatment of olive-derived wastewaters, and this is perceived to be a promising and exciting avenue of future research.

This project has set the stage for further research in several directions. Because of the broad scope of this project, there remains the need for much further detailed research. Such research will add to an already large and growing body of knowledge, and will aid in the development of comprehensive treatment and beneficiation systems for olive-derived wastewaters. In relation to this work, the following recommendations are made:

In the recovery of valuable antioxidants from olive wastewaters, the composition and possible extraction recoveries from all four of the primary wastewater sources (mill wastewaters, black- and green olive brines, and green olive pre-treatment wastewaters) need to be further investigated. The membrane-assisted extraction procedure has potential advantages over conventional extraction methods, but needs to be further developed using more suitable membrane modules than the one used in this work, and the extraction process needs to be optimised. A pilot-scale extraction system is necessary in order to evaluate the economic feasibility of the process, in comparison to conventional methods. Subsequent processing of a solvent extract, irrespective of how it is obtained, also needs to be investigated, as does purification of the crude extract.

Although the biological degradation of olive-derived wastewaters has been investigated in quite some detail by others, this is not the case for black olive brine wastewaters. This is an area that would benefit from further detailed studies. In particular, for the chosen microbial

species (or consortium), kinetic constants of microbial metabolism need to be evaluated. This would include specific growth and death rates, substrate utilisation and inhibition constants, and yield coefficients. For a given wastewater feed composition, kinetic data needs to be appropriately modelled so that biological processes can be designed accordingly.

Downstream treatment of the biologically degraded wastewater needs to be investigated, as biological degradation is only capable of partial (though considerable) reduction in the organic load of the wastewaters. Non-biological oxidation processes (e.g. ozonation) have been widely used for post treatment of the other olive-derived wastewaters, and would thus be likely candidates for black olive brine wastewater post-treatment. The use of an upstream biological degradation process significantly reduces the organic load imposed on a downstream "polishing" process, and therefore such downstream processes become more economically feasible.

The black table olive wastewaters also contain significant inorganic load, predominantly the salt used during brining. The high salt concentration is particularly troublesome, as this precludes the use of this wastewater for irrigation purposes (or anaerobic digestion), irrespective of what proportion of the organic load has been removed. There are thus two options if these wastewaters are to be re-used: either the salt needs to be removed, or if all the organic components are removed the brine could possibly be recycled as process water. Desalination is a huge field in its own right, and is generally energy intensive and costly. Reverse osmosis, which is rapidly becoming the most common method of desalination, is unlikely to be economically feasible for olive producers. There is, however, the relatively low-cost option of solar distillation, that allows for the removal of salt and recovery of some process water, and would be worthy of investigation. Solar distillation has no energy cost, but there are materials costs and other constraints such as available space for solar stills.

The use of submerged membrane bioreactors for the treatment of olive wastewaters needs considerable further development and investigation. Particularly, these need to be scaled-up and evaluated for sustained long-term degradation efficiency and cost. Significant further system development, operational optimisation and process control will no doubt be required.

The combination of small-scale, stand-alone extraction and biological degradation processes should be investigated, as an integrated approach would be of greatest benefit to the olive and

olive oil producers. The economic benefit of recovering a value-added product would help to pay for the costs involved in the subsequent biological (and other) degradation processes.

On a more general note, water is a scarce commodity in South Africa, and therefore treatment systems that allow for beneficiation, reclamation and reuse are of great importance. Detailed estimations of the total quantities, source and composition of local olive wastewaters need to be made, and ideally be collected into a regularly updated database. The scope and nature of production wastewaters will largely dictate the direction to be followed for treatment and beneficiation systems. Because of the rapidly growing olive industry in South Africa, regional treatment facilities may soon become economically feasible, but even if this is the case, the development of small-scale, stand-alone treatment facilities should be given priority, as there will invariably be isolated olive producers, due to the size of the country and large area suitable for olive cultivation.

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APPENDIX A: THEORY FOR THE DETERMINATION OF THE OVERALL MASS TRANSFER COEFFICIENT DURING BATCH CONTACTING MEMBRANE-ASSISTED SOLVENT EXTRACTION

The following mathematical model for mass transfer in the membrane-assisted solvent extraction system (Chapter 3) was adapted from Gonzalez-Munoz *et al.* (2003).

The equation for mass transfer can be described by:

$$J_s = K_a A_m (c_a - c_a^*) \tag{3.1}$$

where J_s = overall solute flux ($g.s^{-1}$)

K_a = overall mass transfer coefficient ($m.s^{-1}$)

A_m = membrane surface area (m^2)

c_a = solute concentration in the aqueous phase at time t ($mg.L^{-1}$)

c_a^* = solute concentration in aqueous phase in equilibrium with organic phase at time t ($mg.L^{-1}$)

The objective is thus to determine the coefficient K in equation (3.1).

With reference to Figure A.1 (where the dashed line represents the membrane), a differential mass balance for solute in the module in terms of transfer of solute from the aqueous phase into the organic phase can be expressed as:

$$Q_a[c_a(x,t) - c_a(x+dx,t)] = -Q_a dc_a(x,t) = K_a dA_m\left(c_a(x,t) - c_a^*(x,t)\right) \tag{3.2}$$

where Q_a = aqueous phase flow rate ($m^3.s^{-1}$), $c_a(x,t)$ is the solute concentration at axial position x at time t, and $c_a^*(x,t)$ is the concentration in the aqueous phase in equilibrium with that in the organic phase at the same time and location, *i.e.* $c_o(x,t)$.

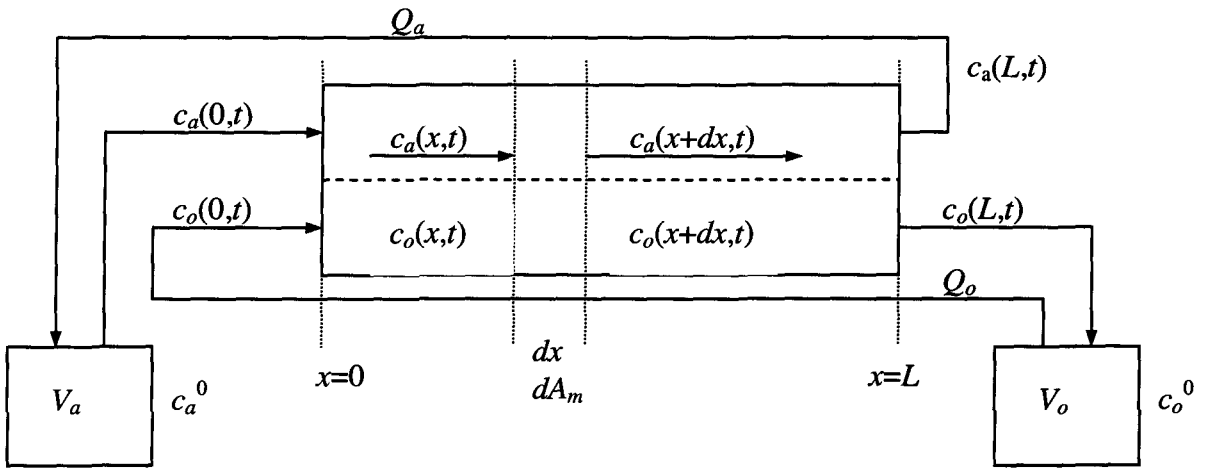

Figure A.1 – Schematic diagram of solute transfer in the membrane extraction system

This equilibrium relationship can be expressed as:

$$c_a^*(x,t) = \frac{c_o(x,t)}{D} \tag{3.3}$$

where D is the equilibrium distribution coefficient of solute between the aqueous and organic phases. Here the subscript o refers to the organic phase.

For an overall mass flow rate balance across the module, the decrease in solute in the aqueous is equal to the increase in the organic phase:

$$Q_a(c_a(0,t) - c_a(L,t)) = Q_o(c_o(L,t) - c_o(0,t)) \tag{3.4}$$

A similar mass balance can be written for any point (x) of the module:

$$Q_a(c_a(x,t) - c_a(L,t)) = Q_o(c_o(L,t) - c_o(x,t)) \tag{3.5}$$

$c_o(x,t)$ from equation (3.3) is then substituted into equation (3.5), and $c_a^*(x,t)$ in equation (3.2). The resulting equation is then integrated between $x = 0$ and $x = L$, and the following expression is obtained:

$$\frac{c_a(L,t)-c_o(L,t)/D}{c_a(0,t)(1+Q)-c_a(L,t)Q-c_o(L,t)/D}=\exp\left[-\frac{K_aA_m}{Q_a}(1+Q)\right] \tag{3.6}$$

where

$$Q=\frac{Q_a}{Q_oD} \tag{3.7}$$

Taking into account the overall mass balance equation (3.4), equation (3.6) can then be expressed as:

$$\frac{c_a(L,t)-c_o(L,t)/D}{c_a(0,t)-c_o(0,t)/D}=\exp\left[-\frac{K_aA_m}{Q_a}(1+Q)\right] \tag{3.8}$$

A simultaneous unsteady-state balance on the aqueous phase feed tank (assuming perfect mixing) gives:

$$-V_a\frac{dc_a(0,t)}{dt}=Q_a[c_a(0,t)-c_a(L,t)] \tag{3.9}$$

where V_a is the volume of the tank (m^3).

At any time the depletion of solute in the aqueous phase should be equal to its increase in the organic phase:

$$V_a(c_a(0,0)-c_a(0,t))=V_o(c_o(0,t)-c_o(0,0)) \tag{3.10}$$

Combining equations (3.4) and (3.10), the concentration of solute in the organic phase at the module outlet can be expressed as a function of the solute concentration in the aqueous phase at the module inlet and outlet:

$$c_o(L,t)=\frac{Q_a(c_a(0,t)-c_a(L,t))}{Q_o}+\frac{V_a(c_a(0,0)-c_a(0,t))}{V_o}+c_o(0,0) \tag{3.11}$$

Combining equations (3.11) and (3.8), an expression is obtained for solute concentration in the aqueous phase at the module outlet as a function of the inlet aqueous phase concentration and some process parameters:

$$c_a(L,t)=\frac{1}{(1+Q)}\left[c_a(0,t)-V(c_a(0,0)-c_a(0,t))+\frac{c_o(0,0)}{D}\right]\exp\left[-\frac{K_aA_m}{Q_a}(1+Q)\right]$$
$$+\frac{1}{(1+Q)}\left[Qc_a(0,t)+V(c_a(0,0)-c_a(0,t))+\frac{c_o(0,0)}{D}\right] \qquad (3.12)$$

where

$$V=\frac{V_a}{V_oD} \qquad (3.13)$$

Substituting equation (3.12) into equation (3.9) and integrating between t = 0 and time t results in:

$$c_a(0,t)=\frac{(c_a(0,0)V-c_o(0,0)/D)}{(1+V)}+$$
$$\frac{(c_a(0,0)V-c_o(0,0)/D)}{(1+V)}\exp\left[-\frac{Q_a}{V_a}\frac{(1+V)}{(1+Q)}\times\left(1-\exp\left(-\frac{K_aA_m}{Q_a}(1+Q)\right)\right)t\right] \qquad (3.14)$$

If the initial concentration in the solute $c_o(0,0)$ is zero, then equation (3.14) can be expressed as:

$$c_a(0,t)=\frac{Vc_a(0,0)}{1+V}+\frac{c_a(0,0)}{1+V}\exp(-ct) \qquad (3.15)$$

where

$$c=\frac{Q_a}{V_a}\frac{(1+V)}{(1+Q)}\left[1-\exp\left[-\frac{A_mK_a}{Q_a}(1+Q)\right]\right] \qquad (3.16)$$

Equation (3.15) can be expressed in the form:

$$c_a(0,t)=a+b\exp(-ct) \qquad (3.17)$$

APPENDIX B: DESIGN DRAWINGS OF THE SUBMERGED MEMBRANE AIRLIFT REACTORS

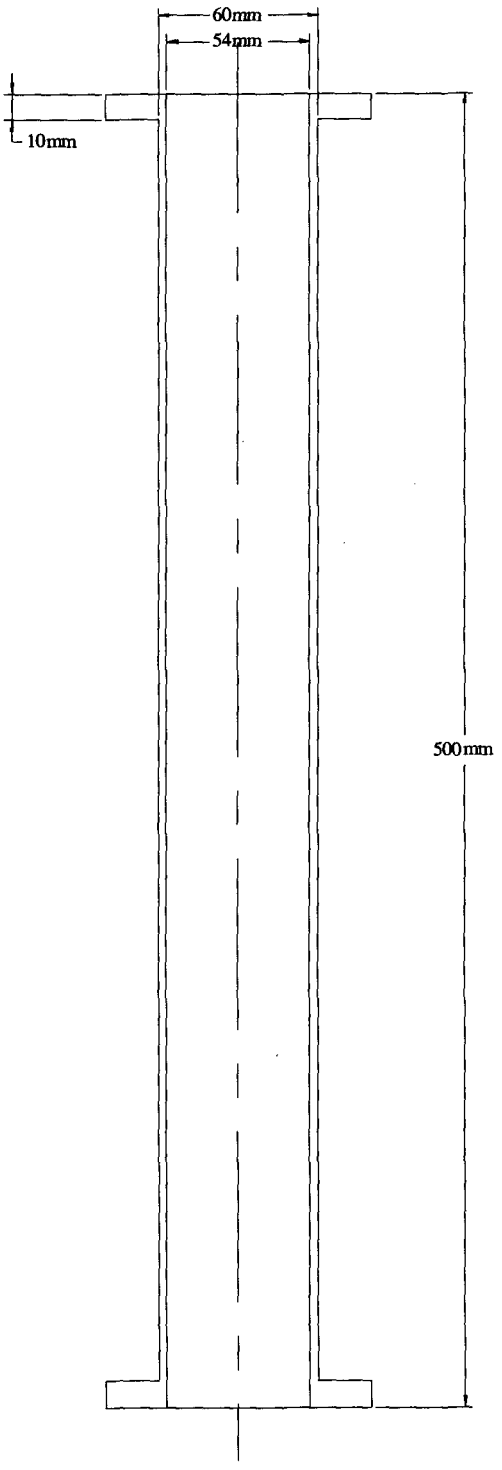

Figure B.1: Shell of the 1 L reactor

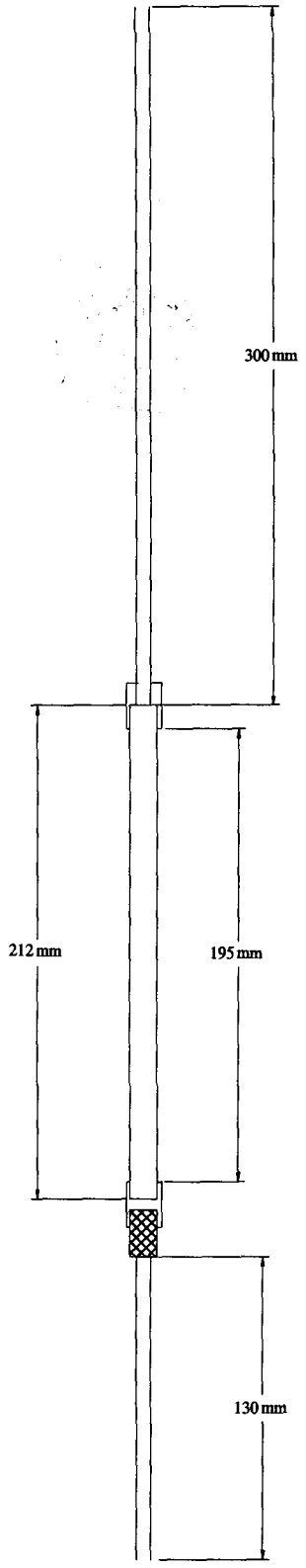

Figure B.2: Membrane and sparger insert

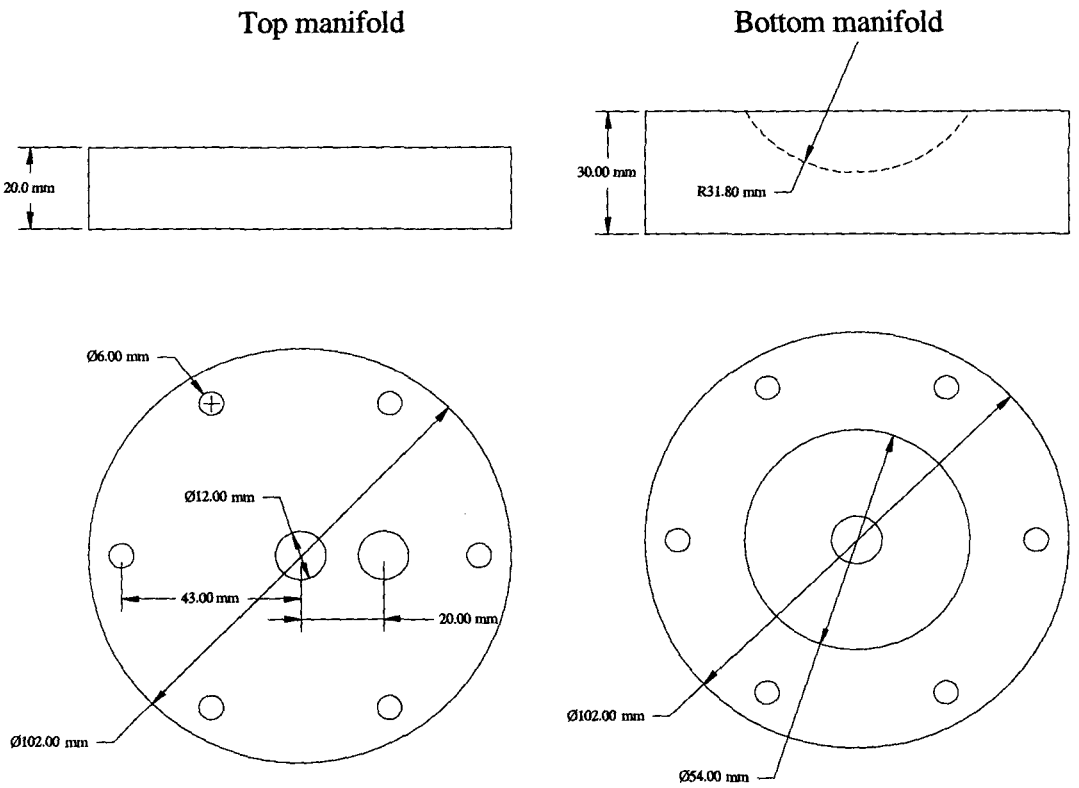

Figure B.3: Top and bottom manifolds for the 1 L reactor

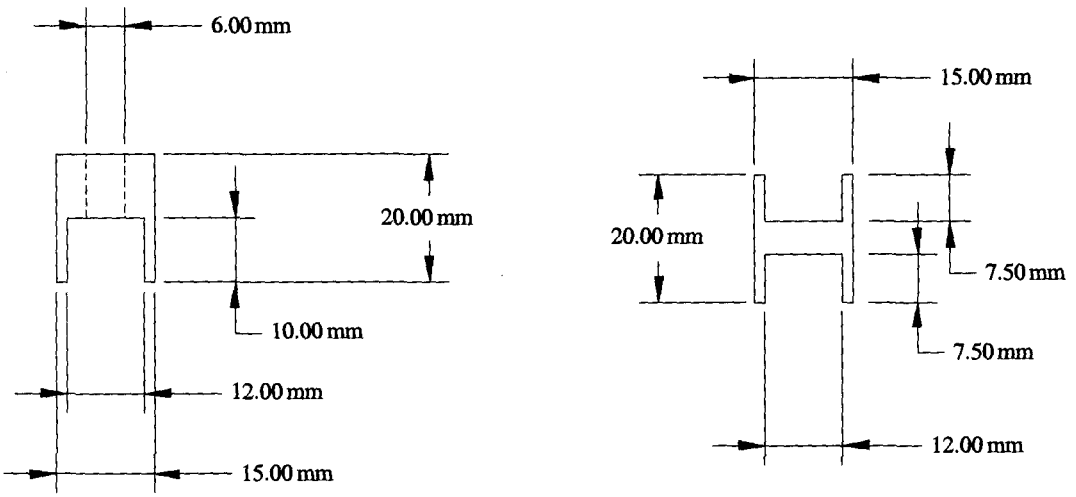

Figure B.4: Top and bottom membrane end fittings

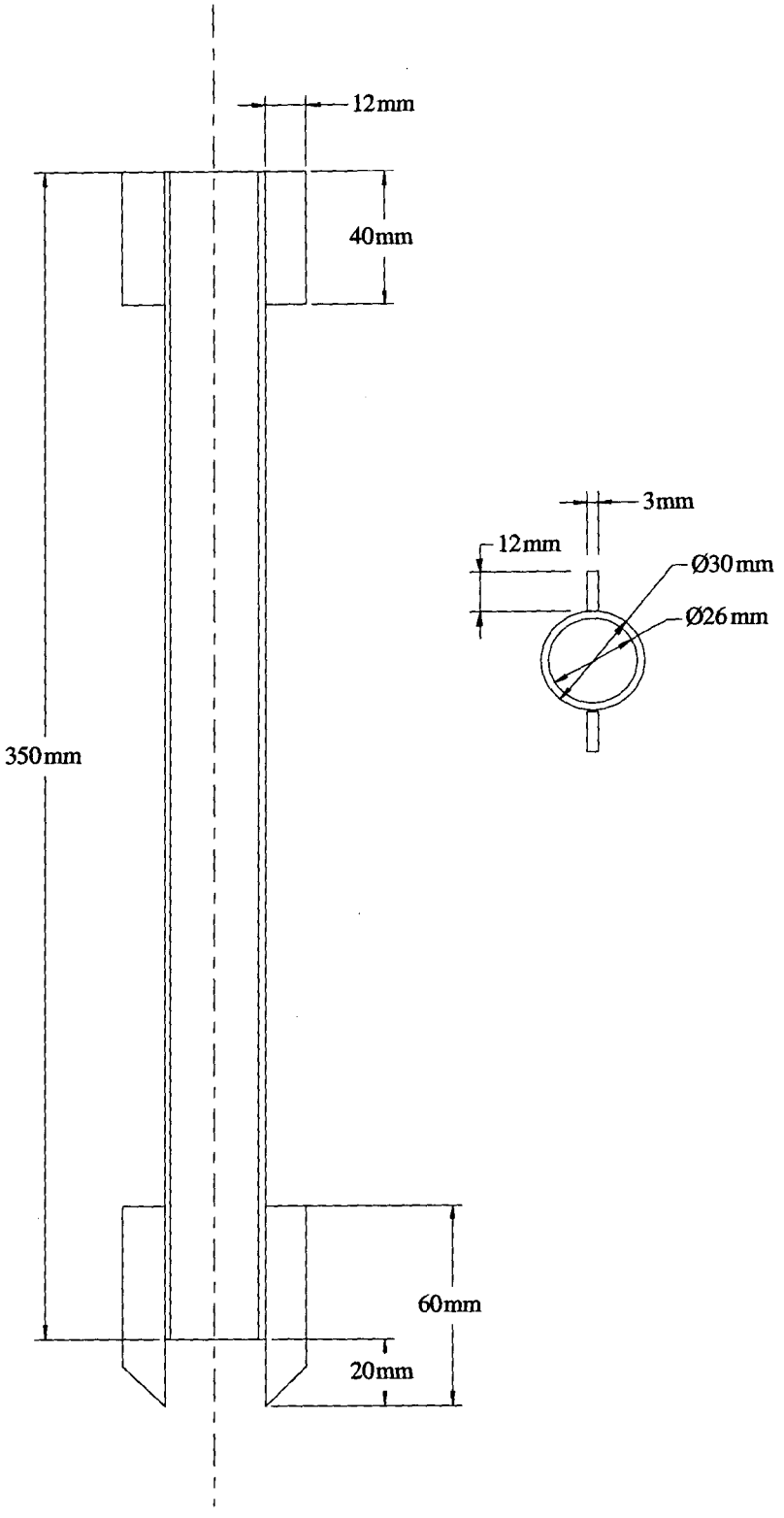

Figure B.5: Narrow draught tube for the 1 L reactor

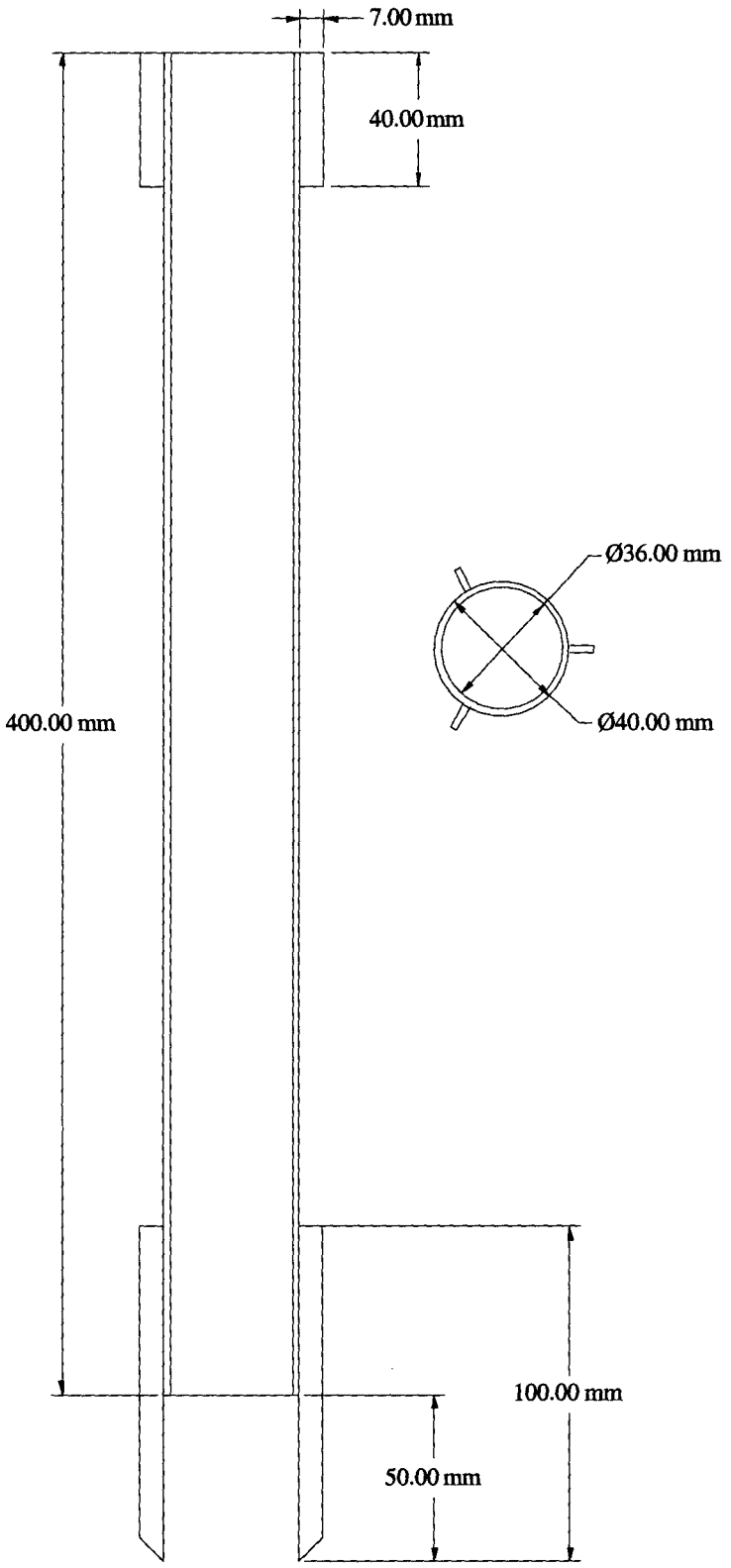

Figure B.6: Wide draught tube for the 1 L reactor

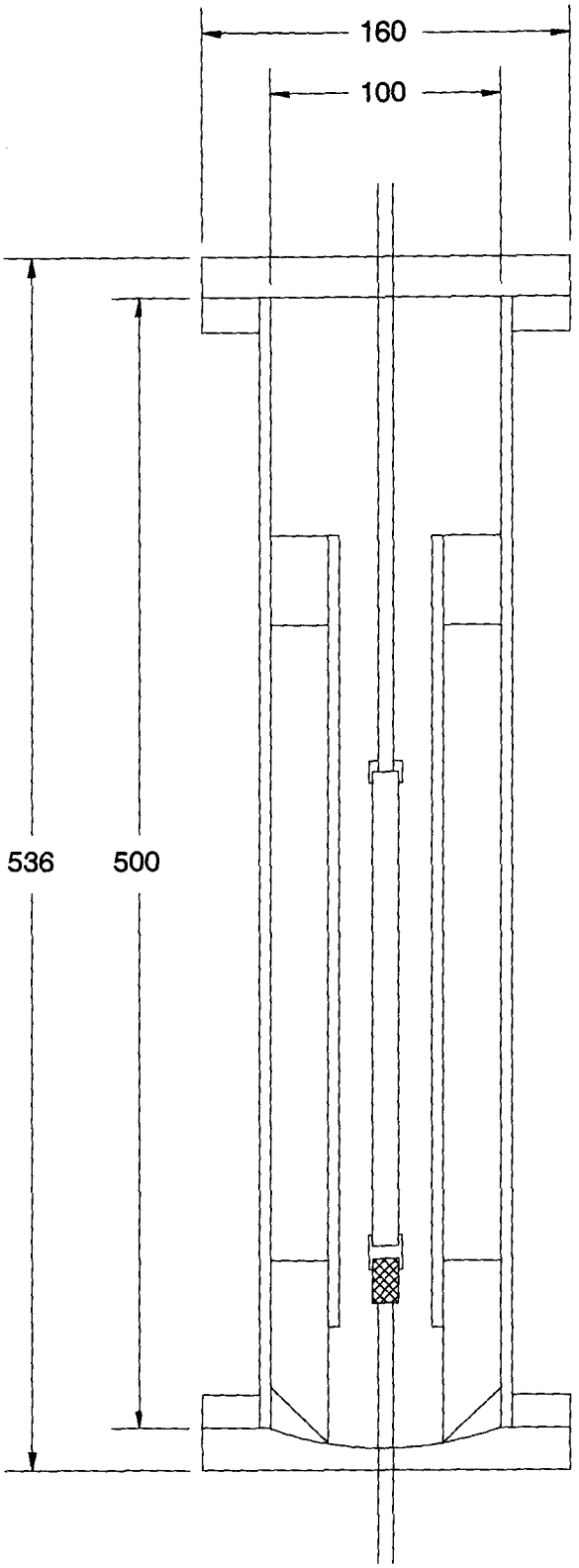

Figure B.7: Scaled-up 4 L reactor

APPENDIX C: CALCULATION FOR THE DETERMINATION OF THEORETICAL LIQUID RISER VELOCITY IN THE 1 L REACTOR

Table C.1 shows calculation of the theoretical riser velocity in the 1 L reactor for the narrow draught tube at increasing airflow. Calculation of the variables, as described in section 5.3.1, is shown below.

Table C.1: Theoretical riser velocity in the 26/30mm draught tube

Air Flow Φ_G (L/min.)	Iterated U_{LR} (m/s)	U_{GR} (m/s)	ε_R	ε_D	ε	h_D	Calculated U_{LR} (m/s)	Measured U_{LR} (m/s)	Std. dev.
0	20	0.0000	0.0000	0.0000	0.0000	0.3950	**0.000**		
0.5	0.2	0.0199	0.0350	0.0315	0.0322	0.4082	0.312		
0.5	0.312	0.0199	0.0275	0.0248	0.0254	0.4053	0.278		
0.5	0.278	0.0199	0.0294	0.0265	0.0271	0.4060	0.287		
0.5	0.287	0.0199	0.0289	0.0260	0.0266	0.4058	0.284		
0.5	0.284	0.0199	0.0291	0.0261	0.0268	0.4059	**0.285**		
1	0.248	0.0399	0.0601	0.0541	0.0554	0.4181	0.404		
1	0.404	0.0399	0.0456	0.0411	0.0420	0.4123	0.355		
1	0.355	0.0399	0.0493	0.0444	0.0455	0.4138	0.368		
1	0.368	0.0399	0.0483	0.0434	0.0445	0.4134	0.364		
1	0.364	0.0399	0.0486	0.0437	0.0448	0.4135	**0.365**	**0.320**	0.000
2	0.317	0.0798	0.0983	0.0885	0.0906	0.4343	0.508		
2	0.508	0.0798	0.0750	0.0675	0.0691	0.4243	0.449		
2	0.449	0.0798	0.0809	0.0728	0.0746	0.4268	0.465		
2	0.465	0.0798	0.0792	0.0713	0.0730	0.4261	0.460		
2	0.460	0.0798	0.0797	0.0717	0.0734	0.4263	**0.461**	**0.465**	0.042
3	0.401	0.1197	0.1226	0.1104	0.1130	0.4453	0.560		
3	0.560	0.1197	0.1011	0.0910	0.0932	0.4356	0.514		
3	0.514	0.1197	0.1065	0.0959	0.0981	0.4380	0.526		
3	0.526	0.1197	0.1050	0.0945	0.0968	0.4373	0.523		
3	0.523	0.1197	0.1054	0.0949	0.0971	0.4375	**0.524**	**0.547**	0.044
4	0.475	0.1596	0.1419	0.1277	0.1308	0.4544	0.597		
4	0.597	0.1596	0.1245	0.1120	0.1147	0.4462	0.564		
4	0.564	0.1596	0.1287	0.1159	0.1186	0.4482	0.572		
4	0.572	0.1596	0.1276	0.1149	0.1176	0.4476	0.570		
4	0.570	0.1596	0.1279	0.1151	0.1179	0.4478	**0.571**	**0.615**	0.061
5	0.571	0.1994	0.1535	0.1382	0.1414	0.4601	0.617		
5	0.617	0.1994	0.1468	0.1321	0.1353	0.4568	0.606		
5	0.606	0.1994	0.1484	0.1336	0.1368	0.4576	0.609		
5	0.609	0.1994	0.1480	0.1332	0.1364	0.4574	0.608		
5	0.608	0.1994	0.1481	0.1333	0.1365	0.4574	**0.608**		

Airflow (L/min.) is the set value supplied to the reactor and measured with a rotameter.

The iterated U_{LR} (liquid velocity in the riser) is initially an assumed value (0.2 $m.s^{-1}$), this is replace by the calculated for the next iteration. Once the calculated U_{LR} has converged, this value is used as the assumed value for the next airflow.

U_{GR} is the superficial gas velocity in the riser ($m.s^{-1}$). From equation (5.3):

$$U_{Gr} = \frac{\phi_G}{A_r}$$

where $\emptyset_G$ = Airflow ($L.min^{-1}$)*10^{-3}(m^3)/60(s) = ($m^3.s^{-1}$)

and $A_R = 4.18 \times 10^{-4}$ (m^2) from Table 5.1

Next the fractional gas hold-up in the riser is calculated according to equation (5.2):

$$\varepsilon_r = \frac{U_{Gr}}{0.24 + 1.35(U_{Gr} + U_{Lr})^{0.93}}$$

then the fraction gas hold-up in the downcomer is given by equation (5.4):

$$\varepsilon_d \approx 0.9\varepsilon_r$$

which allows for the calculation of the overall gas hold-up according to equation (5.5):

$$\varepsilon = \frac{\varepsilon_r A_r + \varepsilon_d A_d}{A_r + A_d}$$

Therefore

$$\varepsilon = \frac{\left(\varepsilon_R \times 4.18 \times 10^{-4}\right) + \left(\varepsilon_D \times 1.58 \times 10^{-3}\right)}{1.99 \times 10^{-3}}$$

Next the liquid dispersion height is calculated according to equation (5.6):

$$h_D = \frac{h_L}{1 - \varepsilon}$$

where h_L is the un-gassed liquid height (0.395 m), therefore

$$h_D = \frac{0.395}{(1 - \varepsilon)}$$

The frictional loss coefficient is then calculated according to equation (5.7):

$$K_B = 11.402\left(\frac{A_d}{A_b}\right)^{0.789} = 11.402\left(\frac{1.58\times10^{-3}}{2.20\times10^{-3}}\right)^{0.789} = 8.48$$

And then all the required values for solving equation (5.1) are known:

$$U_{Lr} = \left[\frac{2gh_D(\varepsilon_r - \varepsilon_d)}{K_B\left(\frac{A_r}{A_d}\right)^2 \frac{1}{(1-\varepsilon_d)^2}}\right]^{0.5} = \left[\frac{2*9.81*h_D(\varepsilon_R - \varepsilon_D)}{8.48*\left(\frac{4.18\times10^{-4}}{1.58\times10^{-3}}\right)^2 \frac{1}{(1-\varepsilon_D)^2}}\right]$$

This calculated value for U_{LR} is then used for the next iteration until the value converges.