

**INTEGRATED ANAEROBIC/AEROBIC
BIOPROCESS ENVIRONMENTS AND THE
BIODEGRADATION OF COMPLEX
HYDROCARBON WASTES**

By

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Abstract

An investigation of the biodegradation of complex hydrocarbon wastes, with emphasis on chlorinated aromatic compounds, in an anaerobic/aerobic bioprocess environment was made. A reactor configuration was developed consisting of linked anaerobic and aerobic reactors which served as the model for a proposed bioremediation strategy targeting subterranean soil/sediment/aquifer chlorinated phenol-contaminated environments. Here oxygen is frequently limited and sulphate is readily available, as occurs especially in marine sediment and intertidal habitats. In the anaerobic system the successful transformation and mobilization of the model contaminant, 2,4,6-trichlorophenol, was shown to rely on reductive dechlorination by a sulphate-reducing-dependent dechlororespiring co-culture. This was followed in the aerobic system by degradation of the pollutant and its metabolites, 2,4-dichlorophenol, 4-chlorophenol and phenol, by immobilized white-rot fungi.

The strategy was initially investigated separately in laboratory bench- and intermediate-scale reactors whereafter reactors were linked to simulate the integrated biodegradation strategy. The application of the fungal reactor to treat an actual waste stream by degrading complex mixtures of hydrocarbons in a waste oil recycling effluent was also investigated. The mineralization of phenol and 2,4,6-TCP by immobilized fungal cultures was studied in pinewood chip and foam glass bead-packed trickling reactors. The reactors were operated in sequencing batch format. Removal efficiency increased over time and elevated influent phenol and TCP (800 and 85 mg.L⁻¹) concentrations were degraded by > 98 % in 24 – 30 h batch cycles. Comparable performance between the packing materials was shown. Uptake by the packing was negligible and stripping of compounds induced by aeration had a minimal effect on biodegradation efficiency.

Reactor performances are discussed in relation to sequencing batch operation and nutrient requirements necessary to sustain fungal activity in inert vs. organic material packed systems.

It was shown that a co-culture consisting of sulphate-reducing and dechlororespiring bacteria established in fed-batch and soil flasks, as well as pine chip-packed fluidized bed reactors. Results showed reductive dechlorination of 2,4,6-TCP to be in strict dependence on the activity of the sulphate-reducing population, sulphate and lactate concentrations. Transformation to 2,4-DCP, 4-CP and phenol was enhanced in sulphate-deficient conditions. Dechlororespiring activity was found to be dependent on the fermentative activity of sulphate-reducing bacteria, and the culture was also shown to mobilize and dechlorinate TCP in soils contaminated with the pollutant. Linking the systems achieved degradation of the compound by > 99 % through fungal mineralization of metabolites produced in the dechlororespiring stage of the system. pH correction to the anaerobic reactor was found to be necessary since acidic effluent from the fungal reactor inhibited sulphate reduction and dechlorination.

The fungal reactor system was evaluated at intermediate-scale using a complex waste oil recycling effluent. Substantial COD reduction (> 96 % in 48 h batch cycles) and removal of specific effluent hydrocarbon components was shown in diluted, undiluted (COD > 37 g.L⁻¹) and 2,4,6-TCP-spiked effluents. Industrial application of the fungal reactor was evaluated in a 14 m³ pilot plant operated on-site at a waste oil processing plant.

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Abbreviations

UASB = Upflow anaerobic sludge blanket
BOD = Biological oxygen demand
CF = Culture fluid
COD = Chemical oxygen demand
CP = Chlorophenol
CSIR = Council for Scientific and Industrial Research
DCP = Dichlorophenol
EDTA = Ethylenediaminetetraacetic acid
FID = Flame-ionization detector
GC = Gas Chromatograph
HPLC = High Pressure Liquid Chromatograph
HRT = Hydraulic retention time
IFR = Immobilized white-rot fungal trickling packed-bed reactor
LiP = Lignin peroxidase
LEL = Lower explosives limit
MnP = Manganese peroxidase
POP = Persistent organic pollutants
PCE = Tetrachloroethylene
PCP = Pentachlorophenol
RV = Recirculation volume
SBR = Sequencing batch reactor
SDR = Sulphidogenic-dependent dechlororespiration reactor
SEM = Scanning electron microscope
SRB = Sulphate-reducing bacteria
TCE = Trichloroethylene
TCP = Trichlorophenol
TEM = Transmission electron microscope
TOC = Total organic carbon
UN = United Nations
UNEP = United Nations Environmental Programme
USEPA = United States Environmental Protection Agency
VOC = Volatile organic compound

WHO = World Health Organisation

Chapter One

The Biodegradation of Chlorinated Aromatic Compounds and Complex Mix Hydrocarbon Wastes

1. Introduction

The International Programme on Chemical Safety (IPCS), a joint venture of the World Health Organisation (WHO), United Nations Environmental Programme (UNEP) and the International Labour Organisation, has established that in 1990 some 100 000 unique chemicals or 4 million mixtures, formulations and blends were in commercial use, and that several hundred new synthetic chemicals were introduced each year (IPCS, 1999). The principle samples of soil and groundwater pollution, and the toxic effects caused by these compounds, are documented in Table 1.1. The production of hazardous waste and the extent of soil, groundwater and surface water contamination in the USA and European Union has been extensively reviewed (Bridges, 1991; Caplan, 1993; Committee on Innovative Remediation Technologies, 1997; Eweis *et al.*, 1998; House of Commons Environmental Committee, 1990; Lee *et al.*, 1998; Moyers, 1991; Olsen and Kavanaugh, 1993; USEPA, 1990).

Reports by the Council for Scientific and Industrial Research (CSIR) in South Africa have established that very little had been known about the nature and extent of contaminated sites and hazardous waste streams generated in this country prior to the early 1990s (CSIR, 1991; Pearce *et al.*, 1995).

In recent years the control and remediation of soil and groundwater aquifers has become a major issue of focus for legislators, environmentalists and civil society alike. Environmental legislation, conceptualized by our understanding of the impact of pollution in the environment, is designed both to correct the adverse effects of past pollution and to require immediate action to minimize impacts when industrial accidents do occur (Hammer, 1993). The scope and enforcement of United States environmental law serves as a model for many other countries and USA legislation governing environmental protection had been the subject of detailed reviewed by Day (1993).

Environmental legislation in the United Kingdom tends towards reaction to particular contamination situations as they come to light, whereas USA legislation reflects a pro-active commitment to search out and respond to contamination. Attewell (1993) discusses UK environmental legislation.

Table 1.1. Classification of major organic soil and groundwater pollution (from Evangelista de Duffard and Duffard, 1996; Eweis *et al.*, 1998 and Knox *et al.*, 1999)

Type of compound	Typical location	Mobility	Effects
Agricultural residues (Halogenated aromatics, Nitroaromatics, Phenols, Pesticides)	Manufacturing plants, chemical distributors, farms, crop duster airports	Generally low	Disease of the nervous system (neurotoxicity), cancers
Petroleum products (Aliphatic and aromatic hydrocarbons)	Service stations, refineries, automobiles and other transportation, oil tankers	Low to moderate	Carcinogenic
Solvents (Halogenated aliphatics)	Degreasing solvents used in garages, electronic plants, plastics manufacturing, former dry-cleaning facilities	Moderate to high	Nerve damage, cancers, visual neurotoxicant, cognitive dysfunction
Polycyclic Aromatic Hydrocarbons (PAHs)	Coal gas manufacturing, asphalt and creosote, automobile emissions, fuels, lubricating oils	Low to moderate	Number of PAHs are suspected carcinogens
Polychlorinated biphenyls (PCBs)	Electrical transformers	Low	Cancers, developmental neurotoxicity, motor activity, cognitive function
Dioxins (Halogenated aromatics)	Chemical manufacture, vehicle exhaust, medical, municipal and hazardous waste incineration	Low	Tumor promotion, chloracne

In South Africa, a shift from dealing with waste materials as an 'end-of-pipe' procedure after they have been generated, to an integrated and comprehensive pollution prevention and waste minimisation strategy represents a transformation in environmental and resources management. Section 24 of the Constitution of South Africa (Bill of Rights) facilitates protection of the environment whereas Section 38 stipulates enforcement of rights as outlined by the Constitution. The Bill of Rights is supported by a series of national, provincial and international laws and conventions that govern the management of the environment and its resources. The Departments of Environmental Affairs and Tourism and Water Affairs and Forestry details environmental legislation on the South African Government Information and State of the Environment Web sites.

The government's commitment to sustainability and promotion of environmentally sound chemical management is driven by the adoption of Agenda 21 at the United Nations (UN) Conference on Environment and Development in Rio de Janeiro (1992) and is one of around 20 other international conventions to which South Africa is signatory, including the Basel Convention (1989, entered into force 1992), Convention of Prior Informed Consent (Rotterdam 1998), Convention of Persistent Organic Pollutants (Stockholm 2001) and World Summit on Sustainable Development (Johannesburg 2002).

Remediation engineering may be described as a next phase of environmental engineering and as the development and implementation of strategies to clean-up (remediate) the environment by removing hazardous contamination (Suthersan, 1997). Bioremediation may be defined as the use of living organisms to reduce or eliminate environmental hazards from the accumulation of toxic chemicals and other hazardous waste (Gibson and Sayler, 1992). Bioremediation represents only one group of the diverse remediation and restoration technologies available. For it to prove commercially attractive, and be employed extensively, bioremediation will need to provide cost-effective solutions for a broad range of complex clean-up problems. Also, bioremediation has to demonstrate advantages over alternative, non-biologically mediated remediation and restoration technologies (Hammer, 1993).

Recent years have witnessed substantial growth in the controlled and practical use of microorganisms for the destruction of chemical pollutants. Biologically-driven technologies rely on the natural degradative capacity of microbes and focus on

processes that bring pollutants into contact with microorganisms in reactor/*ex-situ* or *in-situ* applications allowing for rapid transformation and degradation (Alexander, 1999). A variety of different applications and procedures are now in use, and new and promising technologies have been suggested or have reached advanced stages of development.

With the global increase in industrial (hazardous) waste it has become essential to reduce the waste produced and to give priority to the recovery of valuable materials by the use of appropriate process recovery techniques in sustainable integrated waste management strategies. This also applies to hydrocarbon waste collectors who specialize in the recycling and processing of complex mixtures so as to reclaim materials, produce value-added products and treat waste streams by employing technologies such as bioremediation.

Bioremediation techniques may be divided into three categories *in situ*, *ex-situ* solid and *ex-situ* slurry. *In-situ* techniques refer to soil and associated groundwater contamination when treated in place without excavation or pumping, while it is excavated or pumped prior to treatment with *ex-situ* applications (Blackburn and Hafker, 1993). Bioremediation technologies have been extensively reviewed by Admassu and Korus (1996), Alexander (1999), Blackburn and Hafker (1993), Committee on Innovative Remediation Technologies (1997), Eweis *et al.* (1998), Federal Remediation Technologies Roundtable (2003), King *et al.* (1998) and Suthersan (1997).

Pollutant mobility and availability in soil and groundwater environments affects selection of clean-up technologies and ultimately the effectiveness of bioremediation systems. The soil environment and processes that impact remediation efforts has been the subject of extensive review by Alexander (1991; 1999), Cheng and Mulla (1999), Edgehill (1999), Eweis *et al.* (1998), Morra (1996), Novak *et al.* (1995) and Sadowsky and Turco (1999).

1.2 Phenol and Chlorinated Phenols

Aromatic compounds exist in a variety of biogenic and abiogenic products which are released into the environment (Knox, 1989). Among these, phenols constitute a major

group of compounds which are of particular interest as indicators of the potentially wide range of organic compounds suitable for bioreremediation investigations.

1.2.1 Sources of Phenol Contamination

Phenolics are among the most utilized organic compounds in the world. During the early 1980s, annual production of phenol was 12×10^5 tonnes placing it among the 50 chemicals produced in the highest quantities in the USA (Young and Rivera, 1985). Phenol and phenolic compounds are toxic pollutants and inhibitors of biodegradation (Fedorak and Hrudey, 1984; Holladay *et al.*, 1986). Chlorinated phenols (CP) constitute a series of 19 compounds of mono-, di, tri-, and tetrachloro-isomers and one pentachlorophenol (PCP). CP with less than three chlorines seem to be of limited use today. PCP and lower chlorinated phenols, tetra-, tri-, and dichlorophenols, have been used as biocides to control bacteria, fungi, algae, insects, mollusks and other biota (Häggbloom and Young, 1990; Hale *et al.*, 1994; Madsen and Licht, 1992; Uberoi and Bhattacharaya, 1997). The wood industry has used extensive amounts of chlorophenols for the preservation of timber against blue sapstain fungi (Puhakka and Melin, 1996). They also are widely used as flame retardants, solvents, or in synthetic chemistry as raw materials or intermediates in the manufacturing of explosives and pharmaceuticals (Madsen and Licht, 1992; Montgomery, 1990; Sahm *et al.*, 1986).

CP and other chlorinated compounds are also formed as by-products when chlorine is used for bleaching of paper (Hakulinen and Salkinoja-Salonen, 1982; Kringstad and Lindström, 1984) and for the disinfection of drinking water and wastewater containing phenols (Ahlborg and Thurnberg, 1980; Detrick, 1977).

The annual world-wide production of CP has been estimated at about 200 000 tonnes (Paasivirta, 1978) of which PCP presented about 90 000 tonnes during the late 1970s (Detrick, 1977). The annual use of PCP in the USA has been 23 000 tonnes and in 1978 about 80% of the PCP produced was used for commercial lumber treatment (Crosby, 1981).

The use of CP has been banned or restricted in several countries since the late 1980s but because of poor practices CP are widespread in the environment today (Gesellschaft Deutscher Chemiker, 1985).

According to the WHO drinking water standards allowable for 2,4,6-trichlorophenol and PCP, concentrations are less than 0.1 and 10 $\mu\text{g.L}^{-1}$, respectively (Järvinen and Puhakka, 1994). The impact and distribution of PCP and lower chlorinated phenols in the environment has been extensively documented (Cautreels *et al.*, 1977; Lampi *et al.*, 1992; Paasivirta *et al.*, 1985; Puhakka and Melin, 1996; Valo *et al.*, 1985; Wylie *et al.*, 1975; Xie *et al.*, 1986). Along with other CP, PCP has been placed on the US Environmental Protection Agency (USEPA) list of priority pollutants (Keith and Telliard, 1979).

Much emphasis has been placed on the long-term health effects on man (e.g. carcinogenicity, mutagenicity and toxicity) from the exposure to low concentrations of chemicals such as CP. Impact on the nervous system, including behavioral effects and reproductive capacity, are also being investigated (Evangelista de Duffard and Duffard, 1996; Harris, 1985; Knox *et al.*, 1999). After the UN Conference on Environment and Development (Rio de Janeiro 1992), the UNEP have adopted action to protect human health and the environment through measures which will reduce and/or eliminate persistent organic pollutants (POP). At the UN conference in Stockholm in 2001 a treaty was adopted to control 12 organochlorine chemicals and called for the restriction or elimination of these compounds altogether. Following on that, negotiations at the World Summit on Sustainable Development, Johannesburg 2002, have recommended that countries should aim to minimise production and use of chemicals that leads to significantly adverse effects on human beings and the environment by year 2020.

The South African government has also agreed to support funding for research on the properties of chemicals, impact on the environment and development of clean-up technologies (UN World Summit on Sustainable Development, 2002).

1.2.2 Bioremediation of Chlorinated Phenol-Contaminated Environments

The use of biologically-mediated remediation technology has several advantages over physical and chemical treatment processes. Clean-up costs are considerably lower ($\frac{1}{3}$ to $\frac{1}{2}$ the cost) compared to other alternatives (Eweis *et al.*, 1998). Since bioremediation is the application of the normal metabolic processes of microorganisms to alter the chemical structure of organic materials in solid, liquid or vapour states, it effects the degradation and detoxification of hazardous materials mostly to less hazardous compounds. Other treatment and disposal technologies such as venting, activated carbon adsorption, solidification and landfill disposal, simply transfers contaminants to a different medium or location (Eweis *et al.*, 1998). Bioremediation is also a relatively simple technology with *in-situ* systems managed with minimal site disruption, volatile compound emission and health risks to neighbouring residents and site operators (Eweis *et al.*, 1998; King *et al.*, 1998). However, *in-situ* bioremediation performance is especially difficult to predict and success of clean-up projects depend on the ability of process operators to create and maintain environmental conditions necessary for microbial growth. Also, clean-up standards may not be met and additional technologies may have to be employed (Eweis *et al.*, 1998; Suthersan, 1997).

An extensive variety of organic contaminants including CP are biodegradable. The microbial metabolism of various groups of hydrocarbons, degradation pathways and chemical structure in relation to biodegradation susceptibility have been investigated and extensively reviewed (Alexander, 1999; Bouwer and Zehnder, 1993; Crawford and Crawford, 1996; Eweis *et al.*, 1998 and references therein).

1.3 Microbial Degradation of Chlorinated Phenols

CP may be removed from the environment via volatilization, photodegradation, adsorption onto suspended or bottom sediments and microbial degradation (Hale *et al.*, 1994). Photodecomposition plays a major role in river and surface water bodies, while biodegradation is the principle removal mechanism in soils, groundwater and aquatic sediments. These two mechanisms may also be complimentary (Flora *et al.*, 1994).

With few exceptions, CP are completely or partially degraded by metabolic pathways that have evolved in microorganisms to degrade naturally occurring, non-chlorinated aromatic compounds (Sangodkar *et al.*, 1989).

Two strategies have been identified for the microbial degradation of chlorinated organic compounds: oxygen-dependent and anoxic metabolism (Montgomery *et al.*, 1994). Common to both types of metabolism is a separation into peripheral and central pathways (Fuchs *et al.*, 1994; Harayama and Timmis, 1989; Schink *et al.*, 1992). Peripheral pathways convert a large variety of aromatic compounds into a few central aromatic intermediates. The enzymes of these pathways are often coded by genes on plasmids and are more or less specific for certain growth substrates (Heider and Fuchs, 1997).

The initial goal is to remove substituents from the aromatic ring and introduce hydroxyl functions. Both types of reactions are performed by a variety of monooxygenases and dioxygenases (Heider and Fuchs, 1997). Oxidative reactions may also be involved in the transfer of electrons from the substrate electron donor to the electron acceptor (Montgomery *et al.*, 1994).

Ring cleavage by oxygen can occur either at the *ortho* or *meta* position of the benzene ring. Ring cleavage and subsequent metabolism of the non-aromatic product to common metabolites such as acetyl-CoA or succinate together make up the central pathway (Harayama and Timmis, 1989). The induction of the different enzymes is controlled differently by sequential stimulation by several substrates and degradation intermediates (Assinder and Williams, 1990; Yen and Serdar, 1988).

Under aerobic conditions, the pathways are drawn either from catabolic processes that completely mineralize the compound to produce energy, or from reactions that transform the compound into precursors for biosynthetic pathways (Bollag *et al.*, 1968a;b). A variety of mechanisms have been identified for the aerobic degradation of chlorine-substituted aromatic compounds.

These mechanisms can be differentiated based on the number and position of chlorine substituents: Mono- and dichlorophenols are usually aerobically degraded by

hydroxylation to chlorocatechols followed by spontaneous dechlorination after *ortho*-cleavage of the catechols; and trichloro- and polychlorinated phenols are degraded aerobically via *para*-hydroquinones which are subsequently dechlorinated before ring cleavage (Hale *et al.*, 1994). Anaerobic CP degradation is initiated by reductive dechlorination followed by ring cleavage by a variety of microbial populations (Löffler *et al.*, 1999).

1.3.1 Fungal Degradation of Chlorinated Phenol and Other Haloorganic Pollutants

The white-rot fungal extracellular enzyme free-radical generating mechanism and its potential for pollutant oxidation has been extensively studied (Barr and Aust, 1994; Bumpus and Aust, 1986; Forney *et al.*, 1982; Kirk, 1984). The ability of lignolytic basidiomycetous fungi to mineralize a broad spectrum of structurally diverse organic compounds has been demonstrated in various studies (Anderson and Henrysson, 1996; Chang *et al.*, 1983; Eaton, 1985; Field *et al.*, 1992; Hatakka *et al.*, 2001; Khindaria *et al.*, 1995; Leatham *et al.*, 1983; Morgan *et al.*, 1991; Pal *et al.*, 1995). It has been established that a reductive pathway is active in dehalogenating various chlorinated compounds. Armenante *et al.* (1994), Hammel (1989), Hammel and Tardone (1988), Joshi and Gold (1993), Milseki *et al.* (1988), Pallerla and Chambers (1998), Pérez *et al.* (1997), Reddy *et al.* (1998) and Valli and Gold (1991) reported white-rot fungal-mediated catabolism of CP.

White-rot fungi, for example *Phanerochaete chrysosporium*, *Trametes versicolor* and *Agaricus bisporus*, secrete two extracellular heme peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), as well as an H₂O₂-generating system. They also produce other extracellular enzymes, including laccase and polyphenol oxidases. They constitute the major extracellular components of the degradation system (Barr and Aust, 1994; Bumpus and Aust, 1986; Pallerla and Chambers, 1998; Srinivasan *et al.*, 1995; Trejo-Hernandez *et al.*, 2001).

Researchers have concluded that the degradation of xenobiotics by white-rot fungi is interceded by both extracellular and cellular enzymes (Lin *et al.*, 1990; 1991). Joshi and Gold (1993) showed the metabolism of 2,4,5-trichlorophenol (2,4,5-TCP) by *P. chrysosporium* proceeded through oxidative dechlorination by either lignin peroxidase or

manganese-dependant peroxidase (extracellular) followed by intra- or extracellular reduction resulting in the regeneration of the peroxidase substrate. Armenante *et al.* (1994) concluded that biomass (mycelia) alone cannot degrade 2,4,6-trichlorophenol (2,4,6-TCP) unless extracellular fungal protein is present. The results obtained also showed that with culture supernatant (extracellular protein) alone, no significant mineralization occurred. This was also observed with PCP degradation and the biomass and extracellular protein (peroxidases plus laccase) of *T. versicolor* in batch studies using mycelial pellets with and without extracellular fluid (Pallerla and Chambers, 1998).

1.3.1.1 Elucidating white-rot fungal haloorganic pollutant metabolism mechanisms

The dechlorinating preference and removal of three CP isomers (*ortho*-, *meta*- and *para*-CP) by *P. chrysosporium* was investigated by Pérez *et al.* (1997). It was found that dechlorination rates were in the order *ortho*>*meta*>*para* with the degradation of *meta*- and *para*-CP between 23 and 31% (100 mg.L⁻¹ each of the isomers) compared to 44 % for the *ortho*-isomer in batch investigations. With acclimated mycelia, removal rates of up to 70 % for the *ortho*-isomer and 50 % for the *meta*- and *para*-isomers were shown. They concluded that the ability of this white-rot fungus to dehalogenate and degrade monochlorophenols depended on the position of the chlorine substitute.

Joshi and Gold (1993), Khindaria *et al.* (1995), Okeke *et al.* (1997), Reddy *et al.* (1998) and Valli and Gold (1991) studied the reductive dechlorinating capability of white-rot fungi. Khindaria *et al.* (1995) showed the mineralization of the chlorinated aliphatic hydrocarbons trichloroethylene (TCE) and carbon tetrachloride (CCl₄) by *P. chrysosporium* using ethylenediaminetetraacetic acid (EDTA) and oxalate as electron donors. They concluded that an auxiliary electron donor is necessary to sustain reductive dechlorination and that it is a free-radical-mediated process initiated by the oxidation of veratryl alcohol to the veratryl alcohol cation radical.

The radical, in turn, oxidizes oxalate (excreted extracellularly by the fungus) or EDTA to their respective radicals. Oxalate is decarboxylated with the release of the CO₂⁻ anion radical. The EDTA radical or carboxylate anion radical derived from either EDTA or oxalate can reductively dehalogenate the aliphatic halocarbons.

The application of purified LiP and/or MnP and laccases extracted from *P. chrysosporium*, *Lentinula edodes* and *T. versicolor* was used to elucidate the degradation pathways for CP. Hammel and Tardone (1988) showed that the oxidative 4-dechlorination of 2,4-dichloro-, 2,4,5-trichloro-, 2,4,6-trichlorophenol and PCP is catalyzed by extracellular fungal lignin peroxidases to yield a *p*-benzoquinone. The involvement of reductive dechlorination in metabolism of 2,4,6-TCP and 2,4,5-TCP was studied by Joshi and Gold (1993) and Reddy *et al.* (1998). They proposed that all three chlorine atoms are removed before ring cleavage occurs. The initial oxidative dechlorination by either LiP or MnP forms a dichloroquinone. Subsequent intracellular reduction of the chloroquinone resulted in the formation of 2,6-dichloro- or 2,5-dichlorohydroquinone (from 2,4,6-TCP and 2,4,5-TCP, respectively), which is reductively dechlorinated to 2-chlorohydroquinone. 2-Chlorohydroquinone is either reductively dechlorinated further to hydroquinone, which undergoes orthohydroxylation to form 1,2,4-trihydroxybenzene (THB), or is hydroxylated to form chlorotrihydroxybenzene, which is reductively dechlorinated to THB. THB is then ring cleaved by 1,2,4-THB 1,2-dioxygenase (Rieble *et al.*, 1994).

Mixtures of laccases isolated from *T. versicolor* culture fluids effected between 13.6 and 17.6 % dechlorination of a mixture of CP (Roy-Arcand and Archibald, 1991). Biotransformation products pentachloroanisole, tetrachloroanisole and tetrachlorophenol were identified in soil contaminated with PCP and inoculated with *L. edodes* (Okeke *et al.*, 1997). Dechlorination efficiency was notably greater with monocultures of the fungus (29.5 %) than with mixed cultures (22.4 %).

1.3.1.2 Application of white-rot fungal mineralization in biodegradation studies

Considerable attention has been given to *P. chrysosporium* and other ligninolytic fungi in terms of the nutritional requirements and effect on extracellular enzyme secretion, pH and temperature parameters (Bosco *et al.*, 1999; Dhawale *et al.*, 1992; Pal *et al.*, 1995; Pérez *et al.*, 1997; Yadav and Reddy, 1993; Yum and Peirce, 1998). However, practical applications of fungi have been limited. In the past, research primarily focussed on model pollutants (as single or mixtures of compounds) in bench-scale batch (liquid and soil) and fermentation free-cell reactors (Anderson and Henrysson, 1996; Chiu *et al.*, 1998; Field *et al.*, 1992; Gramss *et al.*, 1999; In der Wiesche *et al.*, 2001; Liao and

Tseng, 1996; Okeke *et al.*, 1997; Yadav and Reddy, 1993); and extracts of the lignolytic enzymes applied to model contaminants (Burton *et al.*, 1998; Hammel and Tardone, 1988; Hofrichter and Hatakka, 2001; Trejo-Hernandez *et al.*, 2001; Valli and Gold, 1991). Fewer studies report investigations of complex effluents.

Reports of white-rot fungi mediated degradation and treatment of wastewaters include decolorisation of paper bleach plant, kraft mill and textile effluent (Archibald *et al.*, 1990; Bergabuer *et al.*, 1991; Esposito *et al.*, 1991; Kunz *et al.*, 2001) and biodegradation of recalcitrant olive-mill wastewater (D'Annibale *et al.*, 1998; Iconomou and Zanganas, 2001; Sayadi and Ellouz, 1995).

It has been shown that degradation capacity and tolerance to toxic pollutant concentrations can be increased when fungal biomass is immobilized opposed to free-cell system operation (Dwyer *et al.*, 1986; Pallerla and Chambers, 1998). Also, the application of fungal enzymes to industrial processes (bioleaching, biopulping, decolorisation, etc.) on a large scale requires the production of high enzyme titres at low cost. Thus, the design of a system permitting high and continuous production of lignolytic enzymes is required (Domínguez *et al.*, 2001). Fixed-cell reactors, also referred to as a semi-solid or solid-state fermentation, involve the attachment of microorganisms on moist solid substrates in the absence or near absence of free liquid. Materials that are employed include inert support (agarose, polyurethane or silicone) and organic support (wood chips, crop wastes). Advantages include prevention of the biomass washout found in free-cell systems thus fixed-cell systems tend to have greater operational flexibility (Domínguez *et al.*, 2001; Sofer *et al.*, 1990). In addition, the fungal cell density in immobilized systems can be greater than that of free-cell systems resulting in higher biodegradation rates. Shim and Kawamoto (2002) concluded immobilized *P. chrysosporium* exhibited improved growth and enhanced protracted periods of ligninolytic enzyme activity.

1.3.2 Anaerobic Chlorinated Phenol Degradation

During anaerobic transformation, chlorine atoms are replaced with hydrogen atoms. The phenol produced by reductive dechlorination may be subsequently transformed either

through cyclohexanol, cyclohexane and adipate to succinate, propionate and acetate (Evans, 1977; Young, 1984), or as presently assumed, through benzoate and acetate to the end products, methane and CO₂ (Knoll and Winter, 1987; 1989). CP degradation under anaerobic degradation usually requires a sequence of transformations involving more than one organism (Zhang and Wiegel, 1990a; b).

Within a family of chlorinated compounds, reductive dechlorination is generally proportional to the degree of chlorination. For example tri-, tetrachlorophenols and PCP is the most highly substituted of the chlorophenols. It has the highest oxidation-reduction potential (redox potential) and is the most favourable electron acceptor as represented by the negative value for change in Gibbs free energy (ϵG^0), therefore dechlorination of these compounds is strongly exergonic (Vogel *et al.*, 1987).

Reductive dechlorination is a cometabolic or respiratory process that is carried out by organochlorine-reducing bacteria. During the respiratory process, microorganisms use chlorinated organic compounds in energy metabolism and growth (Löffler *et al.*, 1999). The common characteristic of organohalogen-reducing bacteria is their ability to couple the energy released during reductive dehalogenation to cell growth in a process known as halo-respiration, or chlororespiration for chlorinated compounds (Löffler *et al.*, 1999). Aryl reductive dehalogenation is the initial and often the rate-limiting reaction for the biodegradation of halogenated aromatic compounds in anoxic environments (DeWeerd *et al.*, 1991).

1.3.2.1 Thermodynamics of dehalogenation reactions

The energetics of the terminal electron accepting process has been shown to control the minimum H₂ concentration that can be consumed (Lovley and Goodwin, 1988).

The H₂ threshold is inversely correlated with changes in the Gibbs free energy (ϵG^0) and the electrochemical potential of the H₂-consuming reaction (Löffler *et al.*, 1999). Lovley and Goodwin (1988) have showed that the steady-state H₂ threshold concentrations are controlled by the physiological state of the hydrogen-consuming microorganisms and are independent of the kinetics of the H₂-producing or H₂-consuming

reactions. Based on the investigation into the fraction of electron consumed in electron acceptor reduction and hydrogen thresholds as indicators of organochlorine-reducing bacteria activity, Löffler *et al.* (1999) predicted that chlororespiring bacteria should consume H_2 so that the threshold concentrations are lower than the threshold concentrations of acetogens, methanogens and sulphate-reducers. Threshold concentrations are close to organisms that exhibit dissimilatory nitrate reduction of nitrate to ammonia.

Dolfling and Harrison (1992) and Dolfling and Janssen (1994) have shown the potential role of halogenated aromatic compounds as electron acceptors in anaerobic environments. The reasoning was based on the calculation of the Gibbs free energy of formation of a series of halogenated aromatic compounds (Ar-X). The estimates indicate that the redox potentials for Ar-X/Ar-H redox couples are in the range 266-478 mV (Table 1.2) which allows microorganisms to use these compounds as electron acceptors under anaerobic conditions (El Fantroussi *et al.*, 1998).

Table 1.2. Gibbs free energy values and redox potential for the reductive dechlorination of halogenated compounds with hydrogen as electron donor (Dolfling and Harrison, 1992; Dolfling and Janssen, 1994)

Substrate	Product	ϵG^0 (kJ/reaction)	E_o' (mV)
PCP	2,3,4,6-Tetrachlorophenol	-167.8	455
2,3,4,6-Tetrachlorophenol	2,4,6-Trichlorophenol	-157.1	400
2,4,6-Trichlorophenol	2,4-Dichlorophenol	-141.5	319
2,4-Dichlorophenol	4-Chlorophenol	-131.3	266
4-Chlorophenol	Phenol	-164.5	438

Due to the favourable thermodynamics of reductive dechlorination (-130 to $-187 \text{ } \epsilon G^0$ (kJ/mol of H_2); Löffler *et al.*, 1999) compared to other terminal electron accepting processes (methanogenesis: $-33.9 \text{ } \epsilon G^0$; sulphate-reduction $SO_4^{2-} + 6H_2S^-$: $-38.0 \text{ } \epsilon G^0$; ammonification $NO_3^- + 6 NH_4^+$: $-149.9 \text{ } \epsilon G^0$; Dolfling and Harrison, 1992; Dolfling and Janssen, 1994), chlororespirers can thus maintain H_2 concentrations below levels that allow sulphate-reduction or methanogenesis. Hence, chlororespiring bacteria can

outcompete methanogens and sulphate-reducing bacteria (SRB) for H_2 and should dominate anaerobic environments as long as reductive dechlorination is not limited by the concentration or bioavailability of the chlorinated electron acceptor (Löffler *et al.* 1999).

Earlier work has shown that aryl dehalogenation reactions are enhanced in anoxic aquifer slurries by the addition of acids and alcohols (Gibson and Suflita, 1990; Linkfield and Tiedje, 1990). It was hypothesised that this stimulation was due to a transient increase in H_2 concentrations in the slurries and that H_2 was an electron donor for dehalogenation (DeWeerd *et al.*, 1991). Based on thermodynamic considerations and confirmed by H_2 -threshold measurements, it has been concluded that halo-respiring bacteria should be able to outcompete hydrogenotrophic sulphate reducers and methanogens in environments where hydrogen is the main source of electrons (Fennell and Gossett, 1998). Close syntrophic interactions of reductively dechlorinating bacteria with H_2 -producing microorganisms are feasible due to thermodynamic limitations dependent on low H_2 -partial pressure (Smidt *et al.*, 2000).

Colberg (1990) suggested that with sulphate as an electron acceptor, there are apparently no thermodynamic barriers to chemical oxidation of a number of aromatic compounds that are considered environmental contaminants. Table 1.3 illustrates the oxidation of chlorinated benzenes and phenols containing one or two chlorine substituent groups and show these reactions to be more thermodynamically favourable under sulphate-reducing conditions. These reactions could, in theory, occur spontaneously although there is no direct evidence for the existence of an abiotic mechanism of sulphate reduction in natural systems.

With the comparison of free-energy calculations for the mineralization of 4-chlorophenol (4-CP) under sulphidogenic conditions with sulphate (SO_4^{2-} , $-71.8 \text{ kJ mol}^{-1}$), sulphite (SO_3^{2-} , $-121.2 \text{ kJ mol}^{-1}$) and thiosulphate ($S_2O_3^{2-}$, $-60.2 \text{ kJ mol}^{-1}$) as electron acceptors and methanogenic conditions with bicarbonate (HCO_3^- , $-39.4 \text{ kJ mol}^{-1}$) serving as an electron acceptor, it is evident that degradation coupled to methane formation is less favourable (Colberg, 1990).

Table 1.3. Thermodynamic calculations for the complete oxidation of chlorinated benzenes and phenols providing sulphate SO_4^{2-} as the terminal electron acceptor (Colberg, 1990)

Compound	ϵG^0 (kJ.mol carbon ⁻¹)	Reaction ^a
Chlorinated benzenes		
Chlorobenzene	-69.0	$2\text{C}_6\text{H}_5\text{Cl} + 7\text{SO}_4^{2-} + 5\text{H}^+ \rightarrow 12\text{CO}_2 + 7\text{HS}^- + 2\text{Cl}^- + 4\text{H}_2\text{O}$ $4\text{C}_6\text{H}_4\text{Cl}_2 + 13\text{SO}_4^{2-} + 5\text{H}^+ \rightarrow 24\text{CO}_2 + 13\text{HS}^- + 8\text{Cl}^- + 4\text{H}_2\text{O}$
1,2-Dichlorobenzene	-68.8	
1,3-Dichlorobenzene	-68.2	
1,4-Dichlorobenzene	-67.9	
Chlorinated phenols		
2-Chlorophenol	-67	$4\text{C}_6\text{H}_4\text{OHCl} + 13\text{SO}_4^{2-} + 9\text{H}^+ \rightarrow 24\text{CO}_2 + 13\text{HS}^- + 4\text{Cl}^- + 8\text{H}_2\text{O}$
3-Chlorophenol	-70.4	
4-Chlorophenol	-71.8	

^aCalculations were made under standard conditions.

This may suggest that there are other non-thermodynamic reasons why chloroaromatic compound transformations have not been as readily observed in sulphidogenic environments as during methanogenesis.

1.3.2.2 Reductive dechlorination in the presence of alternative electron acceptors

Under anaerobic conditions, the fate of halogenated compounds is greatly influenced by the presence or absence of potential electron acceptors (Suflita *et al.*, 1983). Anaerobic dehalogenation reactions have been studied in various habitats and are generally found in environmental samples where methanogenesis occurs but they are found less frequently when sulphate or nitrate-reducing conditions predominate (Colberg, 1990; Gibson and Suflita, 1986, 1990; Kuhn *et al.*, 1990).

Bae *et al.* (2002), Genthner *et al.* (1989a) and Häggblom *et al.* (1993) showed the dechlorination of monochlorinated phenols and benzoates under nitrate-reducing conditions.

A number of reports indicate that sulphate appears to inhibit anaerobic degradation of CP and other chloroaromatic compounds (Colberg, 1990; Gibson and Suflita, 1986; Kuhn *et al.*, 1990; Madsen and Aamand, 1991) but there is also evidence of CP degradation in the presence of sulphate or during sulphate reduction (DeWeerd *et al.*, 1991; Genthner *et al.*, 1989b; Häggblom and Young, 1995; Kohring *et al.*, 1989a;b). Dehalogenation and degradation under methanogenic conditions are well established (Gibson and Suflita, 1986; Häggblom *et al.*, 1993; Mohn and Tiedje, 1992; Zhang and Wiedel, 1990a) but halogenated compounds also often inhibit methanogenesis (Guthrie *et al.*, 1984; Mikesell and Boyd, 1990). King (1988) was the first to report the reductive dechlorination of 2,4-dichlorophenol (2,4-DCP) in marine sediments. At the high sulphate concentrations found in seawater (20 to 30 mM), sulphate reduction is the main pathway for carbon metabolism in marine sediments and sulphate may be the dominant electron acceptor influencing dehalogenation and anaerobic degradation of halogenated compounds (Häggblom and Young, 1995; Monserrate and Häggblom, 1997).

Despite the fact that transformations of many halogenated and non-halogenated aromatic substrates are thermodynamically favourable under sulphate-reducing redox conditions, our understanding of the extent to which alternative electron acceptors may contribute to the turn-over of environmental contaminants is still limited. This may be due, in part, to the long-standing view that anaerobic microorganisms are able to catabolize only a limited number of simple substrates. In addition, the tendency of microbiologists to work with pure cultures has delayed the understanding of many anaerobic transformations (Colberg, 1990).

We now know that many anaerobic transformations are carried out by co-cultures or consortia (Bossert *et al.*, 1986; Dolfing and Tiedje, 1986; Mountford and Bryant, 1982; Shelton and Tiedje, 1984; Tschech and Schink, 1986; Zhang and Wiegel, 1990a).

1.3.2.3 Reductive dechlorination during sulphidogenesis

Studies concluding that sulphate inhibits reductive dechlorination were frequently made by the addition of sulphate to exclusively methanogenic-adapted cultures. These cultures were previously able to transform the chloroorganic compounds under said

conditions but supplementing with SO_4^{2-} obstructed or limited degradation. Since the microorganisms were not acclimatized for sulphidogenic conditions, it was perhaps prematurely presumed that sulphate inhibits dechlorination. Colberg (1990) noted that several of the studies investigating sulphate-supplemented inhibition of dechlorination are from the same laboratory and used methanogenic-acclimated cultures.

Gibson and Suflita (1990) used 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) as a model substrate to investigate dechlorination under sulphidogenic conditions. They made additions of sulphate to methanogenic cultures previously able to degrade 2,4,5-T that resulted in a reduction of degradative activity. They also showed that the addition of sulphate to methanogenic aquifer slurries increased 2,4,5-T acclimation time and decreased the amount of 2,4,5-T degraded relative to control slurries. Similarly, Madsen and Aamand (1991) observed an inhibition of PCP dechlorination in the presence of sulphate, thiosulphate and sulphite in comparison to methanogenic conditions where dechlorination proceeded through 2,3,4,5-tetrachlorophenol, 3,4,5-trichlorophenol and 3,5-dichlorophenol and accumulation of 3-chlorophenol (3-CP). The inhibiting effect on dechlorination was relieved by supplementing the culture with molybdate, a competitive inhibitor of sulphate-reduction.

Häggbloom *et al.* (1993) speculated that dechlorination might be more of a function of competent microbial population than one of inherent molecular structure (position of halogen substituent) since no single redox condition investigated (nitrate-reducing, sulphate-reducing and methanogenesis) permitted metabolism of all monochlorinated phenols and benzoates tested in this study.

However, DeWeerd *et al.* (1990) and Linkfield and Tiedje (1990) showed that sulphate does not inhibit the dehalogenation activity of *Desulfomonile tiedjei*. Kohring *et al.* (1989b) observed reductive dehalogenation of 4-chlorophenol (4-CP) and 2,4-DCP in freshwater sediments while sulphate reduction occurred. In both the presence or absence of added sulphate, 2,4-DCP was stoichiometrically transformed to 4-CP after a temperature dependent acclimation period. In the presence of added sulphate, the adaptation period was longer than observed under methanogenic conditions (Kohring *et al.*, 1989a).

DeWeerd *et al.* (1991) used resting cell suspensions of *D. tiedjei* to monitor both the consumption of H₂ and dehalogenation activity in the presence of various electron donors and acceptors. The cell suspensions consumed H₂ with 3-chloro-, 3-bromo- and 3-iodobenzoate as electron acceptors with rates of 0.50, 0.44 and 0.04 $\Phi\text{mol.h}^{-1}.\text{mg}^{-1}$, respectively. H₂ uptake was at least fourfold faster when sulphate, sulphite or thiosulphate was available as the electron acceptor instead of the haloaromatic substrate but the concentrations of H₂ at the end of the studies were comparably low. The researchers suggested that in the presence of sulphate the organism rapidly lowered the H₂ concentration to a level that is less favourable for dehalogenation. In addition, sulphite and thiosulphate directly inhibited dehalogenation by *D. tiedjei* but sulphate did not, which indicated that the reduction of sulphur oxyanions is favoured over aryl dehalogenation for the removal of reducing equivalents under anaerobic conditions.

It was suggested that the inhibitory effect of sulphate may be caused by the availability of the electron acceptor and competition for electron donors between the SRB and dechlorinating culture, with the SRB able to outcompete dehalogenating bacteria for available hydrogen. This may explain why the process is slower in sulphate-rich environments (DeWeerd *et al.*, 1991; Gibson and Suflita, 1986; Hale *et al.*, 1994; Kuhn *et al.*, 1990).

This conclusion was drawn from the observations that uninhibited reductive dechlorination and sulphate reduction happens simultaneously in the presence of a SRB when a hydrogen atmosphere was supplied; and uninhibited reductive dechlorination occurred in the absence of externally added H₂ in the presence of a SRB when the growth of the sulphate reducer was inhibited with the addition of molybdate (Hale *et al.*, 1994). However, the addition of sulphate and a sulphate-reducer (*Desulfovibrio vulgaris*) facilitated a strong inhibition. Thus, dechlorination may depend on the ratios of affinities for H₂ between the dechlorinating population and SRB.

Studies that employ exclusively sulphidogenic-adapted cultures showed that reductive dechlorination can be coupled to sulphate reduction. Häggblom and Young (1990; 1995) used sediment from an estuarine intertidal channel and showed complete mineralization

of 2-chlorophenol (2-CP), 3-CP and 4-CP under both freshwater and saline conditions. The decrease was coupled to sulphate reduction, corresponding to approximately stoichiometric values expected for complete oxidation of the chlorophenol to CO₂. Sulphide production from sulphate for a 4-CP-degrading culture was confirmed by ³⁵SO₄²⁻ radiotracer assays and inhibition studies using molybdate obstructed both sulphidogenesis and halogenated phenol catabolism. No methane was detected thus it was concluded that sulphate reduction was the main electron sink.

Other sulphur oxyanions, sulphite or thiosulphate, also served as electron acceptors for CP utilization while nitrate, carbonate and fumarate did not. Häggblom and Young (1995) reported 2,4-DCP and 2,6-dichlorophenol (2,6-DCP) transformation using biomass from an anaerobic bioreactor that treated pulp bleaching effluent as inoculum. They observed an accumulation of 4-CP with 2,4-DCP dechlorination and negligible removal of the metabolite under sulphidogenic conditions. 2,6-DCP was subsequently dehalogenated to 2-CP and phenol. Sulphate reduction was not monitored during the study and no methane was detected. Häggblom (1998) also showed 4-chloro-2-fluorophenol and 4-chloro-3-fluorophenol dechlorination by the same estuarine sediment-enriched sulphidogenic culture, resulting in stoichiometric accumulation of 2-fluorophenol and 3-fluorophenol, respectively.

Investigating the hypothesis that reductive dechlorination by chlororespiring bacteria occurs in a synergistic relationship with SRB gained momentum after co-cultures consisting of a sulphate-reducing and dechlorinating species were enriched for and identified in soil contaminated with tetrachloroethylene (PCE) (Drzyzga *et al.*, 2001; Gerritse *et al.*, 1998). This supports the assumption that dechlororespiring bacteria and SRB often share environments that are contaminated with chlorinated compounds.

Studies by Cabirol *et al.* (1998), Drzyzga *et al.* (1994) and Mägli *et al.* (1996) first suggested that a symbiotic relationship between sulphate-reducing and dehalorespiring strains exists and it was concluded that a dependence on growth factors (reducing equivalents) for the dechlorinating bacterium released by the sulphate-reducer drives dechlororespiration. However, it was the work by Drzyzga *et al.* (2001) and Drzyzga and Gottschal (2002) that proved further insight into, and elucidation of, the synergistic

relationship of the sulphate-reducing – dechlororespiring co-culture by employing different sulphate concentrations.

Drzyzga *et al.* (2001) obtained a two-member co-culture consisting of the dehalorespiring *Desulfitobacterium frappieri* TCE1 and the sulphate-reducing *Desulfovibrio* sp. strain SULF1 enriched from soil contaminated with PCE. In a defined chemostat coculture, the combined presence of sulphate and PCE as electron acceptors was reported to effect dechlorination of PCE. With lactate as the primary electron source for both strains along with varying sulphate and PCE concentrations, the *Desulfovibrio* sp. strain outnumbered the dechlorinating strain at high ratios of sulphate : PCE. In the presence of low sulphate : PCE ratios, the *Desulfitobacterium* sp. became the numerically dominant strain within the chemostat co-culture but when both electron acceptors were present in nearly equimolar amounts, stable co-cultures with both present at similar cell densities were observed. The authors proposed a syntrophic association between the sulphate-reducer and dehalorespiring bacterium via interspecies hydrogen transfer. The *Desulfovibrio* sp. was able to sustain growth in the chemostat co-culture by fermenting lactate and using the *Desulfitobacterium* sp. as an electron acceptor where sulphate concentrations were reduced.

Recently, Drzyzga and Gottschal (2002) investigated PCE dechlorination in a continuously operated co-culture of the sulphate-reducer *Desulfovibrio fructosivorans* and the dehalorespiring *D. frappieri* TCE1 at different sulphate concentrations and in the absence of sulphate. Fructose was supplied as an electron donor and could only be used by the *Desulfovibrio* sp. With excess sulphate supplements the dehalogenating strain was outnumbered by the sulphate-reducer and sulphate reduction emerged as the dominant process. At 1mM sulphate, complete sulphate-reduction and PCE dechlorination to *cis*-dichloroethene (*cis*-DCE) was observed. In the absence of sulphate, PCE was also dehalogenated to *cis*-DCE. The authors concluded that the activity of the dehalorespiring anaerobe is in strict dependence on the levels of sulphate and activity of the sulphate reducer with a substrate that is exclusively used by the latter.

Interspecies hydrogen transfer was demonstrated by H₂ measurements of the chemostat head gas phase and shown to depend on the transfer of hydrogen under sulphate depleted conditions since the production of hydrogen occurred only at low sulphate

concentrations or in the absence of sulphate during which the sulphate-reducing strain metabolized fructose.

1.3.2.4 Preference for specific organic compounds

The preference for carbon and energy sources by reductive dehalogenation bacteria have been extensively investigated (Boyle *et al.*, 1999; DeWeerd *et al.*, 1991; Howard and Rogers, 1990; Kuhn *et al.*, 1990; Madsen and Aamand, 1992; Madsen and Licht, 1992; Sanford *et al.*, 1996; Sun *et al.*, 2000; Yeh *et al.*, 1998; 1999). A number of organic substrates such as benzoic acid, lactate, ethanol, methanol, acetate and hydrogen support the process. Inexpensive substrates can also be employed to drive the process in up-scaled applications, such as molasses, cheese whey permeate, grain steep liquor and wastewaters containing fermentable substrates (propionate, acetate) (Lee *et al.*, 1998). If dechlorinating activity is present and well established, almost any fermentable substrate can stimulate activity. However, complex substrates may result in the growth of competing organisms that are not involved in the dechlorination process and lead to biofouling (Johnson *et al.*, 1999; Lee *et al.*, 1998).

1.3.2.5 Application of dehalogenation in biodegradation studies

The concern for contamination of groundwater and subsurface soil, and marine and estuarine sediments by anthropogenic halo-organic compounds has contributed to efforts to elucidate and assess microbial-mediated dehalogenation mechanisms as bioremediation technology applications (Colberg and Young, 1995).

El Fantroussi *et al.* (1998) speculated that anaerobic microorganisms are better suited than aerobes for removing halogenated aromatic compounds from industrial effluents. It was suggested that this could be due to the polymerization of haloaromatic compounds that occurs frequently when they are degraded by aerobic bacteria, the limited susceptibility to electrophilic attack of aromatic structures with a high degree of halogenation and the high affinity for halogenated aromatics by anaerobes (Sahm *et al.*, 1986; Suflita *et al.*, 1982).

Two aspects have been identified by Holliger and Schraa (1994) for the application of dechlorination in treatment operations. The degree of specificity of known dechlorinating

reactions is the first aspect to be considered because metabolic dechlorination reactions have been found to be very substrate specific. The second aspect is dechlorination rate. Degradation rates can be increased for enrichment cultures repeatedly fed complex substrates compared to enrichment cultures fed simple substrates such as acetate or hydrogen (Lee *et al.*, 1998). However, because of variable responses to substrates by microorganisms from different enrichments and environments, further investigation is necessary to confirm a specific substrate will support dehalogenation. No single substrate that reliably supports dechlorination in all applications and conditions has been identified to date (Lee *et al.*, 1998; Puhakka and Melin, 1996).

The application of dehalogenation by anaerobic bacteria is still a relatively new field and not many studies have been undertaken to apply dehalogenation to remediate actual contaminated sites. Nevertheless, there are reports describing the introduction of *de novo* bioremediation abilities in laboratory-scale studies using anaerobic dechlorinating strains. Ahring *et al.* (1992) showed successful 3-chlorobenzoic acid (3-CB) dechlorinating activity of *D. tiedjei* by introducing the strain into upflow anaerobic granular-sludge blanket (UASB) reactors. Christiansen *et al.* (1995) and Christiansen and Ahring (1996) demonstrated favourable PCP dehalogenation by immobilizing DCB-2 (*Desulfitobacterium hafniense*) in an UASB reactor containing sterile sludge granules. The dechlorinating performance was 120 Φmol of PCP.L⁻¹ of reactor volume.day⁻¹ with an HRT of 8h. This is low compared to Puhakka and Järvinen (1992) who showed a CP removal rate of 970 Φmol of CP.L⁻¹ of reactor volume.day⁻¹ in an aerobic fluidized bed reactor (FBR) from a wood preservative-contaminated aquifer.

Wilson *et al.* (1998) observed an almost equimolar (> 99%) concentration transformation of PCP to monochlorophenol in an anaerobic granulated activated carbon (GAC) FBR (200 mg.L⁻¹, influent concentration; 2.3 h, empty bed contact time). They operated the anaerobic GAC FBR in sequence with an aerobic fluidized reactor which removed remaining CP and phenol. Hendriksen and Ahring (1993) and Wu *et al.* (1993) investigated the dehalogenation of PCP to lesser chlorinated isomers and mineralization in fixed-film and UASB reactors, respectively, inoculated with anaerobic granules and sludge.

The anaerobic dechlorination of chlorinated organic compounds with high molecular masses, such as chlorolignins in bleach plant effluent, was investigated with a sample of anaerobic sediment bacteria obtained from a wastewater receiving basin at a pulp mill in Sweden by Fitzsimons *et al.* (1990). A decrease in organically bound chlorine measured as adsorbable organic halogen was found to depend on the molecular mass and presence of readily available carbon sources. The rate and extent of dechlorination of the adsorbable organic halogen decreased with increasing molecular mass and the authors concluded that the process proceeded too slowly to be of any practical use in bleach plant effluent treatment.

Ex-situ PCP-contaminated soil bioremediation in rotating slurry reactors was demonstrated by Lanthier *et al.* (2000). *Desulfitobacterium frappieri* PCP-1, isolated from a methanogenic consortium, was bioaugmented in PCP-contaminated soil (100 – 750 mg PCP.kg⁻¹ of soil). The bacterium was able to dechlorinate PCP (189 mg PCP.kg⁻¹ soil) to 3-CP. In non-inoculated reactors, the indigenous soil microflora was also able to degrade PCP suggesting anaerobic dehalogenation by stimulation or augmentation should be possible *in-situ* and *ex-situ*. *D. frappieri* PCP-1 was also used to augment a mixed bacterial community of an UASB reactor degrading PCP (Tartakovsky *et al.*, 1999). Proliferation of strain PCP-1 allowed for a 99 and $\geq$ 99.5 % PCP removal and dechlorination efficiency with PCP loads between 5 and 80 mg.L⁻¹, respectively (residence time 28 h). The researchers detected 3-CP and phenol as dechlorination intermediates. El Fantroussi *et al.* (1999) assessed pure anaerobic strains for soil bioremediation in a pilot-scale soil reactor which provided a realistic simulation of the actual field situation. Bioaugmentation was carried out by inoculating with *D. tiedjei* in localized areas in the bioreactor and fed continuously with 3-CB as model pollutant. The spatial and temporal distribution of the 3-CB dechlorination activity was investigated with specific biological activity tests. Using sterilized and non-sterilized agricultural soil, the results showed a heterogeneous distribution of the dechlorination activity in the reactor.

Lee *et al.* (1998) reported that only a limited amount of field studies have been conducted for the anaerobic reductive dehalogenation of specifically chlorinated solvents. *In-situ* pilot studies ranged from simple tests in which a single borehole was dosed with substrates (acetate, yeast extract, methanol, casamino acids, ethanol, glucose and surfactants) to systems where the ground water was recirculated.

To assess the potential application of dehalogenation in *in-situ* bioremediation technologies is complex as the direct transfer of results from laboratory batch or column experiments by studying pure cultures or microbial communities to the field is usually not possible or feasible. The natural variability of the soil and subsurface properties cannot be investigated on such small scale. On the other hand, field studies are not only costly and time consuming but also constrained by the fact that neither the contaminant mass nor its spatial distribution and the structure of the subsurface are adequately known nor can be investigated in detail (Kobus, 1996). In that context, pilot-scale reactors will provide a realistic assessment of how dechlorinating microorganisms can be applied to clean up polluted areas (El Fantroussi *et al.*, 1998). In addition, results from laboratory or pilot-scale studies are extrapolated to a variety of habitats so as to provide a basis for environmental risk assessment and regulatory decision making and often suggest ways of remediating the environment (Gibson and Suflita, 1986).

1.3.2.6 Why study reductive dechlorination under sulphidogenic conditions?

The investigation of dehalogenation in the presence of alternative electron acceptors is a subject of some interest given that little is still known about the role that these electron acceptors play in dechlororespiration metabolism and the wider implication of their activity in geochemical microbiology. Furthermore, the successful application of reductive dechlorination in chloro-organic contaminated soil and groundwater or surface water bodies is dependent on the presence of alternative electron acceptors such as sulphate, nitrate or iron in subsurface environments where oxygen is often limited. Sulphate is a predominant electron acceptor in especially marine and intertidal environments and its impact on dechlororespiration metabolism has not been thoroughly appreciated or investigated.

It is clearly important to know whether the availability of sulphate in excess, deficient or depleted concentrations may affect reductive dechlorination given the proposed synergistic relationship between SRB and dechlororespiration bacteria in co-culture. Successful application of reductive dechlorination under sulphate-reducing conditions could provide for further insight into the interactive symbiotic relationship between the co-culture involved. Clarification of these points could enable the evaluation and possibly

the prediction of dechlorination rates when specific site conditions (availability of sulphate and levels thereof) are known and taken into account.

Where this was successfully demonstrated and in the event of limiting sulphate concentrations in contaminated subsurface environments, appropriate sulphate additions could be made to support sulphidogenic-dependent enhanced *in-situ* reductive dechlorination. This presents an opportunity to achieve active biodegradation without further compromising environmental quality such as nitrate supplements for example would do by potentially introducing toxic effects where the selection of nitrate-reducing dependent dechlororespiration is effected.

The application of dechlororespiration reactions in soil/sediment bioremediation has largely been ignored. Pavlostathis *et al.* (2001) have noted that the sequestration of adsorbed polychlorinated organic compounds results in low bioavailability and may affect the rate and extent of pollutant transformation in soils and sediments. The role of sulphate reduction-linked dechlorination in enhancing desorption reactions and increased mobility of the pollutants may be a feature of particular significance in such applications.

1.4 Integration of the Anaerobic/Aerobic Bioprocess Environment for the Bioremediation of Complex Hydrocarbon Wastes

The potential advantages of the aerobic fungal and anaerobic bacterial co-culture systems in hydrocarbon bioremediation have been noted and the potential application highlighted. The potential for linking the anaerobic and aerobic process environments offers the possible combined advantages of each approach.

Sequential anaerobic/aerobic treatment strategies for chlorinated aliphatic and aromatic pollutants have been investigated in the past (Armenante *et al.*, 1992; Ascón and Lebeault, 1999; Atuanya *et al.*, 2000; Field *et al.*, 1995; Flora *et al.*, 1994; Gerritse *et al.*, 1995; 1997; Ronen and Abeliovich, 2000; Salkinoja-Salonen *et al.*, 1983; Shin *et al.*, 1999; Zitomer and Speece, 1993 and references therein).

Armenante *et al.* (1992) stressed that the majority of studies investigating anaerobic-aerobic processes prior to 1992, primarily focussed on the treatment of wastewaters containing high levels of total organic carbon (TOC) and not on the use and actual mechanisms by which each class of microorganism (aerobes vs. anaerobes) assist in the sequential mineralization of halo-organic compounds. Anaerobic stages in treatment processes have been studied widely for the transformation of chlorinated compounds in spent pulp bleaching liquors to less toxic compounds to be successfully mineralized with concomitant reduction in biological oxygen demand (BOD) levels in subsequent aerobic applications (Zitomer and Speece, 1993).

Gerritse *et al.* (1995; 1997) studied the degradation of the chlorinated aliphatic compound, PCE, by combining dechlorinating and methanotrophic cultures in coupled anaerobic-aerobic chemostats and fixed-bed reactors. They showed the transient accumulation of less-chlorinated ethenes with subsequent aerobic bacterial treatment to degrade the metabolites. Porous silica beads were used as packing material in an anaerobic down-flow reactor connected to a packing-free aerobic reactor in continuous operation to dechlorinate 2,4,6-TCP to 2,4-DCP and ultimately 4-CP under methanogenic conditions, and to mineralize 4-CP in the aerobic bacterial reactor (Armenante *et al.*, 1992). Similarly, Shin *et al.* (1999) employed motor tyre chips as support medium in sequential anaerobic-aerobic bacterial biofilm reactors to dechlorinate 2,4-DCP to 4-CP, and degrade the metabolite in a subsequent aerobic system. Atuanya *et al.* (2000) concluded that anaerobic UASB and aerobic suspended growth bioreactors performed more efficiently when operated in-series rather than in parallel to transform and mineralize 2,4-DCP. These studies relied on the specific transformation and degradation capabilities of two classes of bacteria to mineralize chlorinated organic pollutants: the dechlororespiration or reductive dechlorination of polychlorinated molecules by anaerobic bacteria, and oxidative mineralization of the resulting metabolites by aerobic bacteria.

Remediating oxygen-limited deep subsurface chlorinated aromatic compound-contaminated soil, sediment and groundwater currently presents numerous problems.

Due to their hydrophobicity and concomitant reduced availability and accessibility for biologically-driven remediation efforts (Alexander; 1999; Cheng and Mulla, 1999; Morra, 1996; Pavlostathis *et al.*, 2001), currently proposed remediation technologies do not sufficiently address these problems. Chlorinated aromatics polluting soil/aquifer bodies need to be dehalogenated before mineralization can commence. The use of white-rot fungi in remediation techniques may circumvent the bioavailability issue due to highly non-specific and non-stereoselective extracellular enzyme secretion, however, fungi require aerobic conditions to proliferate and produce enzymes. Introducing air into the subterranean environment to induce aerobic conditions is expensive and specific fungal growth stimulation questionable. *Ex-situ* treatment of contaminated soil/sediment followed by slurry- or solid-phase treatment is feasible albeit costly considering the cost of removing the material from below ground environments, and whether this might be achieved at all. In addition, the tendency of polyhydroxylated phenols and CP to undergo oxidative coupling in aerobic environments to form humic-like polymer structures which are highly recalcitrant limits the applicability of aerobic biodegradation (Field *et al.*, 1995; Sahm *et al.*, 1986).

Reductive dechlorination does not demand aerobiosis, however, it is generally an incomplete biodegradative process. Halogenated compounds are not necessarily mineralized but merely transformed through successive steps to lesser-chlorinated and non-chlorinated compounds, therefore, a secondary treatment step is required to facilitate elimination of dechlorinated metabolites. Moreover, compound solubility and availability may be enhanced with reduced chlorine numbers which makes a subsequent treatment step conducive to reductive dechlorination.

1.5 The Bioprocess Environment

It is against a background of the above-mentioned potential that the development of an integrated anaerobic/aerobic bioprocess environment for the remediation of complex hydrocarbon mixtures, including CP-contaminated wastes, was proposed. Applications of such an approach in soil, sediment, aquifers and industrial effluents could be considered. It was suggested that in the case of *in-situ* treatment, enhanced biodegradation via reductive dechlorination supplementing the below-ground arena

could be undertaken with the addition of nutrients (easily fermentable carbon and electron source(s)), sulphate and, if necessary, augmentation with a laboratory acclimated microbial population. Where sulphate concentrations are low, or become insufficient to sustain sulphidogenesis due to accelerated sulphate reduction, the contaminated zone could be supplemented with sulphate to support a continuous sulphate-reducing environment.

Following an initial dechlorination step, more mobile dechlorinated metabolites would be desorbed and removed by pumping to an above ground treatment operation involving an immobilized packed-bed reactor utilising aerobic white-rot fungal activity. Those remaining dechlorinated recalcitrant compounds not amenable to anaerobic metabolism could then be subjected to complete mineralization. A fungal/bacterial consortium should establish as an active mechanism of hydrocarbon degradation as has been observed by Boonchan *et al.* (2000). When acceptable levels of degradation have occurred, the treated water would be returned to the subterranean environment, if required, and could be supplemented with nutrients to stimulate further reductive dechlorination. This model is based on the traditional pump-and-treat and re-injection technology for groundwater remediation. However, in this case treatment would commence *in-situ* and then be concluded above ground, whereas with the pump-and-treat methodology, groundwater processing starts in an *ex-situ* facility. Figure 1.1 shows a schematic representation of the treatment strategy for contaminated soil/groundwater that has been proposed as a model in the study reported here.

The potential benefits of the integrated anaerobic/aerobic strategy would include the following:

- Enhanced CP desorption and mobilization due to transformation by sulphidogenesis-dependent dechlororespiration in anoxic subsurface environments;
- The highly reactive, non-specific degradation capacity and robustness of immobilized white-rot fungal strains are able to rapidly metabolize a variety of organics including CP and complex hydrocarbon mixtures in effluents;

- Complete mineralization by white-rot fungal metabolism of CP and metabolites produced during dechlororespiration;
- Bioremediation is a cost-effective and viable alternative to physical or chemical remediation technologies that often do not detoxify pollutants. *In-situ* enhanced biodegradation by employing reductive dechlorination in contaminated anoxic soil or sediment terrains would provide direct cost benefits in the application of remediation strategies. In this case no air introduction into the subsurface environment is needed.

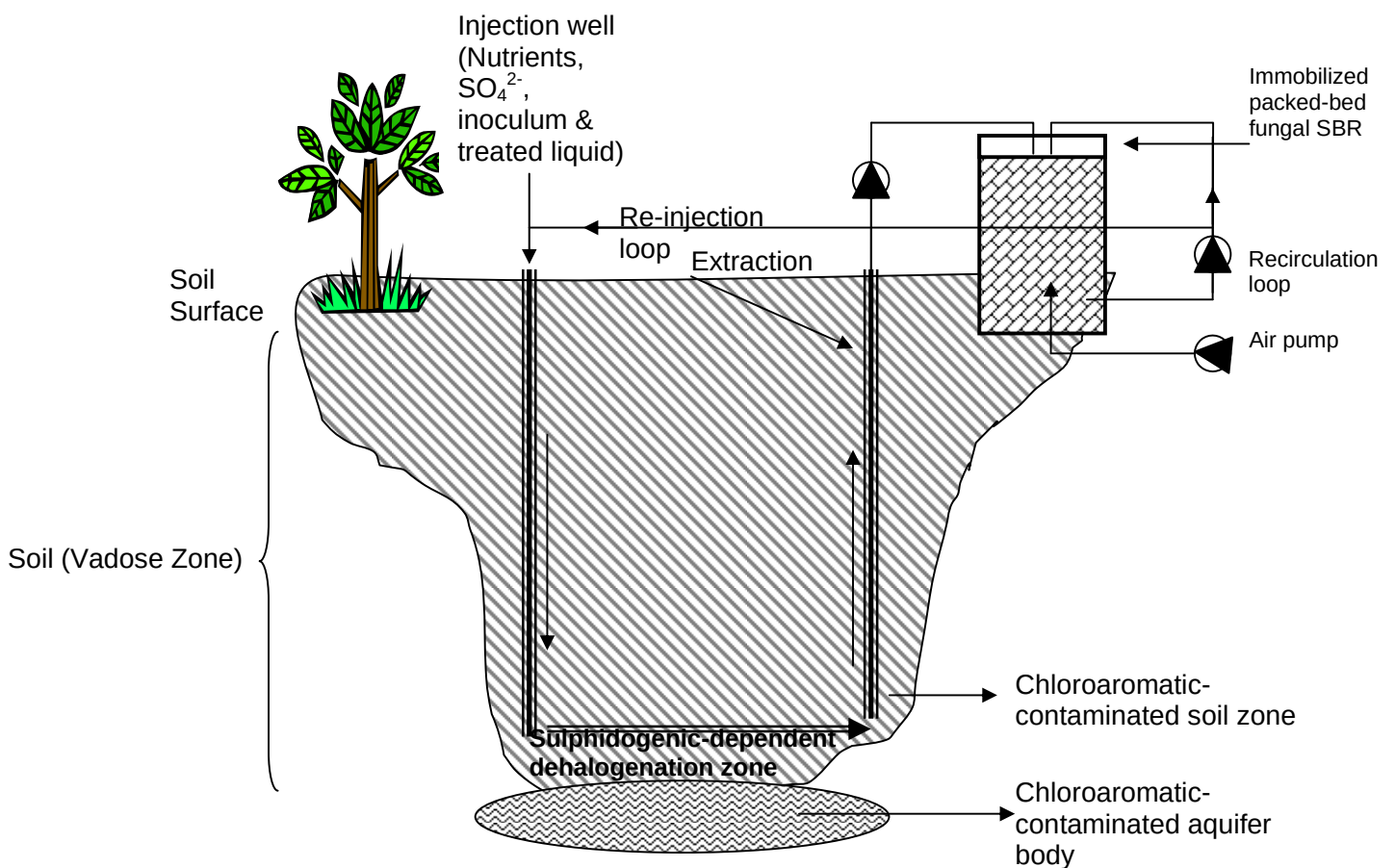


Figure 1.1 Diagrammatic representation of the proposed soil and groundwater biodegradation strategy.

Dual-stage processes have been previously reported in the bioremediation of CP contamination (Armenante *et al.*, 1992; Atuanya *et al.*, 2000; Kafkewitz *et al.*, 1992; Shin *et al.*, 1999; Zitomer and Speece, 1993). These have largely focussed on combinations

of bacterial systems involving anaerobic (methanogenic dechlorination) and aerobic mineralization activities. These have also largely been pump-and-treat operations with bioprocess functions located above ground. Subsurface *in-situ* treatments involving either aerobic or anaerobic bacterial processes have required the introduction of both an energy and carbon source and oxygen, and where strongly adsorbed compounds are involved, addition of surfactants has been considered to achieve removal (Eweis *et al.*, 1998; Lee *et al.*, 1998; Sadowsky and Turco, 1999; Yeh *et al.*, 1999).

Residual metabolites formed during the non-specific breakdown of halogenated pollutants such as polychlorinated biphenyls (PCB), polychlorinated dibenzodioxins (PCDD), polychlorinated dibenzofurans (PCDF) and organochlorine pesticides such as PCP, benzene hexachloride, chlordane, heptachlor and dichlorodiphenyltrichloroethane (DDT) are known carcinogens and endocrine disrupters (Lamb, 1996; Smith and Gangolli, 2002). Endocrine disrupters are chemicals that can adversely affect the male and female reproductive systems by exhibiting estrogenic or anti-androgenic activities (Shinomiya and Shinomiya, 2003; Smith and Gangolli, 2002). These metabolites include chemicals such as 1-chloro-3,4-dihydroxybenzene, a metabolite produced during the degradation of 2,7-dichlorodibenzo-*p*-dioxin (2,7-DCDD) (Sato *et al.*, 2002), hydroxy-dichlorodibenzodioxin and hydroxy-dichlorodibenzofuran from 2,7-DCDD and 2,8-dichlorodibenzofuran, respectively (Mori and Kondo, 2002), refractory PCB congeners from commercial PCB mixtures (Kubatova *et al.*, 2001) and pentachloroanisole, tetrachloroanisole and tetrachlorophenol from PCP degradation (Okeke *et al.*, 1997).

The potential of sulphate reducing bacterial systems in the dechlorination of chlorinated compounds involving dechlororespiration activity has been described (Drzyzga *et al.*, 2001; Drzyzga and Gottschal, 2002) but little is known about the mechanisms involved and the potential applications. The possibility that such activity may be associated with CP desorption and mobilization has also been suggested (Chang *et al.*, 1996; Pavlostathis *et al.*, 2001). However, no reports have been found by this author investigating applications of the *in-situ* dechlorination and desorption activity of bacterial sulphidogenesis used in the release and recovery of CP contaminants from soil bioremediation applications. Likewise, the potential advantages of the recovery of fully dechlorinated compounds rather than potentially toxic chlorinated intermediates have also apparently not been reported.

The white-rot fungal extracellular free-radical enzyme generating mechanism has been shown to be a highly reactive and non-selective mineralization process and the ability of these fungi to mineralize CP has been extensively studied (Armenante *et al.*, 1994; Hammel, 1989, Hammel and Tardone, 1988; Joshi and Gold, 1993; Lewandowski *et al.*, 1990; Milseki *et al.*, 1988; Pallerla and Chambers, 1998; Pérez *et al.*, 1997; Reddy *et al.*, 1998; Valli and Gold, 1991). However, the potential in the linkage of mineralization of dechlorinated products from sulphidogenic-dependent dechlororespiration has not been reported. Also, the linkage of the processes in a subsurface desorption and recovery operation with a final surface located fungal mineralization operation has also apparently not been previously considered.

Nearly the equivalent approach could be followed in the treatment of industrial effluents containing organochlorine chemicals and hydrocarbon mixtures. Here, both aerobic and anaerobic reactors could be managed in separate or linked bioreactor units and operated as above ground conventional water treatment operations. Such an integrated bioreactor approach could be applied to industrial wastewaters containing the above-mentioned pollutants when basidiomycetous fungal degradation might produce residual metabolites with potentially toxic characteristics such as carcinogens and endocrine disrupters. In that case, applying the anaerobic reactor as a primary treatment stage to reduce chlorine numbers will facilitate the mineralization of the dechlorination metabolites without the production of potentially hazardous intermediate compounds in the aerobic reactor. Figure 1.2 shows a schematic representation of the anaerobic/aerobic bioreactor configuration.

1.5.1 Research Hypothesis

The potential available to bioremediation in the linkage of the sulphidogenic and fungal systems led to the hypothesis on which the study reported here has been based:

Effective and complete degradation of chlorinated aromatic compounds and complex hydrocarbon mixtures may be accomplished in a dual-stage anaerobic/aerobic remediation strategy targeting dehalogenation and

mobilization of pollutants under sulphate-reducing conditions in the first stage and then mineralization of the resulting products using white-rot fungal enzyme activity in the second.

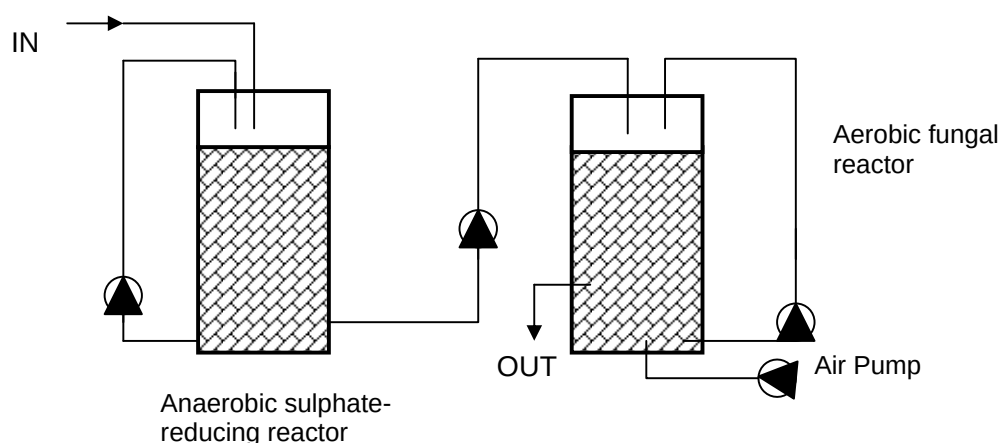


Figure 1.2 Diagrammatic representation of the proposed integrated bioreactor configuration for the treatment of organochlorine-contaminated industrial waste streams.

1.5.2 Research Objectives

- To test the proposal that adsorbed CP may be selectively desorbed in the presence of dechlororespiration activity and that the chlorine numbers may be reduced in that process;
- To investigate the immobilization of white-rot fungi and their action in phenol and CP degradation and mineralization;
- To link a sulphate-reducing dechlororespiration process with immobilized white-rot fungal mineralization activity in dual-stage reactor configuration and follow the biodegradation of CP dechlorinated metabolites and hydrocarbon mixtures;
- To consider the scale-up implications of the proposed processes in the treatment of a real hydrocarbon waste stream.

Chapter Two

Immobilized White-Rot Fungal Mineralization of Phenol and Chlorinated Phenol Compounds

2. Introduction

Biofilm reactors that maintain high biomass concentrations have a specific disadvantage in the uneven biomass development caused by concentration gradients in the direction of flow and leading to stratified microbial growth (Kaballo *et al.*, 1995). Reactor systems must handle variations in load and operational parameters such as pH, oxygen concentration and temperature. Increasing the load concentration in a biofilm reactor with uneven biomass distribution may channel the substrate into areas of the reactor where few or no microorganisms are present. Other restrictions of biofilm reactor configurations include oxygen mass transfer limitation and clogging of the packed-bed by excessive microbial growth at the reactor inlet (King *et al.*, 1998). By employing the SBR operational mode, homogenous biomass distribution throughout the reactor can be obtained and greater tolerance to fluctuating influent concentrations may be achieved (Lewandowski, 1990).

The potential use and application of SBR operation for fungal immobilization has not been extensively studied. The advantages of SBR operation involving highly effective white-rot fungal-mediated metabolism have motivated the examination of a combined strategy for complex hydrocarbon effluent remediation.

The aim of this study was to immobilize four white-rot fungal cultures in trickling packed-bed reactors employing inert (foam glass beads) and organic (pinewood chips) support material and operating the reactors in SBR mode. The application of the bioreactor in degradation of phenol and CP-contaminated liquid was investigated including an assessment of lignolytic white-rot fungal-mediated mineralization as the aerobic stage of the proposed integrated anaerobic/aerobic biodegradation process configuration.

2.2 Materials and Methods

2.2.1 Microorganisms, Culture Conditions and Inoculum Preparation

Phanerochaete chrysosporium BKMF-1767 (ATCC 24725), *Trametes versicolor* (PPRI 3845) and *Lentinula edodes* (PPRI 5769) obtained from the Plant Protection Research Institute, Pretoria; and *Agaricus bisporus* provided by Dr Winston Leukes, Department of Biochemistry, Microbiology and Biotechnology, Rhodes University, were maintained on potato dextrose agar (PDA) plates at 28 °C and routinely inoculated onto fresh PDA every 30 – 60 d.

Four to five 10 mm diameter mycelium agar plugs from the margins of actively growing plate cultures were used as the inoculum for batch fermentations. Growth medium adapted from Rigas *et al.* (2001) was used (see Appendix A). Each fungal strain was inoculated into separate cotton wool plugged 500 ml conical flasks and incubated at 28 °C on a rotary shaker (174 rpm) for up to 5 days and until the medium was sufficiently colonized as determined by visual inspection. The mycelial suspensions were combined and homogenized aseptically for 10 s at low speed in a Waring blender and a 10 % (v/v) blended mycelial inoculum was used for both the reactor and flask studies.

2.2.2 Trickling Packed-Bed Reactors

Two cylindrical trickling packed-bed reactors (schematic diagram presented in Figure 2.1) were constructed with Perspex (6 300 cm³, reactor volume; 10 cm, ID). Reactor One was packed with untreated pinewood (*Pinus* sp.) chips (3-7 cm) and Reactor Two with foam glass beads (PoraverTM; 3.35 – 4.75 mm, diameter; 20 – 1 000 µm, pore size; Alves *et al.*, 1999) to a height of 65 cm (80 % reactor volume). Figure 2.2 shows the pine chip-packed reactor operated in a fume hood in the laboratory.

The carriers were washed three times with distilled water before being randomly packed into the reactors. Humidified air was introduced in the bottom of the reactor through an in-line membrane filter (pore size 0.2 µm) at 1.2 L.min⁻¹. Perforated Perspex beams were placed at the bottom and top of the reactors (5.5 cm from both ends) to suspend and

keep the packing materials intact and separate from the air sparger and to act as a distribution mechanism for the influent feed at the top.

Influent feed consisting of growth medium and mycelial suspension (500 ml) was introduced at constant flow rate ($10.5 \text{ ml} \cdot \text{min}^{-1}$) into the reactors using a Watson-Marlow peristaltic pump (Model 505S). The reactor was provided with an external bottom-to-top recirculation loop (flow rate $5.6 \text{ ml} \cdot \text{min}^{-1}$). Continuous recycling at constant flow was operated for 5 d to facilitate attachment of fungi to carriers. The reactors were operated in the dark at room temperature (min 19°C max 25°C).

The packed volume was $4\,673 \text{ cm}^3$. Void volumes for the pine- and foam glass-packed reactors were $3\,540 \text{ cm}^3$ and $2\,450 \text{ cm}^3$ respectively, and was determined by measuring the total volume of poured liquid from the total reactor column volume after fungal attachment. The void volume, therefore, represents empty (below beam and above packing) plus wood chip packed volume. The reactors were operated in sequencing batch format with feed introduced at the start of a new batch cycle to 20 % of void volume-level (RV, recirculation volume; 15 cm, pine; 12.5 cm, foam glass), thereafter, immediately commencing with recirculation. 15 % and 12 % of packed volume was submerged in Reactor One and Two, respectively. Following a cycle (24 – 30 h), recirculation was stopped and a period was allowed for settling whereafter the liquid was manually discharged via D located $\frac{1}{2}$ -way between the bottom of the reactor and the Perspex beam (Figure 2.1). Hydraulic retention times (HRT) of 126.4 min (pine) and 87.5 min (foam glass) (2.1 and $1.4 \text{ volume} \cdot \text{h}^{-1}$) was calculated. The pH was not controlled during the operation of the reactor.

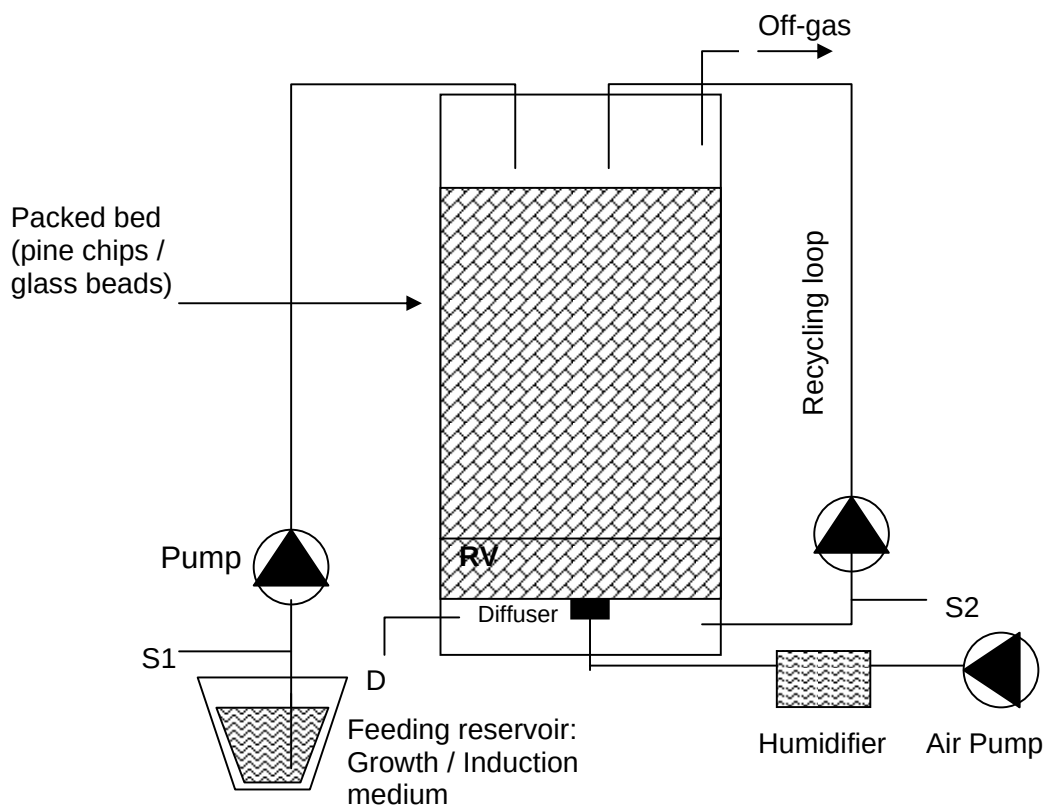


Figure 2.1 Schematic diagram of the trickling packed-bed reactor. S1&2, Sampling points 1&2; D, Drain; RV, recirculation volume.

2.2.3 Reactor Feed

During repeated batch treatments, alternating phases of growth and induction media were administered. Lewandowski *et al.* (1990) reported on a two-phase growth/induction cycle and claimed no fungal growth was expected during the enzyme induction phase which they regarded as a steady-state condition in a plug-flow packed bed reactor. After the 5 d period allowing growth and attachment for steady-state conditions to be reached, the induction medium was introduced. Induction medium was replaced with growth medium after every 5 batch cycles and allowed to operate for a single batch. The cycle was repeated with higher phenol and TCP concentration loads. The induction medium (Appendix A) for the pine-packed reactor was spiked with phenol and 2,4,6-TCP (2.15 g phenol.L⁻¹; 225 mg TCP.L⁻¹) from filter-sterilized stock solutions (0.22 µm pore size cellulose-acetate filters, Osmonics). No glucose was added to the induction medium

since cellulose and hemicellulose were available as carbon source. The nitrogen source was also excluded in order to create appropriate conditions (nitrogen starvation) for ligninolytic enzyme secretion according to Lewandowski *et al.* (1990).



Figure 2.2 The pinewood chip-packed reactor operated in the laboratory.

The foam glass bead-packed reactor received an identical induction feed, however, it contained glucose in reduced amounts (Appendix A). This was done to sustain fungal activity because of the absence of available carbon substrate.

Multiple runs were operated in sequencing batch mode. Samples were collected from the feed inlet and outlet (S1 and S2, Figure 2.1) at specific time intervals and quantified for phenol and 2,4,6-TCP content (results report the means of triplicate samples $\pm$ standard deviations), as well as pH and assayed for extracellular enzyme activity. To determine phenol and TCP stripped due to aeration, the off-gas was passed through three water absorption flask gas traps connected in-series. At specific time intervals, the water in the gas traps was changed and samples were analysed.

To assess the role that adsorption by the packing materials plays 500 ml conical flasks were set-up containing distilled water (250ml), pine chips / glass beads and spiked with

phenol and 2,4,6-TCP from a filter sterilized stock solution (850 mg.L⁻¹, phenol; 200 mg.L⁻¹, TCP) on a rotary shaker set at 165 rpm. Samples were collected every 5 min for 20 min to determine the role adsorption plays on compound removal by the pine or foam glass beads. The study was carried out in triplicate for each carrier.

2.2.4 Role of Uptake and Degradation by Fungal Biomass

For the determination of phenol and 2,4,6-TCP uptake by mycelia, batch cultures were set up in stationary 500 ml conical flasks containing pinewood chips (38 – 43 g) and glass beads (38 – 41 g). Growth medium was inoculated with blended mycelia suspension (10% v/v). The flasks were sealed with Teflon-coated butyl rubber stoppers and aerated (0.75 L.min⁻¹) with humidified air provided by gas-permeable silicone tubing fitted with spargers.

The packing materials were only partially immersed in the liquid medium. The cultures were incubated at room temperature in the dark for up to 5 d until the surface of the pinewood and glass beads appeared to be uniformly colonized. A stock solution of phenol (1.45 g.L⁻¹) and 2,4,6-TCP (225 mg.L⁻¹) was aseptically added to obtain final concentrations of 280 mg.L⁻¹ (phenol) and 45 mg.L⁻¹ (TCP). Samples were collected immediately to determine the exact total feed concentration. After 24 h the phenol and TCP in the liquid phase and biomass phase were quantified. Gas traps were constructed to compensate for air losses.

Packing materials were removed, washed and vortexed in distilled water to remove immobilized mycelia. The culture liquid phases were centrifuged at 6 000 rpm x g for 20 min (Beckman Centrifuge Model J2-21) followed by resuspending the pellets in distilled water and combined with biomass removed from packing materials. This was homogenized in a Waring blender (20 s at low speed) and then centrifuged. The supernatant from the culture liquid phases and biomass phases were analysed for phenol content. A control experiment was done with deactivated mycelia using 4 % (v/v) formaldehyde added 24 h before cultures were supplemented with phenol and TCP. Formaldehyde at 4% (v/v) has been shown to deactivate enzyme secretion in white-rot fungal cultures (W. Leukes, pers. comm.). The results of triplicate studies are reported as means ± standard deviation values.

2.2.5 Analytical Methods

2.2.5.1 Phenol and 2,4,6-TCP

Liquid-liquid phase extractions of samples were performed and the concentration of phenol and TCP were determined by High Pressure Liquid Chromatography (HPLC) (Appendix B).

2.2.5.2 pH

pH of reactor effluent was measured using a Cyberscan 2000 pH meter.

2.2.6 Enzyme Assays

Activities of LiP, MnP and laccase were assayed spectrophotometrically in fluid collected from the reactor outlets. All enzyme activities are expressed in units.L⁻¹ (U.L⁻¹), with one unit equal to 1 μ mol substrate oxidized.min⁻¹.

LiP activity was determined by measuring the oxidation of veratryl alcohol by H₂O₂ to veratraldehyde according to Tien and Kirk (1988). A Shimadzu UV 160-A spectrophotometer fitted with a time scan set at 310 nm was used. Time zero was at the addition of the sample and absorbance readings were taken every 10 s for 300 s. The molar extinction coefficient for the substrate is 9 300 M⁻¹.cm⁻¹. Reagent mixtures contained 2 mM veratryl alcohol (K_m = 60 μ M), 0.4 mM H₂O₂ (K_m = 80 μ M), 50 mM *d*-tartaric acid (pH 2.5) and sample (300 μ l).

One unit MnP activity is defined as the amount of enzyme needed to oxidize 1 μ mol of Mn (II) to Mn (III) in 1 min measured at A₅₉₀ every 10 s for 300 s (Del Pilar Castillo *et al.*, 1994). The reaction of 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 3-(dimethylamino)benzoic acid (DMAB) in the presence of Mn (II) and MnP gives a deep-purple colour with a peak at 590 nm. The extinction coefficient is high (53 000 M⁻¹.cm⁻¹) so low MnP activities can be detected. The reaction mixture contained 0.07 mM MBTH, 0.99 mM DMAB, 0.3 mM MnSO₄, 0.05 mM H₂O₂, 100 mM succinic-lactic acid buffer (pH 4.5) and sample (300 μ l).

Since laccase can also utilize MBTH and DMAB as substrates and to correct for possible laccase interference the H_2O_2 and MnSO_4 were omitted and 0.3 mM EDTA was added to the reaction mixture. MnP activity was determined by subtracting the activity from the total activity.

Laccase activity was determined as described by Niku-Paavola *et al.* (1988) with 2,2'-azinobis(3-ethylbenzathiazoline-6-sulfonic acid) (ABTS) as substrate. The laccase reaction mixture contained 0.1 M lactic acid - 0.1 M sodium acetate buffer (pH 5), 5 mM ABTS and 170 μl sample. The reaction was monitored by measuring the change in A_{436} for 300 s. The extinction coefficient of $29\,300\text{ M}^{-1}\cdot\text{cm}^{-1}$ was used for oxidized ABTS.

2.2.7 Electron Microscopy

Pine chips and glass beads were collected from different heights in the reactors during stages of operation and prepared for scanning and transmission (wood chips only) electron microscopy investigation (Appendix C).

2.3 Results

2.3.1 Role of Adsorption by Pine Chips and Foam Glass Beads

Figures 2.3 and 2.4 show phenol and 2,4,6-TCP in the presence of the packing materials without mycelia to determine the role adsorption plays. Uptake by pine chips (Figure 2.3) and glass beads (Figure 2.4) seem to be negligible for both phenol and TCP, hence microcarrier-mediated adsorption was taken as insignificant and disregarded in subsequent calculations.

2.3.2 Degradation of Phenol and 2,4,6-TCP

The results (Figures 2.5 and 2.6) show mineralization of phenol and 2,4,6-TCP in both the pinewood- and foam glass-packed reactors. Phenol and TCP were introduced in elevated concentrations into the reactors and removal monitored over 24 – 30 h. Samples were collected at each HRT while effluent was continuously recycled (single

batch). At introductory influent concentrations of 430 mg.L⁻¹ (phenol) and 43 mg.L⁻¹ (2,4,6-TCP), complete removal was observed over 24 h for Reactor Two (glass packing) , whereas 99.8 % phenol removal in the pinewood reactor was shown over 30 h with 89.9 % TCP removed in the same period.

Only one flow rate was used during the operation of the reactors and, therefore, first-order rate constants (h⁻¹) were calculated for the two reactors and two influent concentrations (Table 2.1). The rate constants were determined by calculating the natural logarithm for concentration in (Co) minus concentration out (C) for every HRT in a batch cycle. For a particular HRT, Co was regarded as C from the previous HRT and its concentration out was Co for the next HRT and so forth.

Table 2.1. First-rate order constants (h⁻¹) and average biodegradation rates.HRT⁻¹ (mg.L⁻¹.HRT⁻¹) for both reactors with specific feed concentration (mg.L⁻¹) and retention time (h)

		Feed (mg.L ⁻¹)	HRT (h)	First-order constant (h ⁻¹) / R ² ^a	Average degradation.HRT ⁻¹ ^b (mg.L ⁻¹ .HRT ⁻¹)	% (3 HRT) ^c	% (24- 30h) ^d
Pinewood reactor (Reactor One)	Phenol	430	2.1	0.2 / 0.7	91.5	63.8	99.8
	TCP	43	2.1	0.3 / 0.8	8.9	62.1	89.9
	Phenol	800	2.1	0.8 / 0.9	255.0	96.1	99.1
	TCP	85	2.1	0.6 / 0.8	25.6	91.9	98.1
Foam Glass reactor (Reactor Two)	Phenol	430	1.45	0.2 / 0.8	53.2	37.1	100
	TCP	43	1.45	0.5 / 0.6	8.5	59.7	100
	Phenol	800	1.45	0.6 / 0.9	243.9	91.9	99.4
	TCP	85	1.45	0.7 / 0.7	26.1	93.7	100

^aLinear correlation coefficient.

^bAverage biodegradation rate.HRT⁻¹ calculated for initial 3 HRT shown to be optimum at steady-state.

^cPercentage degraded over first 3 HRT of new batch treatment.

^dPercentage degraded over total batch recycle period (24-30 h).

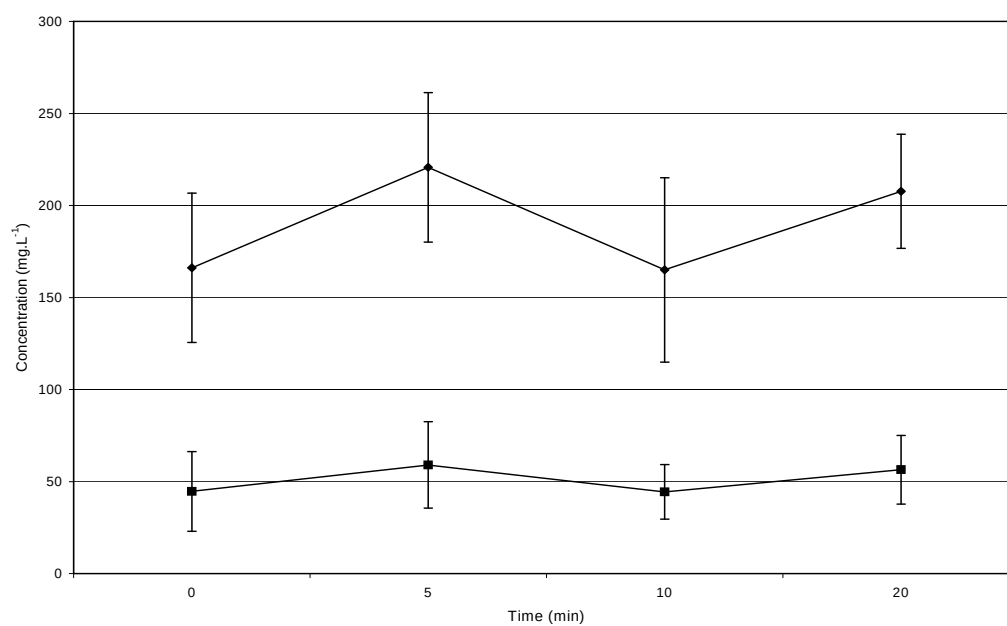


Figure 2.3 Phenol (♦) and 2,4,6-TCP (■) (mg.L⁻¹) in the presence of the pine chips without fungal inoculum to test uptake by the carrier.

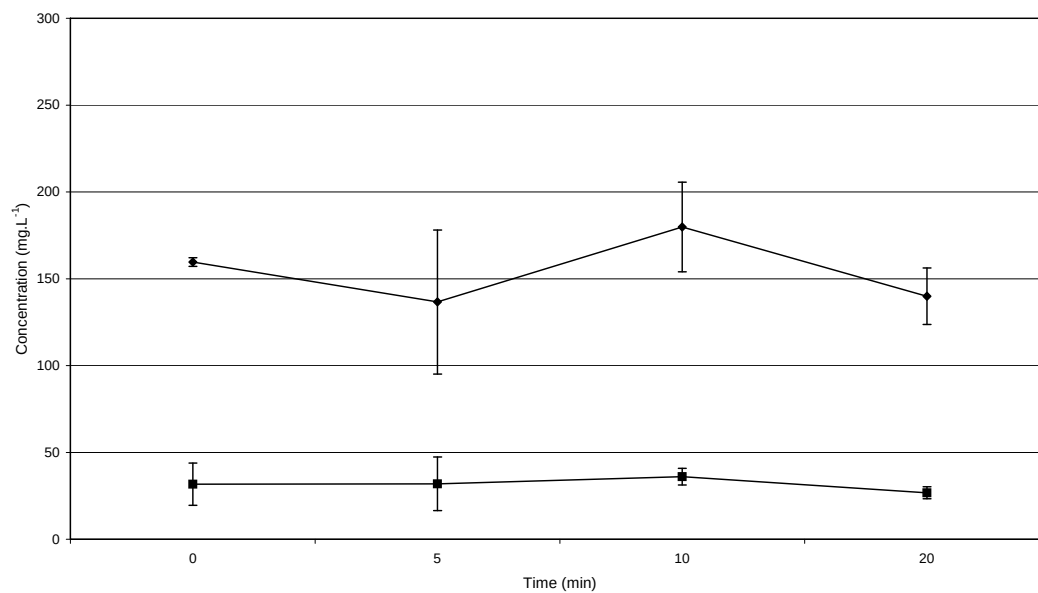


Figure 2.4 Phenol (♦) and 2,4,6-TCP (■) (mg.L⁻¹) in the presence of the glass beads without fungal inoculum to test uptake by the carrier.

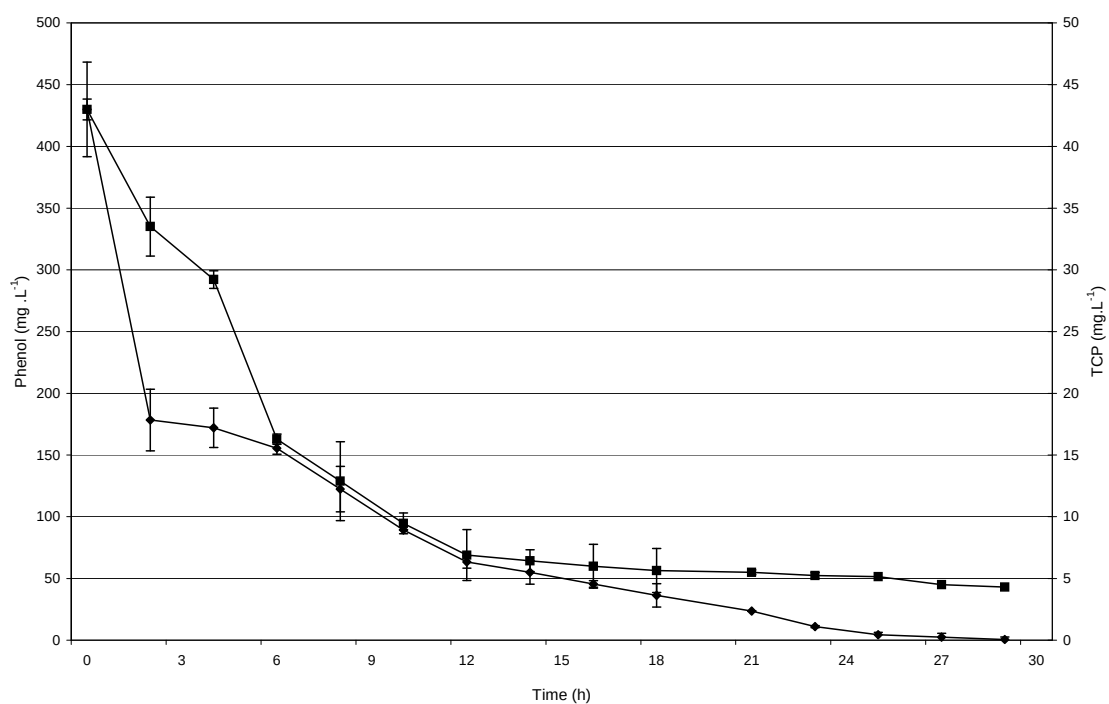


Figure 2.5 Phenol (♦) and 2,4,6-TCP (■) (mg.L⁻¹) removal in pinewood-packed reactor with feed concentrations 430 mg.L⁻¹ and 43 mg.L⁻¹ respectively for a single batch run.

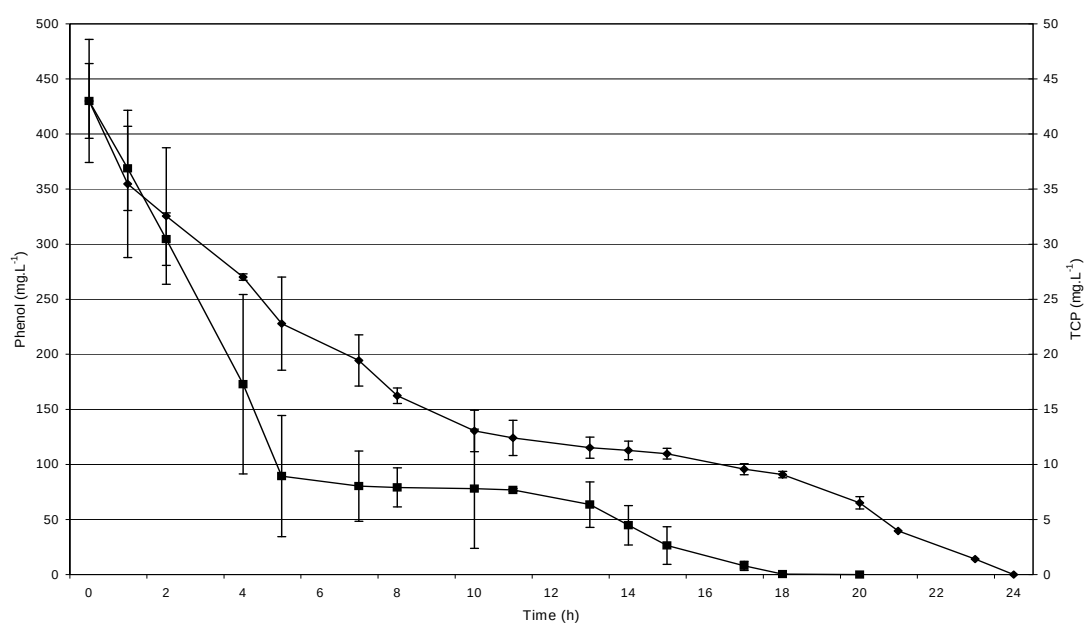


Figure 2.6 Phenol (♦) and 2,4,6-TCP (■) (mg.L⁻¹) removal in foam glass bead-packed reactor with feed concentrations 430 mg.L⁻¹ and 43 mg.L⁻¹ respectively for a single batch run.

By plotting $\ln(C_0 - C)$ vs. time (h) for a batch cycle it was possible to compute a first-order constant by finding the slope of the linear fit for a given reactor and introductory phenol and TCP concentrations. R^2 was used as an accuracy indicator assessing linearity fit trend. This parameter can be related to degradation efficiency by comparing h^{-1} values for the two influent concentrations for each compound and pine chip-packed reactor vs. foam glass beads-packed reactor.

First-order rate constants for Reactor One influent phenol (430 mg.L^{-1}) and TCP (43 mg.L^{-1}) were 0.2 and 0.3 h^{-1} , respectively (Table 2.1). The constants increased to 0.8 and 0.6 h^{-1} for higher compound loads which displays enhanced catalytic efficiency over time after an initial five batch cycle acclimation period. Phenol and TCP did not inhibit fungal metabolism at elevated concentrations investigated.

$> 99 \%$ phenol and 98.1% TCP was eliminated from Reactor One over 24 h, whereas complete TCP plus 99.4% phenol was removed over the same period for Reactor Two (Figures 2.7 and 2.8).

To monitor phenol and TCP removal by stripping, gas traps were connected to collect off-gas from the reactors. Samples were collected and water replenished in the traps after the first HRT, 6, 12 and 24 h. Figure 2.9 shows phenol and TCP concentrations detected in the traps over 24 h. 10.6% and 7.3% of the 800 mg.L^{-1} and 85 mg.L^{-1} phenol and TCP feed were detected in the gas traps for a 24 h period in single batch cycle for Reactor One. 8.5 and 5.2% (phenol and TCP) were measured after the first 2.1 h (one HRT) in the traps. With 383.4 mg.L^{-1} residual phenol and 23.1 mg.L^{-1} TCP detected in the reactor at this time, 51.8% and 72.2% (43.3 and 66.9% if volatilized fraction is considered) of feed concentration was degraded. Loss due to stripping, therefore, had a marginal effect on compound removal relative to biodegradation. A similar trend was observed for Reactor Two.

Comparing periods of optimum catabolism between the introductory and elevated feed concentrations, it was shown that increased percentage degradation occurred at almost double the initial feed during the first three HRT which seemed to be the maximum compound removal period at steady-state conditions. Steady-state was assumed since no fungal growth was expected during the induction feeds and mycelia present in the

reactors was essentially constant during these batch cycles. Lewandowski *et al.* (1990) and Pallerla and Chambers (1998) used similar criteria to assume steady-state conditions in immobilized and plug-flow packed-bed fungal reactors. Moreover, calculating average biodegradation rate. HRT^{-1} over the first three HRT, 63.8 % phenol (feed 430 mg.L^{-1}) was removed compared to 96.1 % (feed 800 mg.L^{-1}) in Reactor One (Table 2.1). The degradation rates were 91.5 and $255.0 \text{ mg.L}^{-1}.\text{HRT}^{-1}$, respectively. Similarly, at feed concentration 43 mg.L^{-1} , TCP was reduced to 16.2 mg.L^{-1} and 6.6 mg.L^{-1} (feed 85 mg.L^{-1}).

Average phenol biodegradation rates. HRT^{-1} calculated for Reactor Two over the first 3 HRT increased from $53.2 \text{ mg.L}^{-1}.\text{HRT}^{-1}$ (feed 430 mg.L^{-1}) to $243.9 \text{ mg.L}^{-1}.\text{HRT}^{-1}$ (feed 800 mg.L^{-1}). Average TCP removal rate was $8.5 \text{ mg.L}^{-1}.\text{HRT}^{-1}$ at introductory feed concentration which increased to $26.1 \text{ mg.L}^{-1}.\text{HRT}^{-1}$ (feed 85 mg.L^{-1}) (Table 2.1). First-order rate constants for introductory vs. higher compound concentrations showed a similar trend for Reactor Two (Table 2.1).

Catalytic efficiencies for Reactors One and Two compared favourably with phenol and 2,4,6-TCP degradation shown over 24 – 30 h for both and for multiple batch cycles, however, TCP degradation efficiency (h^{-1}) was better for the glass-packed reactor for both feed concentrations (0.5 and 0.7 h^{-1}) compared to 0.3 and 0.6 h^{-1} for the pine chip reactor. Pine chip-immobilized fungi showed a higher affinity for phenol as can be derived from the first-order constants.

Outlet pH for Reactor One and Two varied between 3.3 and 5.8 and 4.4 and 6.9, respectively which is a favourable environment for MnP and LiP production (Domínguez *et al.*, 2001), and minimized contamination of the reactors during multiple batch cycle runs.

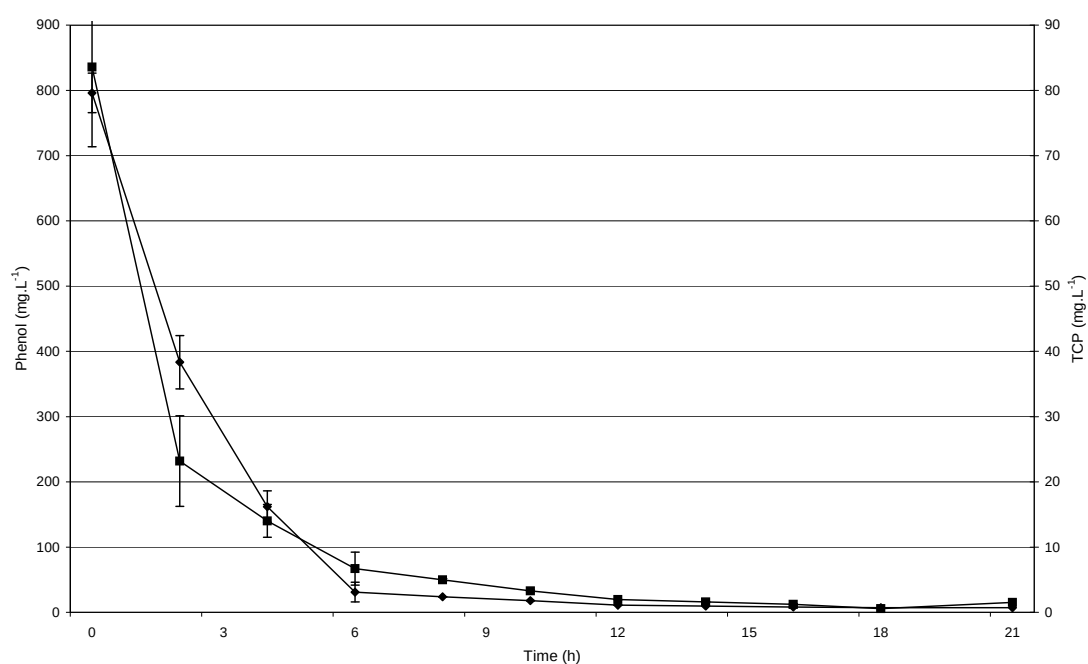


Figure 2.7 Phenol (♦) and 2,4,6-TCP (■) (mg.L⁻¹) removal in pinewood-packed reactor with feed concentrations 800 mg.L⁻¹ and 85 mg.L⁻¹ respectively for a single batch run.

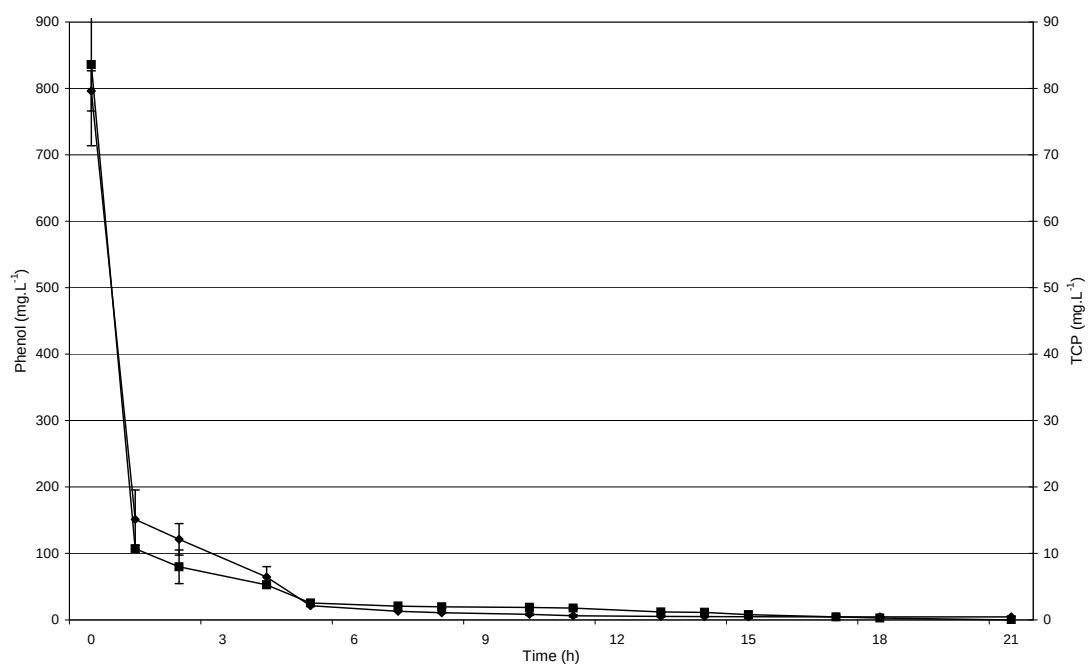


Figure 2.8 Phenol (♦) and 2,4,6-TCP (■) (mg.L⁻¹) removal in foam glass bead-packed reactor with feed concentrations 800 mg.L⁻¹ and 85 mg.L⁻¹ respectively for a single batch run.

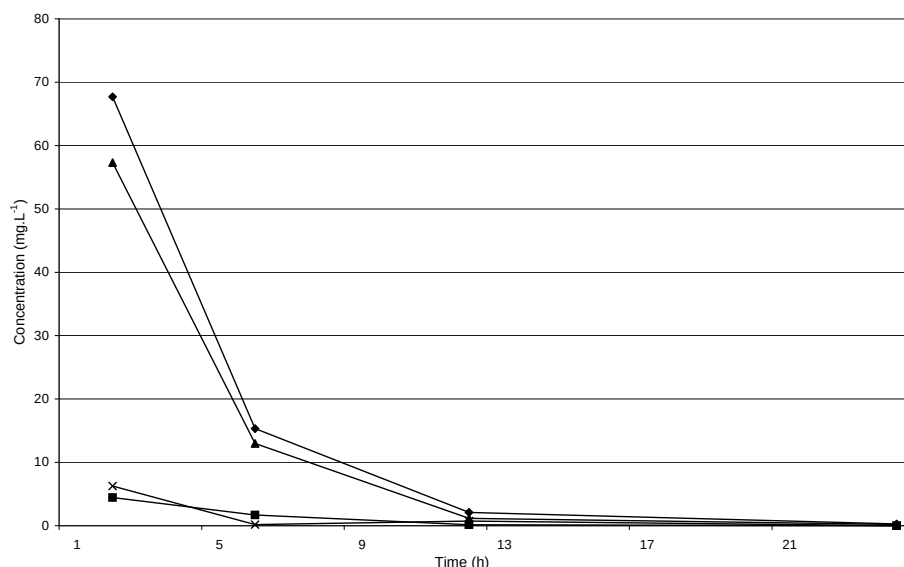


Figure 2.9 Phenol and TCP detected in gas traps over 24 h in a batch cycle with 800 mg.L⁻¹ and 85 mg.L⁻¹ feeds for Reactor One: phenol (♦) and 2,4,6-TCP (■); and Reactor Two: phenol (▲) and 2,4,6-TCP (x).

2.3.3 Role of Fungal Biomass in Phenol and 2,4,6-TCP Uptake and Removal

Static batch flask experiments were constructed to test the uptake of phenol and TCP by the fungal biomass over 24 h. A control experiment containing deactivated mycelia was also monitored for uptake and immobilization. Tables 2.2 and 2.4 present uptake and degradation and Tables 2.3 and 2.5 mass balances calculated for pine chip- and foam glass-immobilized and free, live and deactivated mycelia. The data shows 28.1 and 24.7 % of phenol degraded by live fungi (volatilized fraction taken into account) over 24 h in pine- and glass-packed flasks, respectively. 99.4 and 96.9 % phenol was retained in deactivated fungal flasks. 37.3 % of the TCP feed (42.9 mg.L⁻¹) was taken up by dead biomass compared to 10.0 % retained by live biomass after 24 h (Table 2.2).

84.9 % TCP was retained in the liquid and biomass phases of deactivated foam glass-immobilized cultures, with 25 % (feed 51.1 mg.L⁻¹) taken up by the mycelia; and 51.5 % residual present in the active culture with 4.2 % retained in the biomass after 24 h (Table 2.4). This shows that the compounds were not simply taken up and retained by the biomass but live mycelia was able to metabolize the adsorbed phenol and TCP fractions.

Table 2.2. Uptake by live vs. deactivated biomass (mg.L⁻¹) in pine chip-packed flasks after 24 h

Phenol (mg.L ⁻¹)							
Live mycelia				Deactivated mycelia ^b			
Feed ^a	Liquid phase ^a	Biomass phase ^a	Total ^a	Feed ^a	Liquid phase ^a	Biomass phase ^a	Total ^a
286.1 ± 31.2	176.6 ± 33.7	7.9 ± 1.5	184.5	278.3 ± 50.7	200.5 ± 53.9	57.8 ± 2.8	258.3
% ^c	61.7	2.7			72	20.7	
2,4,6-TCP (mg.L ⁻¹)							
Feed ^a				Feed ^a			
47.6 ± 12.7	20.2 ± 1.5	4.8 ± 0.1	25.0	42.9 ± 7.8	21.4 ± 3.0	15.9 ± 0.9	37.3
% ^c	42.4	10.0			49.8	37.0	

^aConcentration per mean triplicate flasks ± standard deviation.

^bDeactivation of mycelia using 4 % (v/v) formaldehyde.

^c% of feed concentration.

Table 2.3. Stripped phenol and 2,4,6-TCP fraction captured in traps and total concentrations (mg.L⁻¹) calculated for compounds in liquid and biomass phases plus volatilized fraction for pine chip-packed flasks

mg.L ⁻¹	Feed	Fraction ^a	Total ^b	% ^c
Live mycelia				
Phenol	286.1	21.3	205.8	71.9
2,4,6-TCP	47.6	2.6	27.6	57.9
Deactivated mycelia				
Phenol	278.3	18.6	276.9	99.4
2,4,6-TCP	42.9	2.9	40.2	93.7

^aFraction collected from gas traps (calculated as per mean for traps) after 24h.

^bTotal equals liquid plus biomass phases plus average fraction concentrations.

^c% of feed concentration.

The fractions of phenol and 2,4,6-TCP volatilized due to continuous aeration were captured in water traps. Tables 2.3 and 2.5 show these fractions, with between 4 and 8.1 % phenol and between 5.3 and 7.3 % TCP of the feed concentration detected in the traps.

Table 2.4. Uptake by live vs. deactivated biomass (mg.L⁻¹) in foam glass beads-packed flasks after 24 h

Phenol (mg.L ⁻¹)							
Live mycelia				Deactivated mycelia ^b			
Feed ^a	Liquid phase ^a	Biomass phase ^a	Total ^a	Feed ^a	Liquid phase ^a	Biomass phase ^a	Total ^a
285.4 ± 30.4	195.1 ± 77.0	8.5 ± 1.6	203.6	293.0 ± 25.1	247.1 ± 1.3	13.1 ± 3.7	260.2
% ^c	68.3	2.9			84.3	4.4	
2,4,6-TCP (mg.L ⁻¹)							
Feed ^a							
52.0 ± 4.9	24.6 ± 4.2	2.2 ± 0.03	26.8	51.1 ± 9.0	30.6 ± 1.9	12.8 ± 1.9	43.4
% ^c	47.3	4.2			59.8	25	

^aConcentration per mean triplicate flasks ± standard deviation.

^bDeactivation of mycelia using 4 % (v/v) formaldehyde.

^c% of feed concentration.

Table 2.5. Stripped phenol and 2,4,6-TCP fraction captured in traps and total concentrations (mg.L⁻¹) calculated for compounds in liquid and biomass phases plus volatilized fraction for foam glass packed-packed flasks

mg.L ⁻¹	Feed	Fraction ^a	Total ^b	% ^c
Live mycelia				
Phenol	285.4	11.4	215.0	75.3
2,4,6-TCP	52.0	2.8	29.6	56.9
Deactivated mycelia				
Phenol	293.0	24.0	284.2	96.9
2,4,6-TCP	51.1	3.0	46.4	90.8

^aFraction collected from gas traps (calculated as per mean for traps) after 24 h

^bTotal equals liquid plus biomass phases plus average fraction concentrations

^c% of feed concentration

2.3.4 Extracellular Enzyme Profiles

The immobilized mycelia turned brown on days 5–6 after introduction of mycelial suspension. Browning is considered to be associated with the onset of secondary metabolism and coincides with the production of lignin-degrading enzymes (Dhawale *et al.*, 1992). Lignin peroxidase activity was measured in fluid collected from the outlets for both reactors with the elevated phenol and 2,4,6-TCP loads. LiP activity showed an

increase with the introduction of phenol and TCP and highest values were measured over the first 12 h after feed (Figures 2.10 and 2.11). An increase from 0.2 to 5 U.L⁻¹ over 5-6 h was detected in the pinewood reactor which corresponds to optimum removal period (> 95 % for phenol and > 97.5% for TCP) (Figure 2.10).

Maximum LiP activity (between 16.7 and 19 U.L⁻¹) was detected in the glass bead-packed reactor over the same period (Figure 2.11). The presence of available carbon (cellulose and hemicellulose) sustained ligninolytic activity in Reactor One between batch cycle feeds (0.1 – 0.2 U.L⁻¹). The higher LiP activity detected for Reactor Two could have been due to the easy availability of glucose in the induction feed, however, it showed periods of negligible activity (0 – 0.008 U.L⁻¹) between feeds probably due to the depletion of glucose. The absence of a carbon source between induction feeds resulted in zero to low activity periods.

Both MnP and laccase activity was detected in the reactors. MnP activity (between 0.3 and 5.8 U.L⁻¹) and laccase activity (0.2 – 0.3 U.L⁻¹) were detected only at intermediate periods in Reactor One (Figure 2.10). In contrast, MnP activity for Reactor Two was between 0.1 and 0.4 U.L⁻¹; and laccase, 0.2 – 0.4 U.L⁻¹ (Figure 2.11). Mn²⁺ was found to play a crucial role in bioconversions involving MnP (Hofrichter and Hatakka, 2001). The growth and induction media did not contain Mn²⁺, however, the measured MnP activity in the pinewood-packed reactor compared to the glass-packed reactor possibly indicates the presence of available Mn for bioconversions.

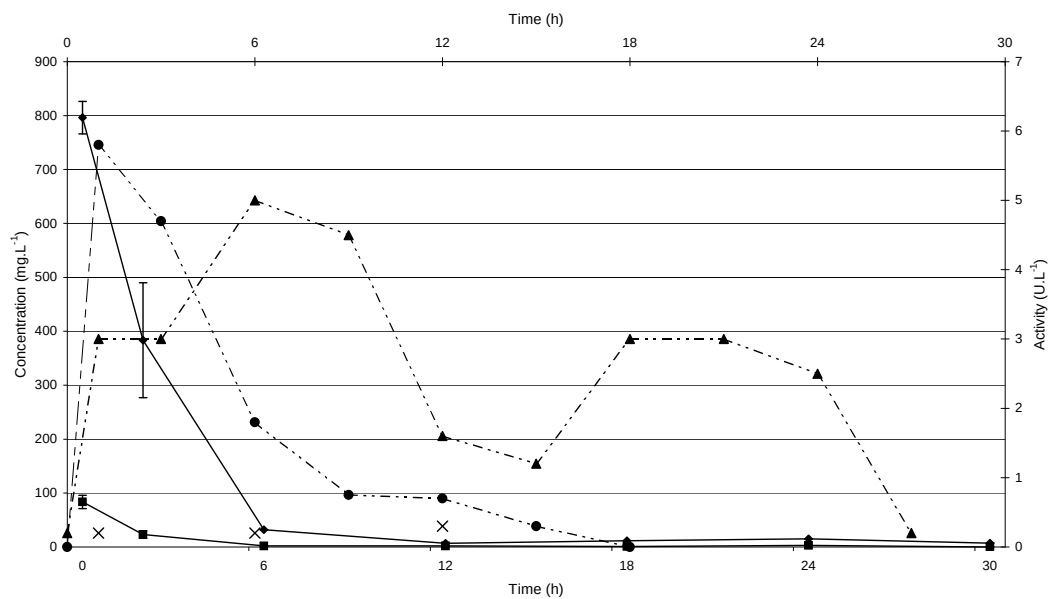


Figure 2.10 Phenol (♦) and 2,4,6-TCP (■) (mg.L⁻¹) removal in Reactor One with extracellular enzyme activity (U.L⁻¹) detected in reactor outlet: LiP (▲), MnP (●) and laccase (x).

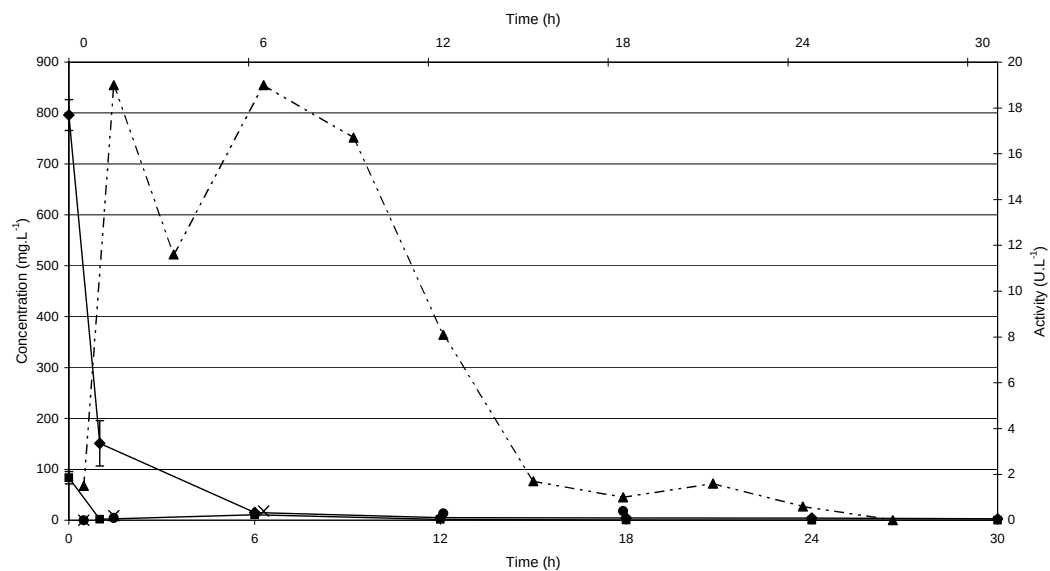


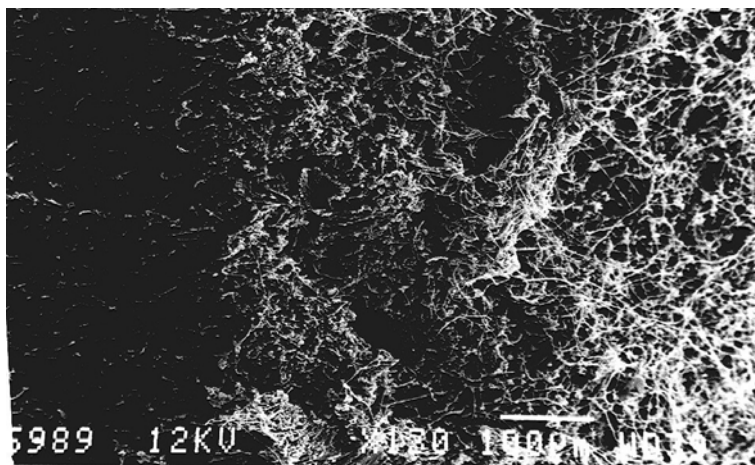
Figure 2.11 Phenol (♦) and 2,4,6-TCP (■) (mg.L⁻¹) removal in Reactor Two with extracellular enzyme activity (U.L⁻¹) detected in reactor outlet: LiP (▲), MnP (●) and laccase (x).

2.3.5 Electron Microscopy

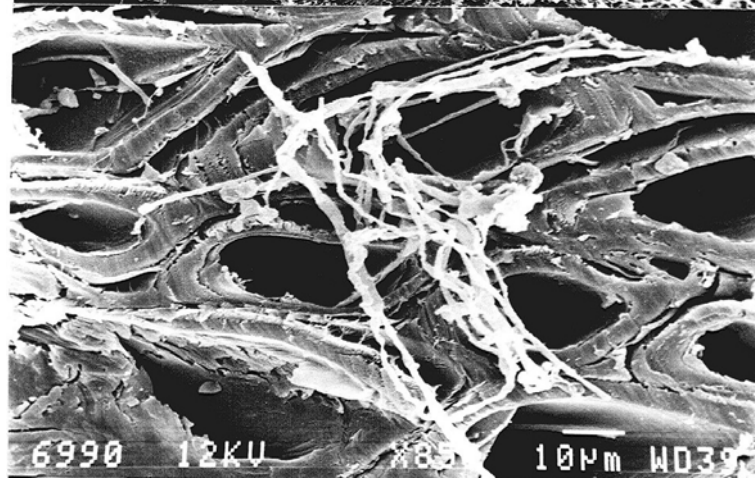
Figures 2.12 and 2.13 show scanning electron microscope (SEM) micrographs of pine chips and foam glass bead-immobilized fungi. Figure 2.12 (A) shows the colonization of the surface of a single wood chip by the mycelia sampled from the trickling packed-bed reactors after successful operation. Magnified sections of wood chips exhibiting surface colonization and penetration are presented in Figure 2.12 (B;C). Effective attachment of fungal mycelia to the glass beads on the outer surface (Figure 2.13 B;C), as well as inner surface area (Figure 2.13 A) was found.

Transmission electron microscope (TEM) micrographs present vertical (Figure 2.14 A) and horizontal (Figures 2.14 B) cross sections of fungal hyphae penetrating the wood material. This shows that the fungi not only used the chips as immobilization substrate but also penetrated the wood tissue possibly extracting cellulose and hemicellulose to be used as carbon source.

A



B



C

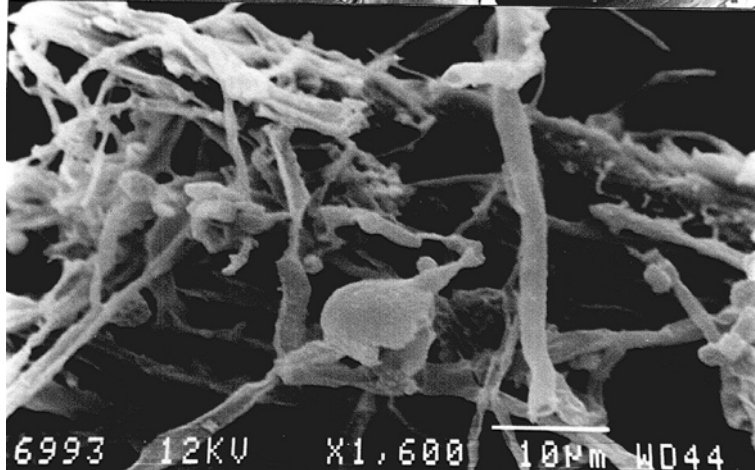
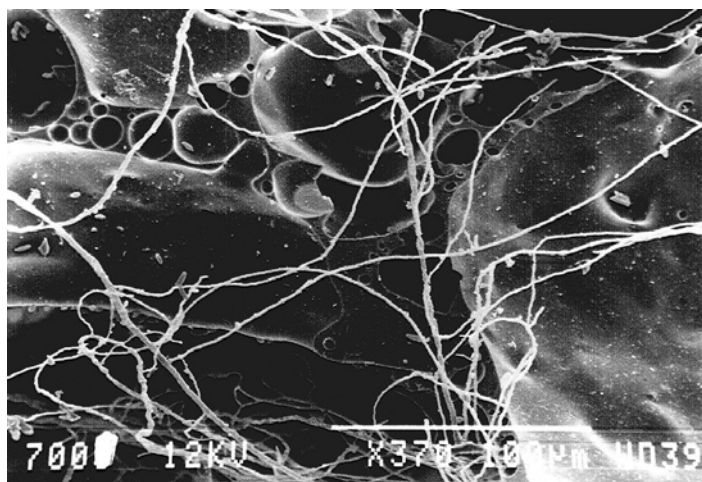
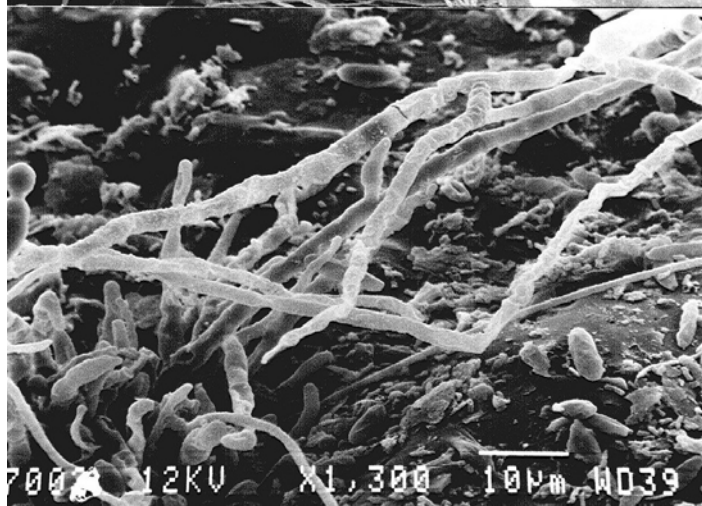


Figure 2.12 SEM micrographs of pine chips collected from the packed-bed reactor showing the colonization of the surface of a single wood chip by the mycelia.

A



B



C

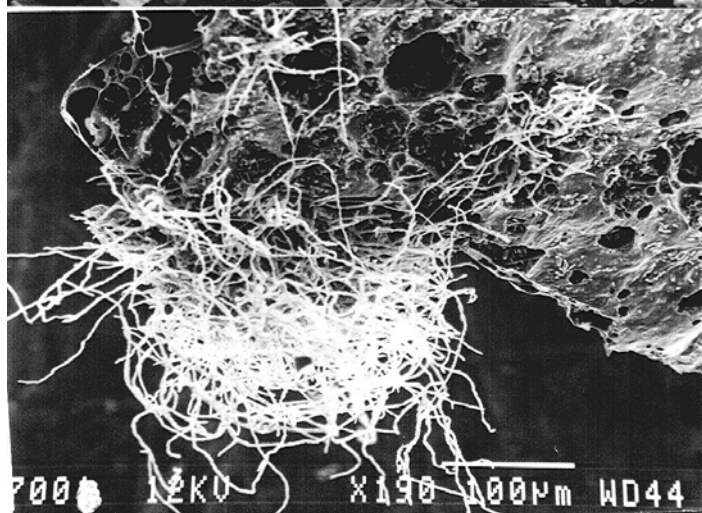


Figure 2.13 SEM micrographs of foam glass beads collected from the packed-bed reactor showing attachment of fungal mycelia to the glass beads.

A



B

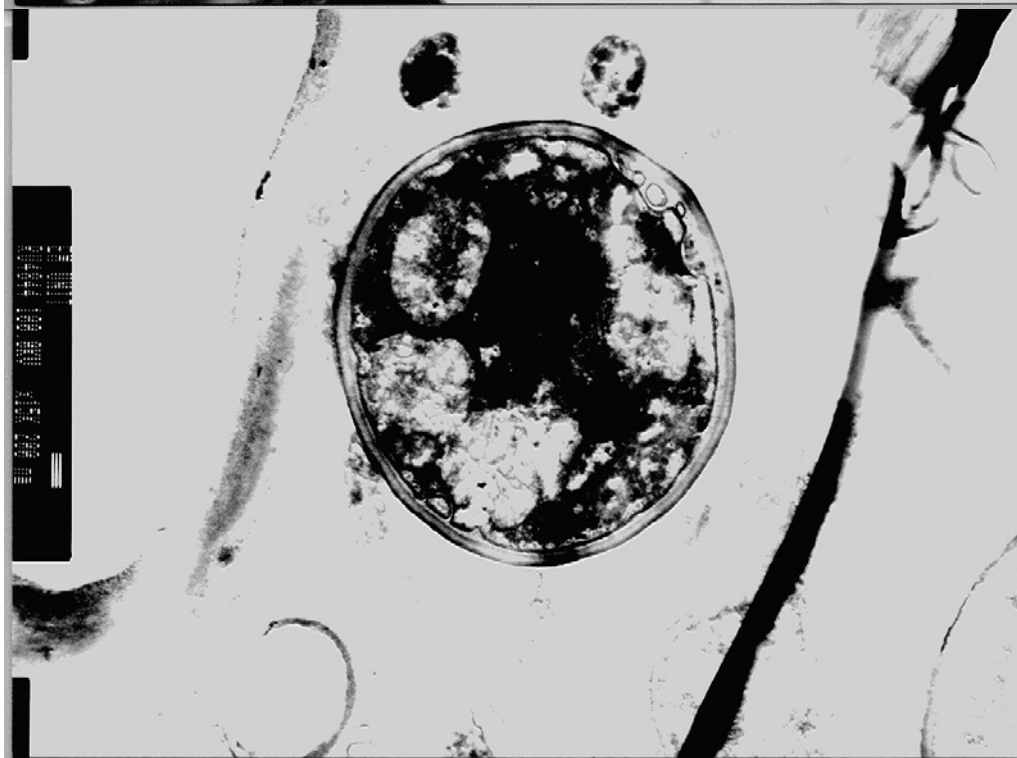


Figure 2.14 TEM micrographs of pine chips collected from packed-bed reactor showing fungal hyphae penetrating the wood material.

2.4 Discussion

The investigation demonstrated the mineralization of two priority pollutants, phenol and 2,4,6-TCP, by immobilized white-rot fungi in trickling packed-bed reactors. This constitutes the aerobic compartment of the proposed integrated anaerobic/aerobic bioprocess environment for CP-contaminated soil/aquifer bodies as discussed above. Since dechlororespiration reactions occurring in anaerobic zones is not a mineralization process, a secondary step is necessary to mineralize mobilized parental pollutant and dechlorination metabolites. This study, however, also shows the potential use of the immobilized fungal reactor as a free-standing technology for the treatment of phenol-contaminated industrial effluents. This potential was followed up and is reported later.

An organic substrate (pinewood) and inert support medium (foam glass beads) were compared as packing materials. Pine chips, a waste product from the forest products industry, can potentially provide the necessary carbon source (cellulose and hemicellulose) for white-rot fungal growth plus it has the physical integrity to serve as immobilization surface in reactor applications. This makes the addition of auxiliary carbon and nutrient sources to reactor studies and bioremediation strategies unnecessary. This study indicated that both microcarriers support immobilization, extracellular fungal enzyme secretion and metabolization of the pollutants.

Comparable performance between wood chip and glass bead packing was shown. Glucose was excluded in the pine chip-packed induction feed since cellulose was available as a carbon source, therefore, it is expected that the life of the packing would be limited. However, supplementing with additional nutrients may assist in reducing the rate at which wood tissue is consumed. Using an immobilized system provided a greater degree of stability for the fungi and a high tolerance for elevated pollutant concentrations since SBR operation ensured a concentration gradient established with subsequent recycling inflows. SBR mode increased operational flexibility with the prevention of mycelia and extracellular enzyme wash-out that may occur in continuous operation and supported the immobilization and ligninolytic enzyme secretion of the white-rot fungal strains. Previous investigators noted the necessity of mycelia and extracellular protein presence in biodegradation studies (Armenante *et al.*, 1994; Pallerla and Chambers,

1998). Both are required simultaneously to facilitate substantial reductions in pollutant concentrations.

The overall increase in degradation rates with higher phenol and 2,4,6-TCP concentration loads administered after the initial five batch cycles is similar to the observations made by Pérez *et al.* (1997). An increase in CP degradation was shown by acclimated *P. chrysosporium* mycelium with up to 70 % increase in degradation for *ortho*-CP (250 mg.L⁻¹) and 50 % for *meta*- and *para*-isomers (150 mg.L⁻¹ each) with respect to non-acclimated mycelium.

Lewandowski *et al.* (1990) operated immobilized *P. chrysosporium* packed-bed reactors treating a 2-CP (300 and 460 mg.L⁻¹) solution using silica-based porous beads, ceramic saddles and balsawood chips as immobilization media, and a well-mixed reactor with fungal mycelia immobilized onto alginate beads. First-order rate constants calculated for these reactors cannot be directly compared to values obtained for the current study since reactor design and operation is different. In addition, rate constants were calculated in reactors with unknown biomass content. Biomass concentration was difficult to measure for both reactors. Lewandowski *et al.* (1990) quoted 2-CP first-rate order constants of 0.19 h⁻¹ (balsawood) and 0.15 h⁻¹ (silica beads) for the packed-bed reactors. Degradation rates increased by a factor of 3 and 4.6 for the pine chip- and glass bead-packed reactors was shown when comparing 2,4,6-TCP rate order constants at the higher influent concentration to values for the above mentioned study that used a 400 cm³ packed-bed volume. A rate constant of 0.51 h⁻¹ was calculated for the well-mixed reactor (alginate beads) in the same study.

Kaballo *et al.* (1995) employed clay balls (0.8 - 1.6 cm, diameter) to immobilize activated sludge enriched microorganisms in two fixed-film reactors (214 L, fixed bed volume) using continuous and sequencing batch operation to treat 4-CP contaminated effluent. The authors declared uniformly developed biomass in the SBR compared to stratification of biomass in continuous operation plus resistance to shock loading (45 mg.L⁻¹). After a cycle duration (period before sample is taken) of 6 h, 89 % of the influent was eliminated compared to 72 % in the continuous biofilm reactor per mean residence time. A specific elimination rate per biomass unit in the SBR was approximately 5-fold higher than that observed in continuous mode. It was concluded that more efficient transport of

substrates caused by smaller internal mass transport resistance performed by a thinner biofilm in sequencing batch operation could have contributed to this.

The role that uptake by the mycelia play was shown to be insignificant. The compounds adsorbed by live biomass were shown to be degraded and do not accumulate intracellularly. Comparing the removal efficiency in the reactors with stationary flasks for both pine and glass beads (Tables 2.1, 2.3 and 2.5) over a similar period, the reactors performed better. It can be concluded that the reactor configuration enhanced biodegradation, with submerged, partially submerged and non-submerged (semi and solid state fermentation) microcarriers increasing the contact between compounds and mycelia, in contrast to partially submerged packing materials in stationary flasks.

The induction media administered to Reactor One (no glucose / nitrogen source) and Reactor Two (glucose in reduced amount / no nitrogen source) facilitated the secretion of LiP, which showed an increase in activity after the feed and during the optimum period of phenol and TCP reduction. The higher LiP activity detected for Reactor Two may be ascribed to the glucose as an easily available carbon source in the induction medium. This could have facilitated the more rapid degradation of the pollutants compared to the rates of removal monitored for Reactor One (Table 2.1). 51.8 and 71.3 % removal was detected for phenol and 2,4,6-TCP during the initial HRT in the pine chip-packed reactor whereas 81 and 97.4 % (phenol and TCP) removal was detected in the glass bead-packed reactor for the initial 1.4 h. The increased elimination rates of the compounds correspond to increased LiP activity and enhanced removal in Reactor Two to the higher enzyme activity detected. MnP and laccase activity was measured at intermediate periods in both reactors. The possible presence of manganese in the pine material supported the release of MnP activity as opposed to low to negligible activity in the glass bead-packed reactor. The possibility that the enhanced LiP activity suppressed the secretion of MnP in Reactor Two and the effect of enzyme induction and activity on pollutant degradation rates require further investigation and will be followed up in later scale-up studies. Nevertheless, conditions favouring lignolytic enzyme secretion prevailed in both reactors with concomitant degradation of the compounds. The fact that no additional carbon source was added to Reactor One and conditions were optimal to stimulate and sustain white-rot activity indicates the unnecessary addition of expensive culture growth medium constituents.

Though enzyme titres detected in this study were generally low it has been speculated that LiP and MnP while possibly not absolutely essential, nevertheless, have a decided affect on pollutant degradation (Armenante *et al.*, 1994; Dhawale *et al.*, 1992). Shim and Kawamoto (2002) reported on comparative LiP activity in various reactor systems for the immobilization of *P. chrysosporium*. The authors, however, advise that comparative activity evaluation and assessment must be approached with circumspection since experimental culture conditions (pH, temperature, supplements made and oxygen/aeration) affect enzyme yields.

Chapter Three

The Reductive Dechlorination of Chlorinated Phenol under Sulphate-Reducing Conditions

3. Introduction

Sulphidogenic-dependent dechlororespiration is the desired reaction in the anaerobic compartment of the proposed integrated anaerobic/aerobic bioprocess environment for the remediation of CP-contaminated soil, sediment and groundwater bodies where oxygen is frequently limited. Though the aerobic stage was shown to mineralize phenol and 2,4,6-TCP, fungi require aerobic conditions and introduction of air into subsurface environments or excavation of contaminated material would be costly or impossible due to depth and risky since target removal rates might not be achieved. Since sulphate is an ubiquitous electron acceptor found in marine sediments and intertidal environments and has a likely impact on dehalogenation reactions in habitats contaminated with chlorinated compounds, the potentially successful introduction of *in-situ* enhanced dechlororespiration and effect on pollutant bioavailability during sulphate reduction was investigated.

The objectives of the study were to enrich for a chlororespiring bacterial consortium capable of dechlorinating 2,4,6-TCP under sulphidogenic conditions and investigate the relationship with SRB in batch and continuous laboratory-scale packed-bed reactors. The impact of sulphate-deficient and excess concentrations was investigated as this has been reported to be a critical factor affecting the synergetic relationship between chlororespiring bacteria and SRB (Drzyza *et al.*, 2001). The potential effect of the sulphidogenic component of the proposed dual-stage biodegradation strategy for chloroaromatic-contaminated groundwater and soils was investigated for two soil types. The role of sulphate reduction activity in mobilizing adsorbed CP soil contaminants was considered. Exogenous culture-augmented synthetic TCP-contaminated soil was used to monitor dechlorination effects on pollutant mobility and availability in batch studies.

3.2 Materials and Methods

3.2.1 Enrichment Procedure

Anaerobic cell generators and enrichment cultures were set up. The inoculum was prepared from sludge collected from an UASB reactor located at the Environmental Biotechnology Field Station at the Grahamstown Disposal Works and fresh leachate from the Aloes hazardous waste co-disposal landfill site, Port Elizabeth. The UASB is a reactor in which screened, raw sewage is blended with an artificial, sulphate rich mine water and is one of the components of research investigating the co-treatment of acid mine drainage and sewage.

The cell generators were constructed using 1 L glass flasks each containing a mixture of sludge and leachate (1:1) as 12 % (v/v) inoculum. Sterile Postgate medium B (adapted from Parks, 1993; Appendix A) was introduced into the flasks. The medium had a total SO_4^{2-} concentration of 900 mg.L^{-1} . At this concentration, SO_4^{2-} is at nearly twice the equimolar concentration necessary to carry out incomplete oxidation of the total amount of lactate (10 mM) according to the equation: $2\text{CH}_3\text{CHOHCOO}^- + \text{SO}_4^{2-} \rightarrow 2\text{CH}_3\text{COO}^- + 2\text{HCO}_3^- + \text{HS}^- + \text{H}^+$ (Drzyzga *et al.*, 2002). 2,4,6-TCP additions were made from a filter sterilized concentrated stock solution, typically to a final concentration of 20 mg.L^{-1} . Flask headspaces were overgassed with oxygen-free nitrogen, sealed with butyl rubber stoppers and incubated statically in the dark at 28°C . Zinc acetate (12.4 g.L^{-1}) traps were attached to the cell generators to capture liberated hydrogen sulphide to precipitate as insoluble zinc sulphide. To avoid the accumulation of hydrogen sulphide, the gas phase of the flasks were regularly flushed with N_2 .

The gas traps were routinely checked for zinc sulphide formation and samples were drawn through sampling outlets from the cell generators for sulphate analysis. Upon zero sulphate detection in a flask, it was resupplemented with $1.3 \text{ g Na}_2\text{SO}_4$. Sodium lactate (1.1 g) and yeast extract (1 g) was also regularly added to the flasks when sulphate depletion was unsatisfactory. Sulphate was, therefore, provided in excess and on a continuous basis to promote a sulphidogenic consortium from the onset of the enrichment. The cell generators were operated over a 6 month period.

From the initial enrichment cultures, stable sulphidogenic consortia were obtained from repeated dilutions (1:5 v/v) into growth medium and by spiking each dilution with 2,4,6-TCP. These cultures were made in N₂-purged 125-ml serum bottles sealed with butyl rubber stoppers and aluminum crimp seals incubated in the dark at 28 °C. Deoxygenated plastic syringes (5 ml) connected to sterile hypodermic needles were used to draw samples through the stoppers and were analysed for sulphate and TCP content.

The dilution procedure was repeated until such time that products of TCP dechlorination were first observed and identified by comparison with the retention times of known metabolites according to literature and pure standards on the HPLC. The culture was used to inoculate batch and reactor studies.

3.2.2 Batch Flasks Study

Reductive dechlorination was executed in stationary fed-batch flask operation to assess the influence of different sulphate concentrations on the overall reaction performance in 1 L glass flasks. Flasks were inoculated with a 12 % (v/v) suspension from the serum bottle enrichments. The flasks were filled with Postgate medium B in two sulphate concentrations: 900 and 110 mg.L⁻¹ ($\pm$ 25 % of equimolar SO₄²⁻ requirement) and spiked with TCP from a concentrated stock solution to a final concentration of 30 mg.L⁻¹ into duplicate flasks. The flasks were sealed with rubber stoppers and incubated in the dark at 28 °C. Zinc acetate traps were connected and headspaces purged with N₂.

Samples were routinely drawn from the flasks through sampling outlets and analysed for sulphate, sulphide, pH, TCP and dechlorination metabolites content. Upon zero or negligible sulphate concentrations, the flasks were resupplemented with Na₂SO₄ to its introductory concentration level working under a constant N₂ flow to maintain anaerobiosis. The flasks were also supplemented with lactic acid and yeast extract during periods when sulphate removal rates were reduced.

A control flask was set up to investigate abiotic removal of the compound. Inoculum (12% v/v) and growth medium was autoclaved at 121 °C for 20 min on two consecutive days before spiking with 2,4,6-TCP from the stock solution. The flask was sealed and

incubated in the dark at 28 °C. Samples were collected and sulphate and TCP quantified. To investigate the dependency of dechlororespiration on sulphate reduction, a duplicate inhibition experiment using molybdate was also set up. Molybdate is a sulphate analog and competitive inhibitor of sulphate reduction (Oremland and Capone, 1988). The cultures were amended with 20 mM molybdate (Bedessem *et al.*, 1997) as Na₂MoO₄ and Na₂SO₄ after SO₄²⁻ was reduced to negligible levels.

3.2.3 Soil Flasks Study

To investigate adsorption and desorption phenomena in sulphate-reducing environments, soil was spiked with 2,4,6-TCP, amended with growth medium and augmented with the consortium. Two soil types were investigated: clay and a soil with reasonable organic carbon content. Kaolin was obtained from Makana Brick, Grahamstown, and soil with higher organic content was sampled from a site in the industrial zone in Grahamstown at a depth of > 30 cm by employing an auger. The soil was stored at 4 °C before use. The Analytical Services Department of the Döhne Agricultural Development Institute, Stutterheim performed soil texture, organic carbon content and soil fertility analyses. The protocol for adsorption, bioavailability and biodegradation studies in soil was adapted from Tabak *et al.* (1997).

The soil was air dried and sieved to pass 2 mm. 25 g of sieved soil / kaolin was introduced into a 250 ml bottle and 140 ml of a filter-sterilized TCP solution (70 mg.L⁻¹) was added. The bottles were purged with N₂, capped and left in the dark at room temperature for 24 h. After this period, destructive sampling was adopted to filter the soil slurry through a Whatmann number 42 filter paper employing a glass Büchner funnel to remove gravitational and interstitial liquid under vacuum pressure. The soil / kaolin and filtrate fractions were solvent extracted and analysed for TCP content.

Two sets of soil and kaolin slurry experiments were set up: a control and an inoculated set in triplicate for destructive sampling at 24, 168, 336 and 504 h. For the inoculated set, the soil/kaolin fraction was returned to the bottles after filtering for the 168, 336 and 504 h sampling in triplicates. 140 ml of growth medium plus 12 % (v/v) inoculum from serum bottle enrichments was introduced into each of the bottles (total SO₄²⁻ concentration 110 mg.L⁻¹), purged with N₂, capped and left in the dark at room

temperature. The bottles were shaken by hand on a daily basis. The control set did not receive an inoculum or growth medium. The 168, 336 and 504 h triplicates were left until their respective sampling times arrived. The moisture content of the kaolin and soil was determined by weighing 25 g sieved soil/kaolin and left overnight at 100 °C in a drying oven.

At the specific time intervals, the soil slurries of the set of triplicates were separated into soil/kaolin fraction and liquid fraction by filtration. The liquid and soil portions were extracted into an organic solvent for TCP quantification. The pH was also measured. For the inoculated bottles, sulphate and sulphide concentrations were determined for the liquid fraction. Liquid and soil samples were also used for total bacteria colony plate counts using a selective medium.

3.2.4 Fluidized Packed-Bed Reactor Studies

Two pinewood chip-packed reactors of similar dimension as outlined in 2.2.2 were used. Perspex beams were placed inside at both ends (5.5 cm from bottom/top of reactor) to keep packing material intact. The reactors were sealed with silicone sealant. The reactors were inoculated using a 12 % (v/v) inoculum sampled from the serum bottles in Postgate medium B spiked with 2,4,6-TCP from a concentrated sterile stock solution to final concentration of 25 mg.L⁻¹ introduced at flow rate 282 ml.h⁻¹. The influent inoculum/medium and pine chip-packed reactors were first purged with oxygen-free N₂. The reactors were filled with the inoculation medium and recycling commenced through a bottom-to-top loop at constant flow 35 ml.h⁻¹. The recycling continued for 20 d to facilitate the establishment and attachment of the consortium inside the reactor and onto the wood chips.

Following the inoculation procedure, the reactors were operated in continuous flow-through mode. Feeding solution was supplied from an influent reservoir consisting of TCP-spiked growth medium. The headspace of the reservoir was continuously flushed with N₂ at 30 ml.h⁻¹ to remove O₂. The influent was introduced into the top and discharged from the bottom at 35 ml.h⁻¹. The void fraction (void volume to reactor volume) due to the wood chip packing was 0.57. The HRT was 102.6 h.

The reactors were operated identically, however, reactor outflow was continuously recycled (bottom-to-top) at 35 ml.h⁻¹ in conjunction with discharge in Reactor Two. Off-gas from the reactors was trapped in zinc acetate (10.4 g.L⁻¹) traps and headspaces were regularly flushed with N₂ to evacuate hydrogen sulphide.

Samples collected from inlet sampling ports were quantified for sulphate and TCP content; and outlet ports for sulphate, sulphide, pH, TCP and its dechlorination products. Effluent samples were also collected for total bacteria colony plate counts using a selective medium. Equilibrated steady-state conditions were established by allowing for at least three reactor culture volume changes before monitoring was commenced. Initially, the reactors were fed with a total sulphate concentration of 110 mg.L⁻¹ and stable conditions were maintained for 20 d which, according to the retention time, equates to four to five complete volume changes of the liquid in the reactors. Thereafter, the sulphate level in the influent feed was increased to 900 mg.L⁻¹ and after allowing stable conditions to establish, operation was continued for another 20 d. Figure 3.1 shows a diagrammatic representation of the anaerobic packed-bed reactors.

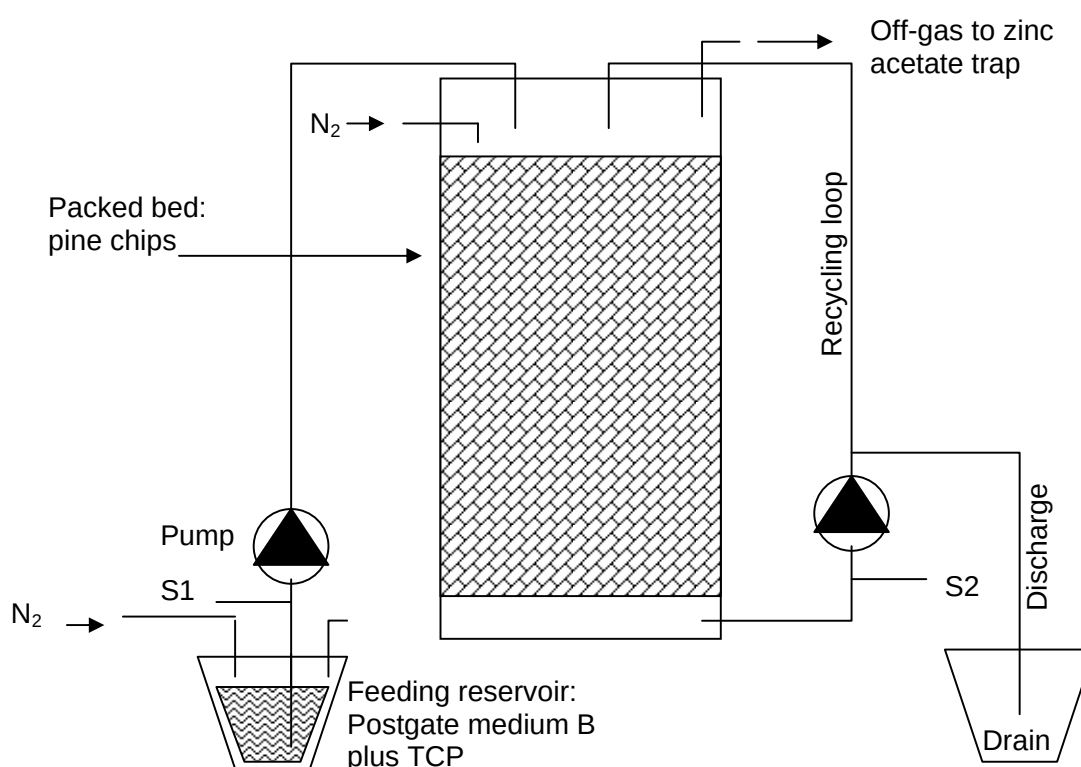


Figure 3.1 Schematic diagram of anaerobic pine chip-packed reactors operated in continuous mode (Reactor One) and recycling and continuous mode (Reactor Two). S1&2, sampling points 1 & 2.

The reactors were operated at room temperature (min 20 °C max 25 °C) in the dark.

3.2.5 Analytical Methods

3.2.5.1 Soil extraction

The ultrasonic soil extraction method used in this study was modified from the US EPA 3550B method (US EPA Test methods for evaluating solid waste, physical/chemical methods, 2003). The filtered soil/kaolin was weighed and qualitatively removed from the filter paper and transferred into a 250 ml glass flask with stopper and placed on ice. A sample of known weight was collected from the filter paper and oven dried for moisture content determination. The filter paper was also introduced into the flask for any residual sorbed soil to be extracted. Anhydrous Na₂SO₄ (1:1 m/m) was added to the soil/clay and mixed to make the soil material more freely flowing. The soil/kaolin was extracted in 25 ml ethyl acetate to which 5 ml of a 1 mg.ml⁻¹ naphthalene (internal standard in ethyl acetate) solution was added by sonicating on ice for 5 min using a Virtis Virisonic 100 probe sonicator (4 watts RMS, 50 Hz). The extracted organic phase was removed using a Pasteur pipette and stored on ice while the procedure was repeated for the soil/kaolin (sonicated for 2 min). The extracted phases were combined and centrifuged using a bench-top microfuge (Eppendorf 541D) at 11 000 rpm x g for 2 min. 20 ml of the solvent supernatant collected was evaporated under reducing pressure using a rotary evaporator (Büchi). The extractives were resuspended in HPLC mobile phase (10 ml) and filtered through a 0.45 µm nylon filter (Osmonics) before injection into the HPLC.

The culture liquid phases were extracted as outlined in Appendix B.

3.2.5.2 TCP and metabolites

2,4,6-TCP and its dechlorination products were extracted and quantified by HPLC (Appendix B). However, the internal standard used was naphthalene (1 mg.ml⁻¹) so as to not interfere with metabolite peak retention times. Metabolites were identified by comparison with the retention times of known standards. Pure 2-CP, 4-CP, 2,6-DCP and 2,4-DCP were used to identify reaction products on the premises that dechlorination of *ortho*-substituted chlorines precedes *para*-dechlorination (Armenante *et al.*, 1992;

Kennes *et al.*, 1996; Madsen and Aamand, 1992). A mixture of pure compounds (30 mg.L⁻¹ each) was successfully separated by employing a gradient which consisted of phase A (MiliQ distilled water plus 1 % acetic acid) and phase B (100 % acetonitrile). A linear gradient of 100 % phase A to 100 % phase B over 25 min was used at 1 ml.min⁻¹. Transient peaks in samples from the reactors were identified using the same gradient operation.

3.2.5.3 Sulphate

Two methods were used to quantify sulphate levels in reactor effluent and batch and inoculated soil flasks: HPLC anion exchange chromatography and turbidometric sulphate determination (Appendix D).

3.2.5.4 Sulphide

Sulphide was quantified using the methylene blue method (Truper and Schlegel, 1964; Appendix E).

3.2.6 Colony Plate Counts

Conventional plate counts were performed for the effluent from the packed-bed reactors by sampling from both the reactor outlet feeds at different times during which the systems were fed with the elevated and lower sulphate concentrations. Plate counts were also performed for the soil batch flask experiments to record proliferation of microbes after augmentation.

Madsen and Licht (1992) used a culture fluid (CF) medium to isolate a dechlorinating bacterium enriched from municipal sludge by combining a basal salts medium and fluid from an actively dechlorinating culture. The authors assumed that the dechlorinating bacteria depended on the metabolic activity of other members of the consortium. A similar approach was used to enumerate the total bacteria in the current study.

Liquid samples from the reactors and supernatant from the soil flasks (inoculated and controls) were collected in N₂-purged glass bottles and sealed. The samples were

diluted serially (10^{-3} to 10^{-7}) and spread-plated onto CF medium. The CF medium consisted of autoclaved Postgate medium B to which 3 % agar was added and was diluted 2:1 (v/v) with filter sterilized fluid collected from the reactors. The culture medium was flushed with N_2 and boiled. The petri dishes were incubated at 30 °C in an anaerobic chamber (Forma Scientific, model 1025 S/N) under an atmosphere of 85 % N_2 , 10 % H_2 and 5 % CO_2 . After a minimum period of 21 d, plates were inspected for the appearance of colonies.

3 g Soil/kaolin was transferred aseptically from the filter paper into 50 ml N_2 -purged sterile buffer saline with gelatin solution (8.5 g, NaCl; 0.3 g, KH_2PO_4 ; 0.6 g, Na_2HPO_4 ; 10 ml of 1 % m/v gelatin solution) in a Schott bottle. The bottle was sealed and shaken for 30 min at 50 rpm. Thereafter, serial dilutions were performed and plated out onto CF medium.

Soil/kaolin samples from both the inoculated and control flasks were collected for plate counts, as well as fresh air dried soil which was plated out onto CF medium and Postgate medium B alone.

3.3 Results

3.3.1 Fed-Batch Flask Study

A consortium actively engaged in dechlorination of 2,4,6-TCP under sulphidogenic conditions was successfully enriched. Potential abiotic loss of TCP was also investigated in a sterile control and the interdependency of SRB and chlororespiring bacteria was shown by inhibiting sulphate-reduction using molybdate

Fed-batch conditions were operated for 56 d during which the flasks were analysed for sulphate and sulphide content, and also quantified for TCP and dechlorination products (2,4-DCP, 4-CP and phenol) (Figures 3.2 and 3.3). Upon zero to negligible sulphate concentrations detected, flasks were resupplemented with Na_2SO_4 to either 110 or 900 mg.L⁻¹ to maintain a sulphate deficient or excess environment. Lactate and yeast extract was also reintroduced when sulphate reduction rates were reduced. 2,4,6-TCP was reduced by 62.1 % during the operation of the sulphate-deficient flasks compared to

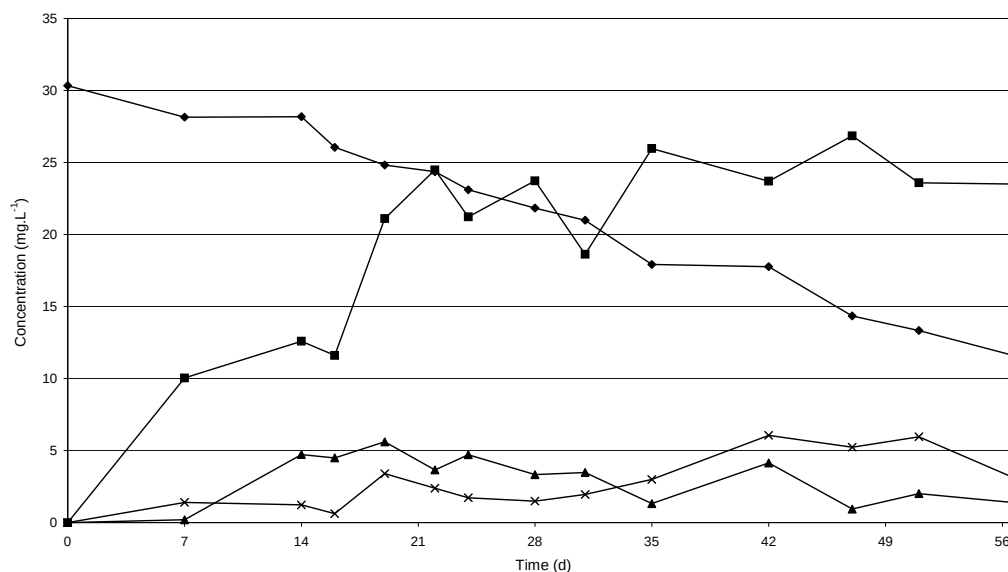


Figure 3.2 Fed-batch flask investigation showing 2,4,6-TCP (♦), 2,4-DCP (■), 4-CP (▲) and phenol (X) concentrations (mg.L^{-1}) detected in flasks supplemented with $110 \text{ mg.L}^{-1} \text{SO}_4^{2-}$. Concentrations are the average for duplicate samples from duplicate flasks.

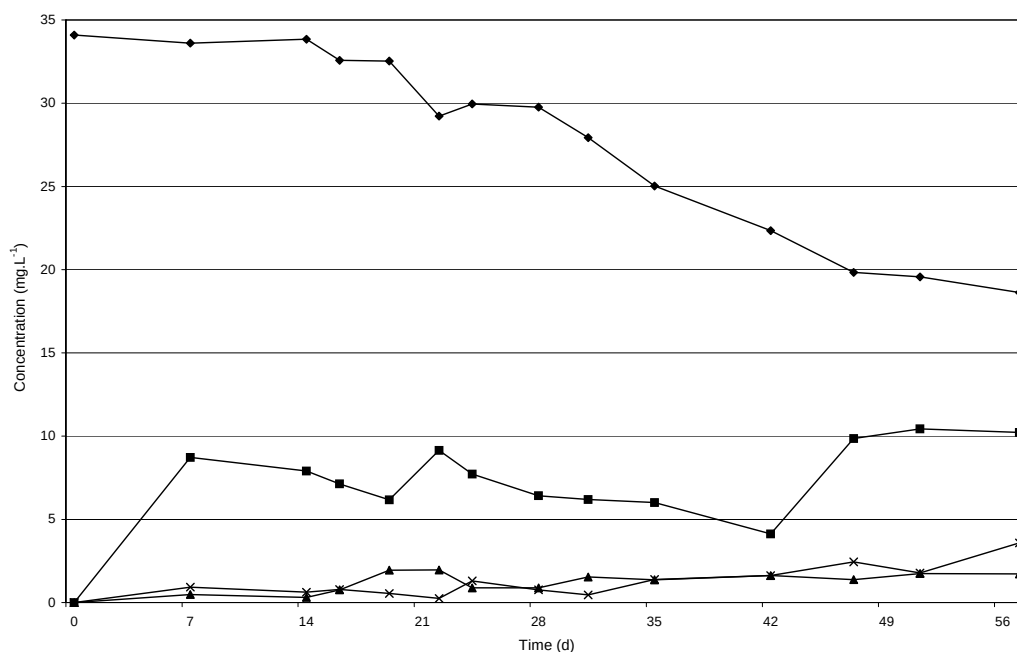


Figure 3.3 Fed-batch flask investigation showing 2,4,6-TCP (♦), 2,4-DCP (■), 4-CP (▲) and phenol (X) concentrations (mg.L^{-1}) detected in flasks supplemented with $900 \text{ mg.L}^{-1} \text{SO}_4^{2-}$. Concentrations are the average for duplicate samples from duplicate flasks.

45.3 % in sulphate-sufficient flasks. Dechlorination products were detected in culture fluids. 2,4-DCP accumulation was evident especially in flasks with a sulphate-deficient load.

Sulphide accumulated in the flasks with concentrations up to 247.0 mg.L^{-1} and 537.1 mg.L^{-1} detected by days 31-35 in sulphate-deficient and sulphate-sufficient flasks, respectively. No further HS^- increase was monitored beyond 35 d for sulphate-sufficient culture fluids since sulphide levels were above detectable limits (1 ppm) even with high sample dilution.

The loss of TCP by abiotic processes was investigated with sterile inoculated medium amended with 2,4,6-TCP. 4.0 % of TCP concentration was lost during a 30-d period. No dechlorinated metabolites were detected (data not shown). Molybdate-spiked flasks showed no further sulphate reduction or TCP transformation for a 35-d period (Figure 3.4). Molybdate inhibited sulphate reduction and with no TCP dechlorination and appearance of 2,4-DCP and other metabolites, the experiment indicated the possible presence of a synergistic relationship that exists between the SRB and dechlororespiring cultures.

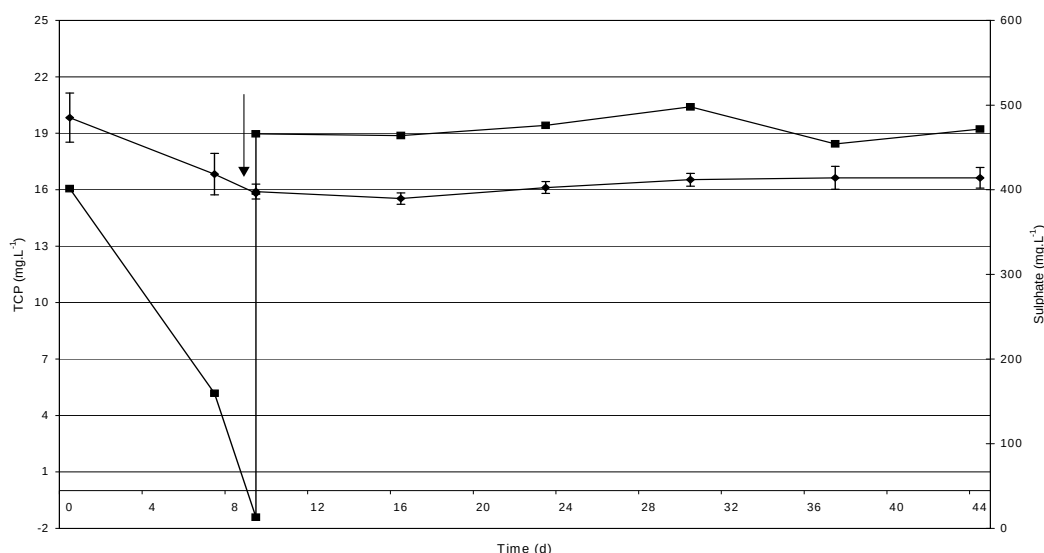


Figure 3.4 Inhibition study showing 2,4,6-TCP (◆) and sulphate (■) concentrations (mg.L^{-1}) detected in molybdate supplemented flasks. Arrow indicates addition of 20 mM molybdate and Na_2SO_4 . Concentrations are the average for duplicate samples from duplicate flasks.

3.3.2 Soil Flasks Study

Two soil types were used in this experiment: clay and sandy loam. Table 3.1 presents inorganic composition, organic carbon and texture analyses of the soils. The soils were spiked with 2,4,6-TCP and after a period allowing for immobilization and desorption to equilibrate, the liquid phases were removed and the soils were supplemented with Postgate medium B ($110 \text{ mg.L}^{-1} \text{ SO}_4^{2-}$). The flasks were augmented with the sulphate reducing–dechlororespiring consortium. Controls were also operated (no medium or culture introduction).

Figures 3.5 and 3.6 present TCP detected in the clay and liquid fractions during the 3 week operation for the control and augmented flasks, respectively. Table 3.2 summarizes the recordings and includes the percentage 2,4-DCP, 4-CP and phenol detected of the immobilized TCP (at 24 h) in clay and liquid culture components.

Table 3.1. Inorganic ion (mg.L^{-1}), pH, organic carbon content (%) and texture data for the two soils

	Inorganic ions (mg.L^{-1})					pH	Organic carbon (%)	Texture
	P	K	Ca	Mg	Zn			
Kaolin	3.1	225	626	389	1.1	6.1	0.02	Clay
Soil	19.9	292	1265	207	5.0	6.8	0.11	Sandy loam

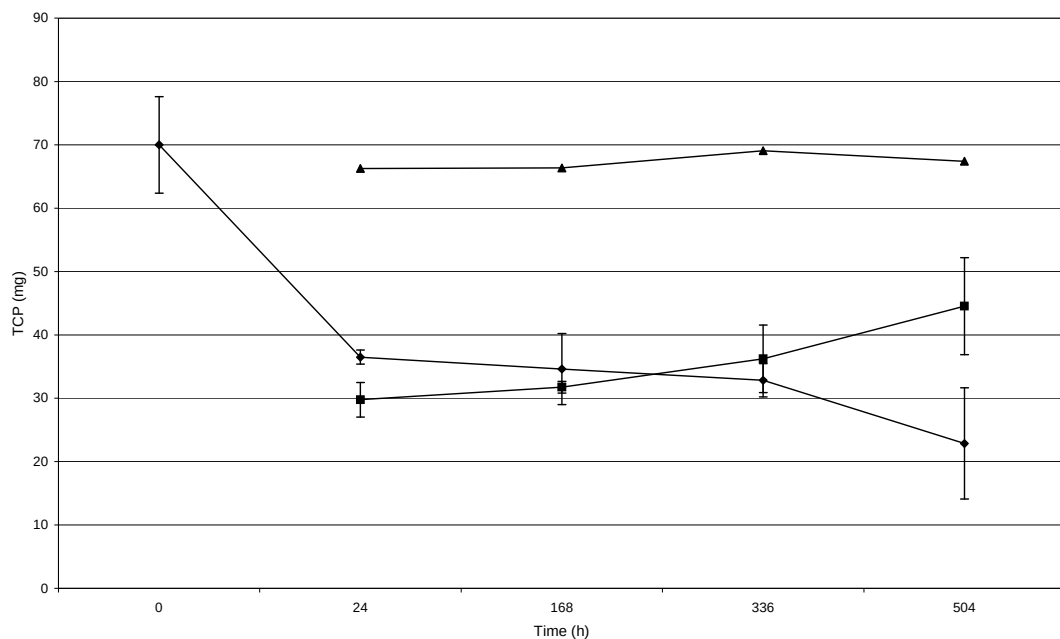


Figure 3.5 Liquid (mg) (♦) and clay (mg per dry weight) (■) fractions and total recovered mass (▲) of TCP detected in control flasks.

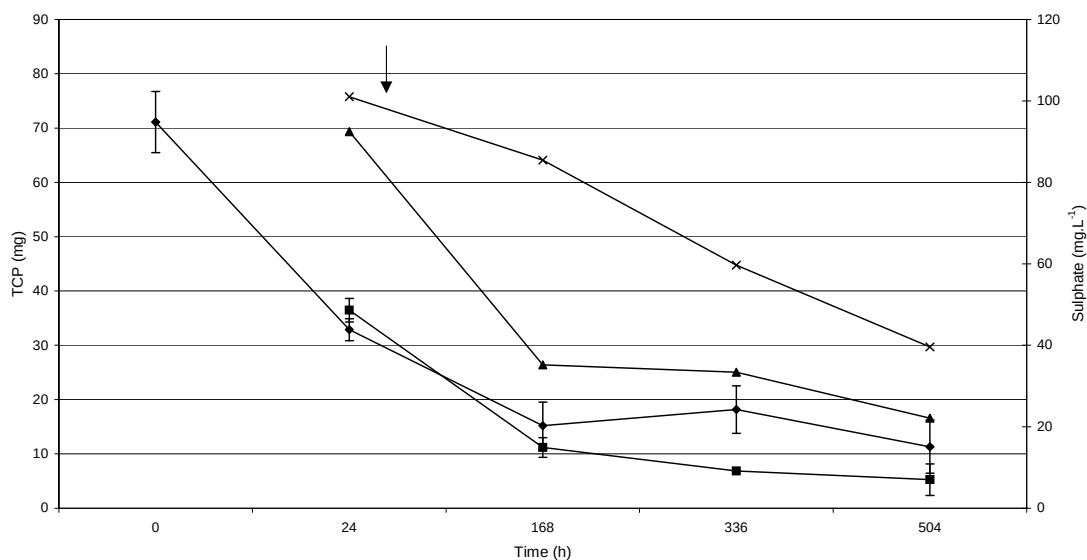


Figure 3.6 Liquid (mg) (♦) and clay (mg per dry weight) (■) fractions, total recovered mass (▲) of TCP and sulphate (mg.L⁻¹) (X) detected in inoculated flasks. Arrow indicates introduction of Postgate medium B plus consortium.

Table 3.2. 2,4,6-TCP load (mg), mass of liquid and clay components (mg), total mass recovered (mg) plus percentages of each component and total recovered fraction for control and inoculated flasks monitored for 3 weeks; including percentage of 2,4-DCP, 4-CP and phenol of immobilized TCP in inoculated flasks

Load ^a (mg)	Time (h)	Supernatant ^a (mg) /%	Clay ^a (mg) /%	Total recovery (mg) /%
Control				
69.9 ± 7.6	24	36.4 ± 1.1 /52.0	29.7 ± 2.7 /42.4	66.1 /94.5
	168 (7 d)	34.6 ± 5.6 /49.4	31.7 ± 0.9 /45.3	66.3 /94.8
	336 (14 d)	32.8 ± 2.6 /46.9	36.2 ± 5.3 /51.7	69.0 /98.7
	504 (21 d)	22.8 ± 8.7 /32.6	44.5 ± 7.6 /63.6	67.3 /96.2
Inoculated				
71.0 ± 5.6	24	32.8 ± 2.0 /46.1	36.4 ± 2.1 /51.2	69.2 /97.4
2,4-DCP 4-CP Phenol ^b (%)	168	15.2 ± 4.3 /41.7 6.8 3.2 1.3	11.1 ± 1.8 /30.4 4.4 9.0 -	26.3 /72.2
2,4-DCP 4-CP Phenol ^b (%)	336	18.1 ± 4.3 /49.7 3.1 7.4 8.4	6.8 ± 0.04 /18.6 5.2 1.3 2.3	24.9 /68.4
2,4-DCP 4-CP Phenol ^b (%)	504	11.3 ± 4.8 /31.0 32.5 2.2 4.6	5.2 ± 2.8 /14.2 5.7 5.9 -	16.5 /45.3

^aMass quoted as mean for results from triplicate flasks ± standard deviation.

^b% 2,4-DCP, 4-CP and phenol of clay-immobilized TCP (36.4 mg) at 24 h.

The control flasks showed 42.4 % of the TCP load (69.9 mg.L⁻¹) retained in the clay after 24 h. TCP in the liquid phase was, thereafter, further removed and adsorbed onto clay particles over a period of 480 h to 63.6 %, with 22.8 mg retained in the liquid. The total recovered TCP mass in the clay and liquid fractions were between 94.5 and 98.7 % during the operational period (Table 3.2). The inoculated flasks showed 51.2 % TCP

(36.4 mg) retained in the clay material at 24 h. At this point the liquid fraction of the flasks were removed and the clay was supplemented with the medium and inoculated. After the first week, 15.2 and 11.1 mg TCP was detected in the liquid and soil components respectively, thus a fraction of immobilized TCP was desorbed and 41.7 % was detected in the liquid. TCP loss of the total recovered fractions (in clay plus liquid) was 27.8 %. The liquid TCP fraction increased to 49.7 % at the end of the second week which indicates that further desorption occurred. However, 24.9 mg TCP was recovered from both fractions indicating 31.6 % TCP transformation. During the last week, TCP in the liquid and clay components decreased to 11.3 and 5.2 mg, respectively. This equates to a 54.7 % reduction of adsorbed TCP on inoculation. Dechlorination metabolites were detected in the liquid and clay components after the first week of incubation (Table 3.2). Higher 2,4-DCP, 4-CP and phenol masses were found in the supernatants which was especially evident after the 3rd week. No metabolites were detected in the control flasks.

The supernatant pH of the control flasks fluctuated between 6.83 and 6.95. pH measured in the inoculated flasks increased from 6.9 to 8.6 during the operational period. Active sulphate reduction was detected in the inoculated flasks (Figure 3.6) as indicated by blackening of the clay due to the precipitation of metal sulphides. Total bacteria colony counts were 13.3×10^7 cells.g⁻¹ soil (dry weight) at 168 h, thereafter, total counts increased to 25.3 and 66.7×10^7 cells.g⁻¹ soil for 336 and 504 h. No colonies were detected for the liquid fraction of the inoculated flasks, as well as the control flasks and starting clay material (plated out on Postgate and CF medium).

The sandy loam soil immobilized 60.9 and 64.2 % of the TCP load after 24 h (Table 3.3). The adsorbed fraction is, therefore, higher for this soil compared to the clay. It is possible that the higher organic carbon content (Table 3.1) of the sandy loam could have exerted more influence on the retention of TCP compared to clay particles. Figure 3.7 presents the adsorbed and free TCP masses in the control flasks, including the mass balance calculated from total recovered fractions per sampling time. Negligible adsorption or desorption was detected during the next 480 h.

Table 3.3. 2,4,6-TCP load (mg), mass of liquid and soil (sandy loam) fractions (mg), total mass recovered (mg) and percentages of each fraction plus recovered fraction for control and inoculated flasks monitored for 3 weeks; including percentage of 2,4-DCP, 4-CP and phenol of immobilized TCP in inoculated flasks

Load ^a (mg)	Time (h)	Supernatant ^a (mg) /%	Soil ^a (mg) /%	Total recovery (mg) %
Control				
67.1 ± 2.4	24	20.7 ± 6.8 /30.8	43.1 ± 5.2 /64.2	63.8 /95.0
	168 (7 d)	25.6 ± 4.3 /38.1	37.7 ± 2.0 /56.1	63.3 /94.3
	336 (14 d)	20.2 ± 5.6 /30.1	43.1 ± 3.6 /64.2	63.3 /94.3
	504 (21 d)	22.5 ± 4.6 /33.5	38.5 ± 2.6 /57.3	61.0 /90.9
Inoculated				
68.2 ± 3.9	24	22.0 ± 1.1 /32.2	41.6 ± 4.7 /60.9	63.6 /93.2
2,4-DCP 4-CP Phenol ^b (%)	168	28.2 ± 8.5 /67.7 - - -	12.6 ± 1.7 /30.2 3.5 0.9 -	40.8 /98.0
2,4-DCP 4-CP Phenol ^b (%)	336	10.7 ± 4.8 /25.7 38.9 1.3 -	9.8 ± 3.2 /23.5 18.6 3.7 1.1	20.5 /49.2
2,4-DCP 4-CP Phenol ^b (%)	504	4.6 ± 5.5 /11.0 1.0 41.4 0.8	7.4 ± 3.6 /17.7 0.8 20.3 2.9	12.0 /28.8

^aMass quoted as mean for results from triplicate flasks ± standard deviation.

^b% 2,4-DCP, 4-CP and phenol of soil-immobilized TCP (41.6 mg) at 24 h.

The inoculated flasks (Figure 3.8) showed TCP desorption occurring after week 1. Of the 41.6 mg TCP immobilized at 24 h, 67.6 % TCP mobilized. During the subsequent two weeks, TCP was reduced to 4.6 and 7.4 mg in the liquid and soil components, respectively (Table 3.3). This equates to 28.8 % of the adsorbed fraction detected at 24 h.

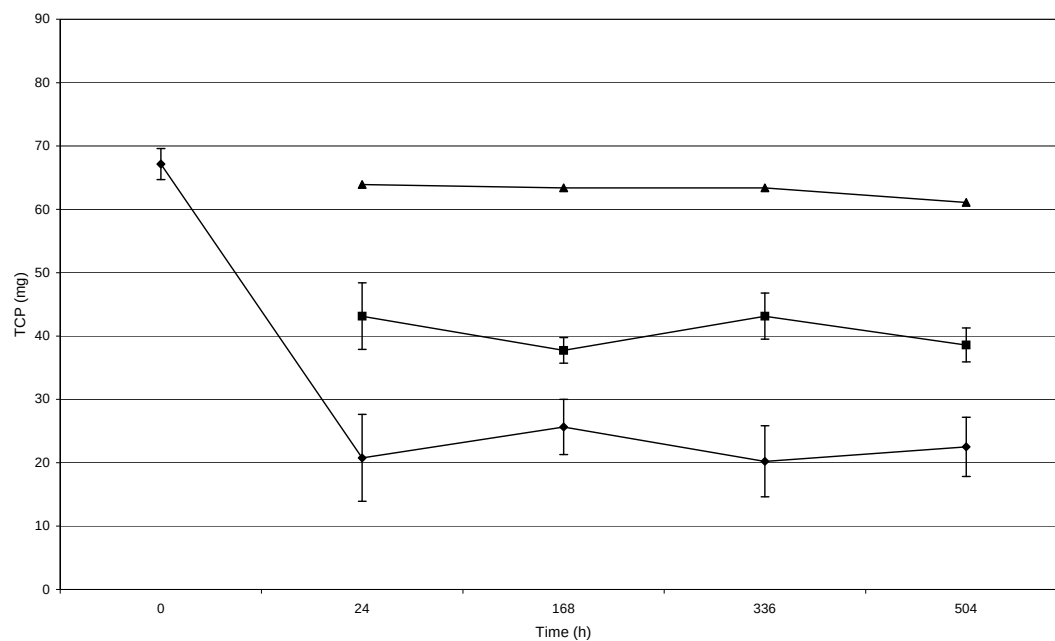


Figure 3.7 Liquid (mg) (◆) and sandy loam soil (mg per dry weight) (■) fractions and total recovered mass (▲) of TCP detected in control flasks.

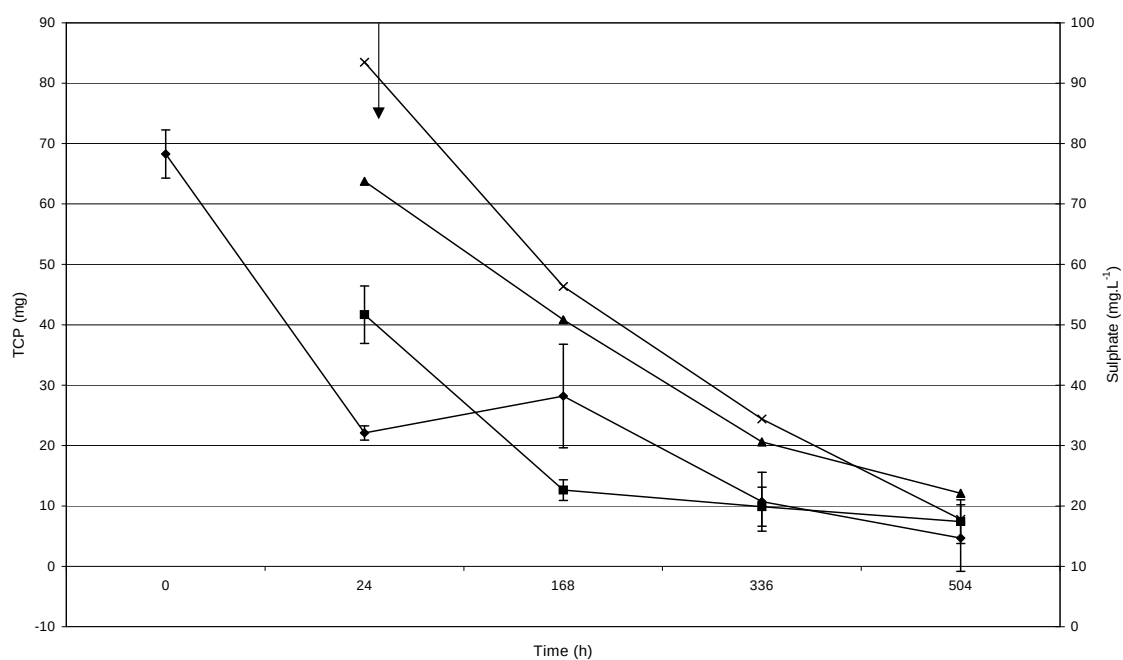


Figure 3.8 Liquid (mg) (◆) and sandy loam soil (mg per dry weight) (■) fractions, total recovered mass (▲) of TCP and sulphate (mg.L⁻¹) (X) detected in inoculated flasks. Arrow indicates introduction of Postgate medium B plus consortium.

An increase in dechlorination metabolites was seen during the final 336 h of the study. 38.9 % and 18.6 % 2,4-DCP of original immobilized TCP was detected in the liquid and soil fractions at 336 h, respectively. 4-CP concentration in both the liquid and sorbed fraction increased to 41.4 and 20.3 % after 3 weeks.

Sulphate reduction is documented in Figure 3.8. No sulphide was detected in the inoculated flasks possibly due to the precipitation of metal sulphides. Supernatant pH for control and inoculated flasks varied between 6.4 and 7.0 and 8.1 and 8.7, with total colony count increasing from 3×10^6 to 36×10^6 cells.g⁻¹ soil (dry weight) for the latter. Colony counts may be below actual soil numbers since complete bacterial mobilization from the surface of the soil was probably impaired by strong microorganism–soil-binding forces. No colonies were detected in the supernatant phases, control flasks or in fresh sandy loam soil using Postgate and CF medium.

3.3.3 Fluidized Packed-Bed Reactors

Two sulphate concentrations, 110 and 900 mg.L⁻¹, were introduced into two fluidized pine chip-packed bed reactors. The reactors were, therefore, operated in sulphate-deficient and sulphate-sufficient environments to assess the effect on the interdependent metabolism of the two cultures. The reactors were first fed with 110 mg.L⁻¹ sulphate. Samples from the outlets were quantified for effluent 2,4,6-TCP, 2,4-DCP, 4-CP and phenol, sulphate and sulphide, as well as pH.

Figures 3.9 and 3.10 present TCP and SO₄²⁻ concentrations introduced and TCP, SO₄²⁻ and sulphide concentrations measured in Reactor One (continuous flow) outlet for 110 and 900 mg.L⁻¹ SO₄²⁻, respectively. Tables 3.4 and 3.5 provide data contrasting average TCP and sulphate reduction detected for both reactors. Table 3.4 shows average TCP feed for Reactor One was 21.8 mg.L⁻¹, with an average 47.7 % reduction during 4 - 5 reactor culture volume changes in a sulphate-deficient environment. This contrasts with an 11.6 % reduction for the sulphate-sufficient concentration feed.

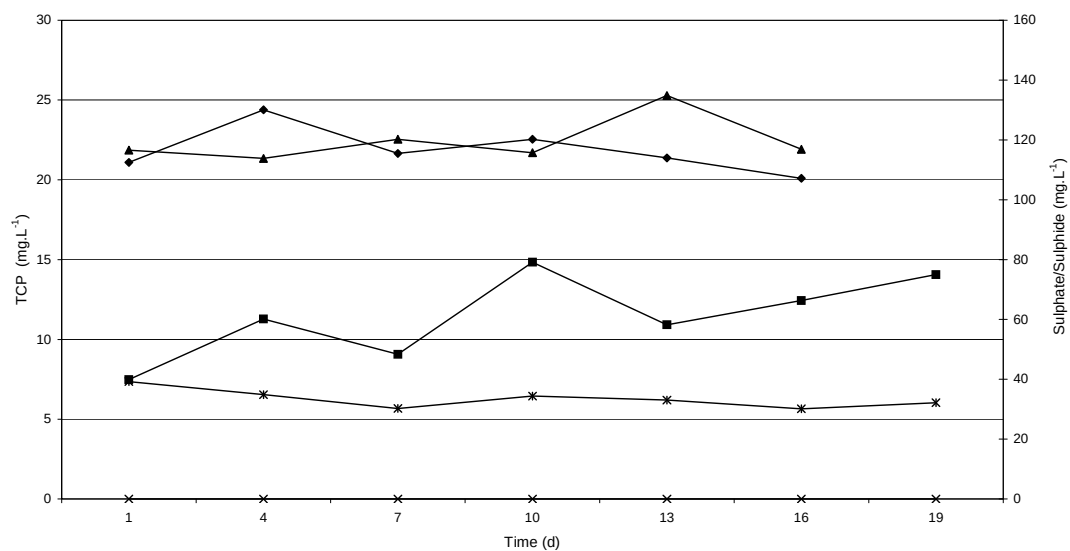


Figure 3.9 Influent (♦) and outlet (■) 2,4,6-TCP, influent (▲) and outlet sulphate (X) concentrations and culture fluid sulphide (✕) concentrations (mg.L⁻¹) for Reactor One perfused with 110 mg.L⁻¹ SO₄²⁻ during 4 to 5 culture volume changes.

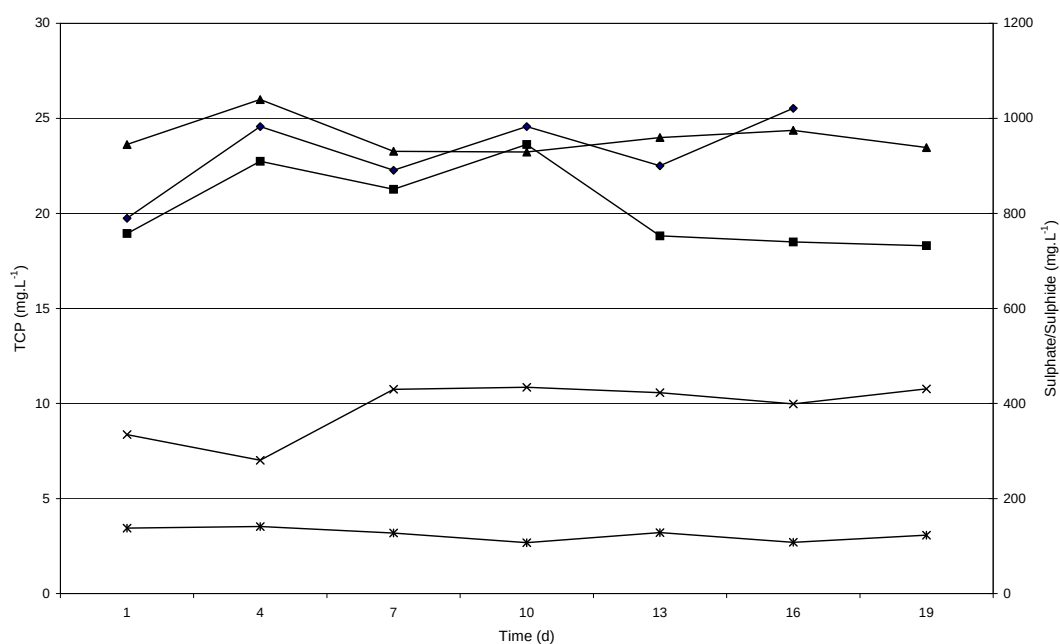


Figure 3.10 Influent (♦) and outlet (■) 2,4,6-TCP, influent (▲) and outlet sulphate (X) concentrations and culture fluid sulphide (✕) concentrations (mg.L⁻¹) for Reactor One perfused with 900 mg.L⁻¹ SO₄²⁻ during 4 to 5 culture volume changes.

Table 3.4. Average TCP load and TCP, 2,4-DCP, 4-CP and phenol outlet concentrations (mg.L⁻¹) for Reactors One and Two (110 and 900 mg.L⁻¹ SO₄²⁻)

Reactor	Average TCP in ^a (mg.L ⁻¹)	Average TCP out ^a (mg.L ⁻¹) /% ^b	Average DCP ^a (mg.L ⁻¹)	Theoretical DCP ^c (mg.L ⁻¹)	Average CP ^a (mg.L ⁻¹)	Theoretical CP ^c (mg.L ⁻¹)	Average phenol ^a (mg.L ⁻¹)	Theoretical phenol ^c (mg.L ⁻¹)
One (110)	21.8 ± 1.4	11.4 ± 2.6 /47.7	16.2 ± 1.6	18.0	5.5 ± 0.8	14.2	3.9 ± 1.3	10.4
One (900)	23.1 ± 2.1	20.4 ± 2.2 /11.6	7.0 ± 0.9	19.1	2.1 ± 0.4	15.1	0.9 ± 0.2	11.0
Two (110)	22.8 ± 1.1	11.4 ± 2.0 /50.0	18.6 ± 1.0	18.8	5.3 ± 1.4	14.9	4.0 ± 0.6	10.8
Two (900)	22.8 ± 1.1	18.4 ± 1.1 /19.2	7.4 ± 0.9	18.9	2.6 ± 1.1	14.9	1.1± 0.3	10.9

^aAverage for triplicate samples ± standard deviation.

^b% TCP reduction.

^cTheoretical metabolite concentrations calculated for total transformation of 2,4,6 TCP → 2,4-DCP → 4-CP → phenol by molecular weight loss.

Table 3.5. Average sulphate loads and sulphate and sulphide outlet concentrations (mg.L⁻¹) for Reactors One and Two (110 and 900 mg.L⁻¹ SO₄²⁻)

Reactor	Average sulphate in ^a (mg.L ⁻¹)	Average sulphate out ^a (mg.L ⁻¹) /% ^b	Average sulphide in culture fluid ^a (mg.L ⁻¹)	Theoretical sulphide ^c (mg.L ⁻¹)
One (110)	119.6 ± 7.7	0	33.4 ± 3.1	41.2
One (900)	959.7 ± 38.8	390.2 ± 29.3 (59.3)	124.2 ± 13.2	196.1
Two (110)	117.9 ± 3.9	0	24.1 ± 5.5	40.6
Two (900)	907.5 ± 31.9	375.3 ± 55.4 (58.6)	173.5 ± 20.2	183.3

^aAverage for triplicate samples ± standard deviation.

^b% sulphate reduction.

^cTheoretical HS⁻ concentration calculated from $2\text{CH}_3\text{CHOHCOO}^- + \text{SO}_4^{2-} \rightarrow 2\text{CH}_3\text{COO}^- + 2\text{HCO}_3^- + \text{HS}^- + \text{H}^+$.

Average sulphate-deficient feed outlet 2,4-DCP concentration for Reactor One was 16.2 mg.L⁻¹ which equates to 90 % of the theoretical DCP outlet concentration (18.0 mg.L⁻¹) calculated on the premises of complete transformation of influent 2,4,6-TCP (21.8 mg.L⁻¹) to 2,4-DCP, in agreement with molecular weight loss. The sulphate-sufficient feed effected 36.6 % of theoretical 2,4-DCP concentration (19.1 mg.L⁻¹) which averaged 7.0 mg.L⁻¹ during the reactor operation. 4-CP and phenol was also detected in Reactor One outlet for both sulphate-deficient and sufficient feeds, however, the measured concentrations were lower than expected values if total conversion (2,4-DCP to 4-CP and 4-CP to phenol) was to be forecasted (Table 3.4). The transition from 2,4-DCP to 4-CP was relatively slow, with 38.7 and 13.9 % of expected 4-CP concentrations for 110 and 900 mg.L⁻¹ introductory SO₄²⁻ concentrations, respectively. Average phenol concentrations detected were 3.9 and 0.9 mg.L⁻¹ (110 and 900 mg.L⁻¹ SO₄²⁻). Figures 3.11 and 3.12 document the dechlorination products detected for Reactor One.

As can be seen from Figures 3.9 and 3.10 and Table 3.5, sulphate was completely consumed in the sulphate-deficient feed but not entirely reduced in the sufficient feed, with a maximum of 575.8 - 610.5 mg.L⁻¹ consumed (average 59.3 %). 5 mM SO₄²⁻ was needed for the incomplete oxidation of lactate (10 mM) by the SRB, calculated from the following equation: $2\text{CH}_3\text{CHOHCOO}^- + \text{SO}_4^{2-} \rightarrow 2\text{CH}_3\text{COO}^- + 2\text{HCO}_3^- + \text{HS}^- + \text{H}^+$ (Drzyzga *et al.*, 2002).

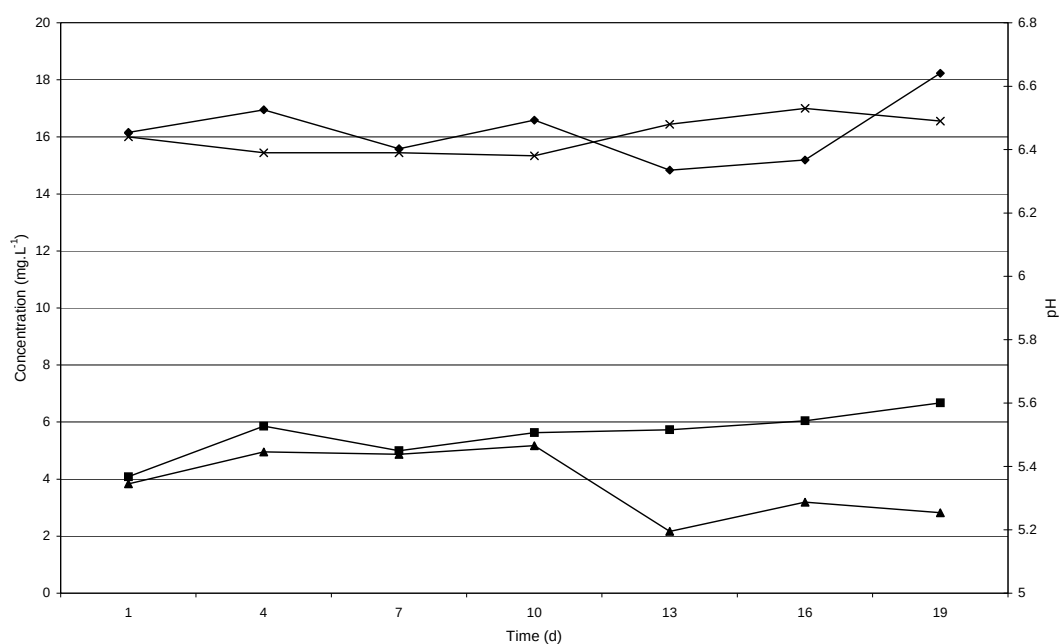


Figure 3.11 Reactor One culture fluid 2,4-DCP (♦), 4-CP (■) and phenol (▲) concentrations (mg.L⁻¹) and pH (X), perfused with 110 mg.L⁻¹ SO₄²⁻ during 4 to 5 culture volume changes.

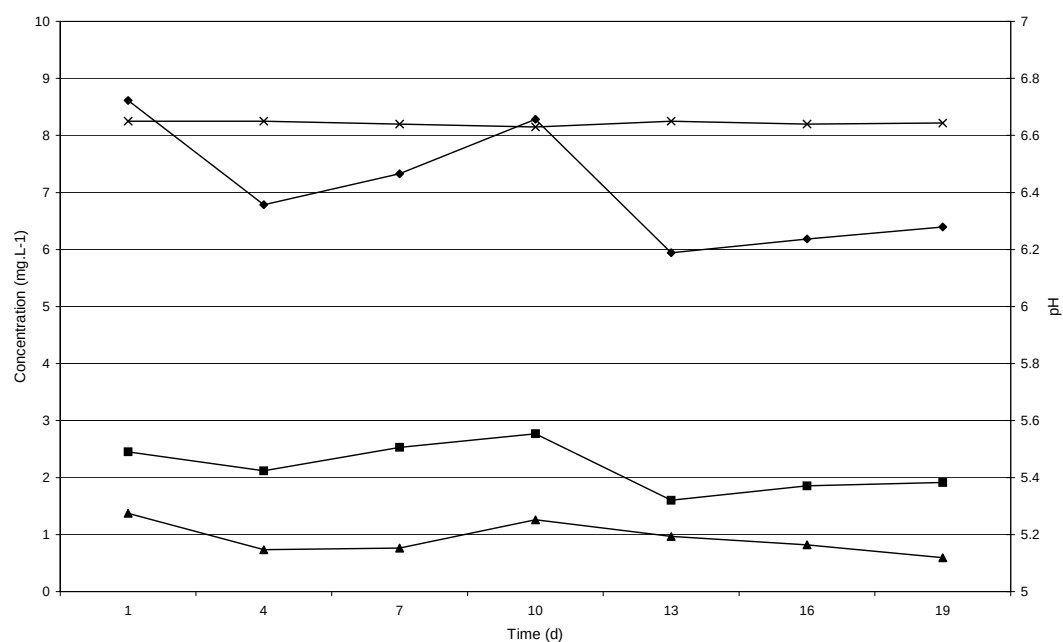


Figure 3.12 Reactor One culture fluid 2,4-DCP (♦), 4-CP (■) and phenol (▲) concentrations (mg.L⁻¹) and pH (X), perfused with 900 mg.L⁻¹ SO₄²⁻ during 4 to 5 culture volume changes.

With a measured average SO_4^{2-} feed concentration of 959.7 mg.L^{-1} ($\approx 10 \text{ mM SO}_4^{2-}$) and average outlet concentration 390.2 mg.L^{-1} (4.1 mM SO_4^{2-}), 5.9 mM SO_4^{2-} was consumed in the reactor. Complete depletion of 10 mM lactate was, therefore, expected. With average $119.6 \text{ mg.L}^{-1} \text{ SO}_4^{2-}$ in feed solution, 100% was consumed and since sulphate was only available at $\approx 25 \%$ of required concentration to bring about complete lactate consumption, therefore, it can be expected that lactate was not completely used up by sulphate reduction.

Sulphide levels measured in reactor culture fluid were below that to be predicted in relation to sulphate reduction (Table 3.5). This can be expected since hydrogen sulphide was evacuated via the off-gas of the reactor headspace. Upon visual inspection of the zinc acetate traps, the presence of precipitates indicated the formation of insoluble zinc sulphides. The accumulation of black metal precipitates on the surface of the wood chips inside the reactor (Figure 3.13) could have contributed to lower than expected culture fluid sulphide concentrations.

Total bacteria colony counts for the reactor effluent were $2.8 \times 10^7 \text{ cells.ml}^{-1}$ and $80 \times 10^7 \text{ cells.ml}^{-1}$ (110 mg.L^{-1} and $900 \text{ mg.L}^{-1} \text{ SO}_4^{2-}$).



Figure 3.13 Black metal precipitates on the surfaces of wood chips due to sulphate reduction in the reactor.

Recycling together with continuous operation showed similar results for Reactor Two (Figures 3.14 and 3.15). Average TCP concentration detected in reactor outflow was lower (11.4 mg.L^{-1}) for sulphate-deficient feed compared to sulphate-sufficient operation (18.4 mg.L^{-1}). 2,4-DCP, 4-CP and phenol were also detected (Figures 3.16 and 3.17). When comparing reductive dechlorination performance, it can be seen that average TCP reduction was somewhat lower in Reactor One with 47.7 % removed compared to 50 % in Reactor Two for the $110 \text{ mg.L}^{-1} \text{ SO}_4^{2-}$ feed (Table 3.4). A notable difference is more evident for the $900 \text{ mg.L}^{-1} \text{ SO}_4^{2-}$ feed. 19.2 % TCP reduction was detected in Reactor Two outlet compared to 11.6% (Reactor One)

18.6 mg.L^{-1} 2,4-DCP was detected ($110 \text{ mg.L}^{-1} \text{ SO}_4^{2-}$ influent feed) which translates to > 98 % of the theoretically expected concentration if total dechlorination of influent TCP to DCP occurred. This, however, did not take place as can be seen in Table 3.4. 4-CP and phenol concentrations matched those detected in Reactor One.

Similar sulphate concentration decreases were detected for Reactor Two (Table 3.5). Incomplete sulphate consumption was detected for sulphate-sufficient feed (Figure 3.15). Total plate counts for Reactor Two were 4.5×10^7 and $231 \times 10^7 \text{ cells.ml}^{-1}$ for the 110 and $900 \text{ mg.L}^{-1} \text{ SO}_4^{2-}$ feeds, respectively.

Figures 3.18 and 3.19 present a time course of the percentage concentrations of 2,4,6-TCP and dechlorination metabolites detected in reactor culture liquid relative to the inlet TCP concentrations during perfusion with two sulphate concentrations for Reactors One and Two, respectively. The corresponding TCP effluent and displayed 2,4-DCP, 4-CP and phenol concentrations demonstrate the performance of dechlororespiration relative to sulphate concentration and occurrence of sulphate reduction.

Figure 3.20 (A; B) shows scanning electron microscope (SEM) micrographs of the surfaces of pine chips collected from the reactors. The attachment to and colonization of the wood chips by bacterial cells can be seen. Figure 3.20 (C) displays a SEM micrograph of the reactor liquid consortium captured onto the surface of $0.22 \text{ }\mu\text{m}$ filter paper.

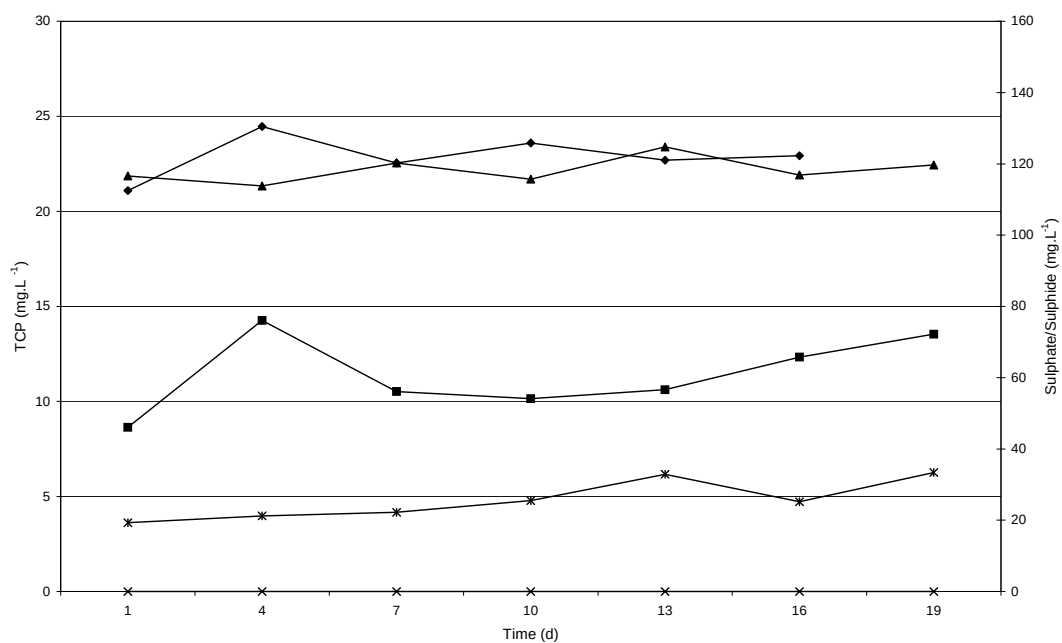


Figure 3.14 Influent (♦) and outlet (■) 2,4,6-TCP, influent (▲) and outlet (X) sulphate concentrations and culture fluid sulphide (✱) concentrations (mg.L⁻¹) for Reactor Two perfused with 110 mg.L⁻¹ SO₄²⁻ during 4 to 5 culture volume changes.

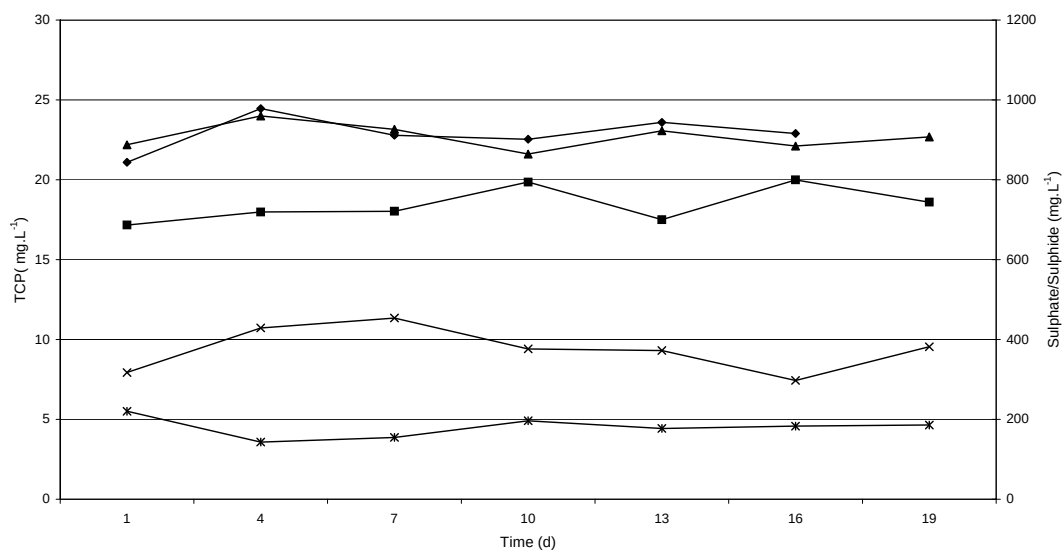


Figure 3.15 Influent (♦) and outlet (■) 2,4,6-TCP, influent (▲) and outlet (X) sulphate concentrations and culture fluid sulphide (✱) concentrations (mg.L⁻¹) for Reactor Two perfused with 900 mg.L⁻¹ SO₄²⁻ during 4 to 5 culture volume changes.

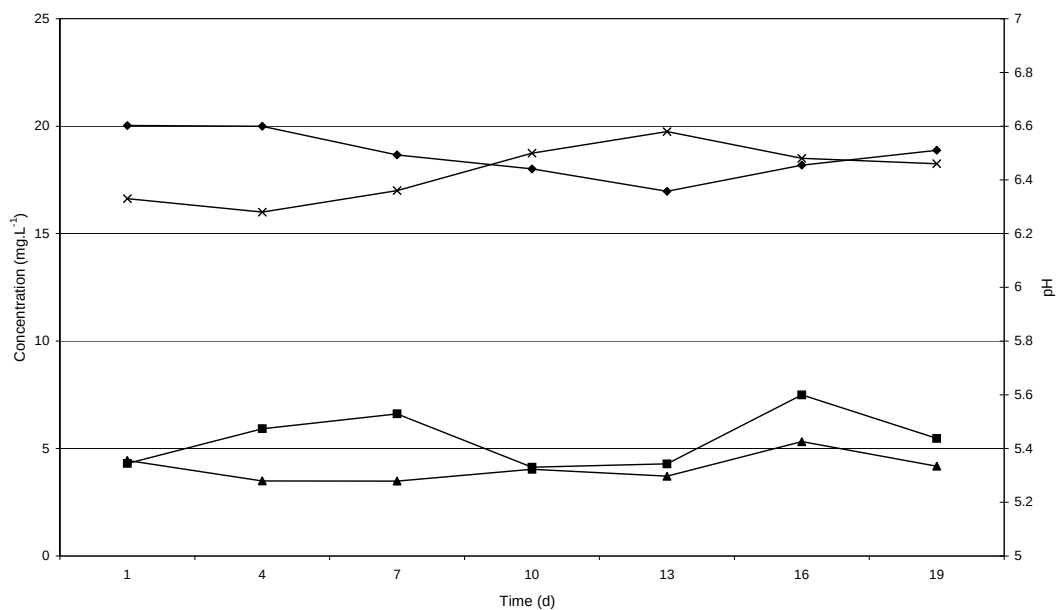


Figure 3.16 Reactor Two culture fluid 2,4-DCP (◆), 4-CP (■) and phenol (▲) concentrations (mg.L⁻¹) and pH (X), perfused with 110 mg.L⁻¹ SO₄²⁻ during 4 to 5 culture volume changes.

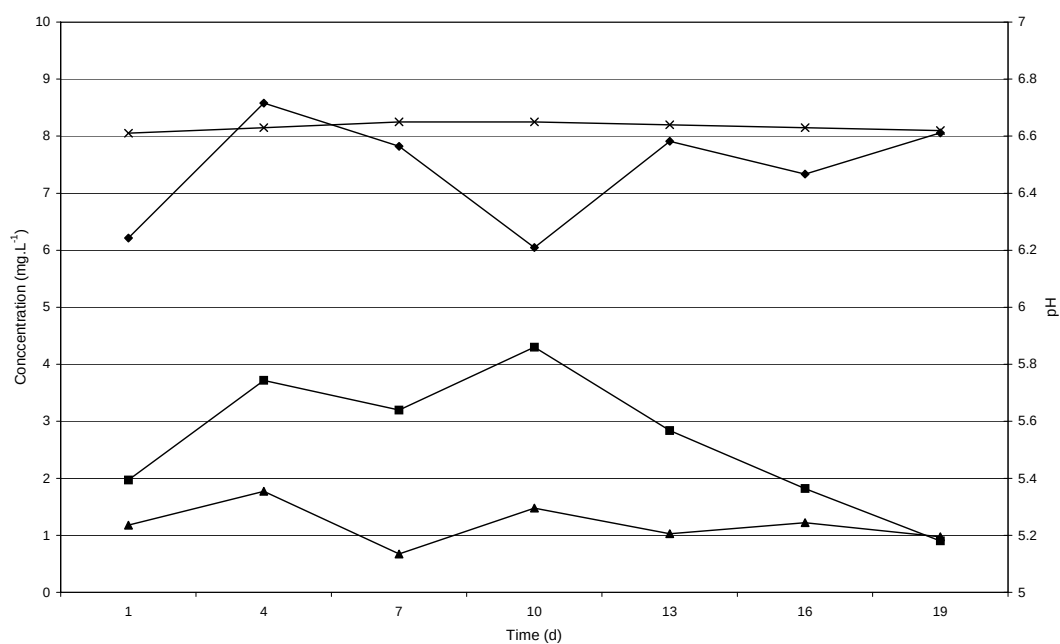


Figure 3.17 Reactor Two culture fluid 2,4-DCP (◆), 4-CP (■) and phenol (▲) concentrations (mg.L⁻¹) and pH (X), perfused with 900 mg.L⁻¹ SO₄²⁻ during 4 to 5 culture volume changes.

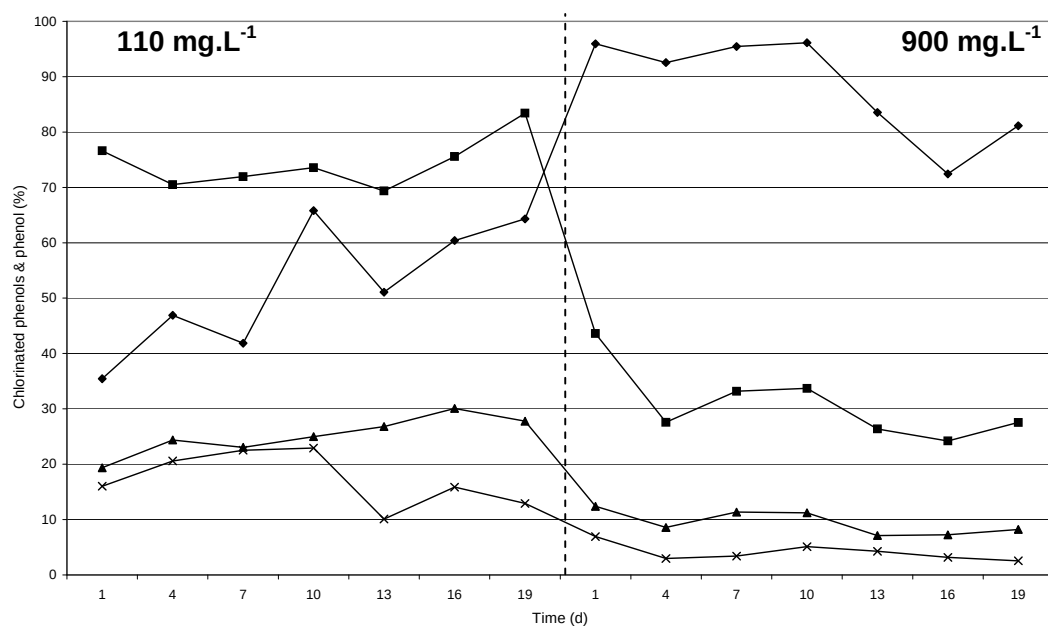


Figure 3.18 Time course of the percentage 2,4,6-TCP (◆), 2,4-DCP (■), 4-CP (▲) and phenol (X) concentrations detected in Reactor One culture liquid relative to the inlet TCP concentrations perfused with 110 and 900 mg.L⁻¹ SO_4^{2-} .

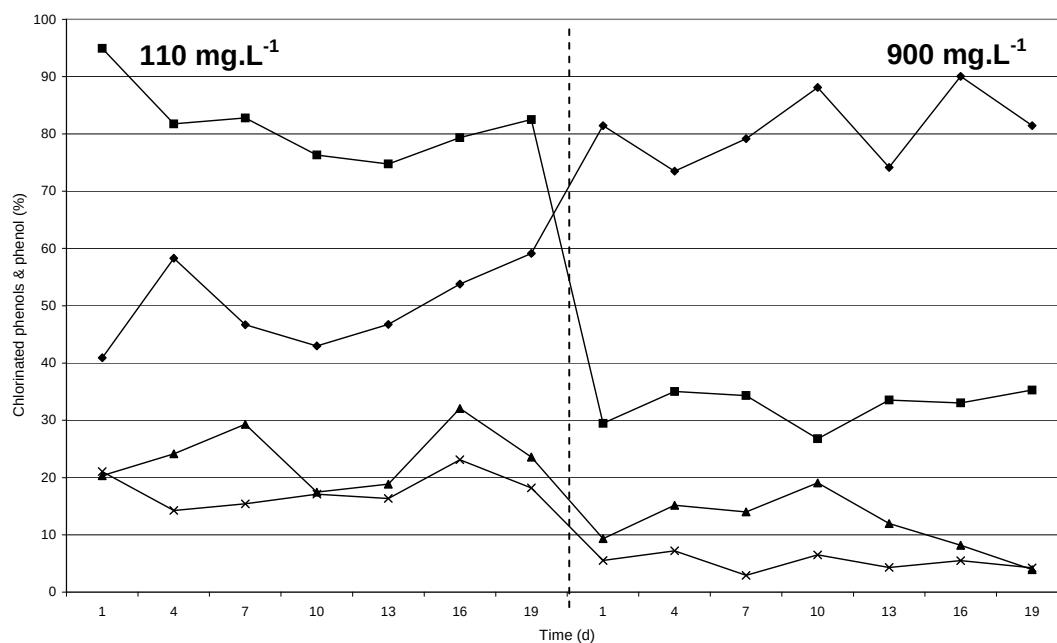
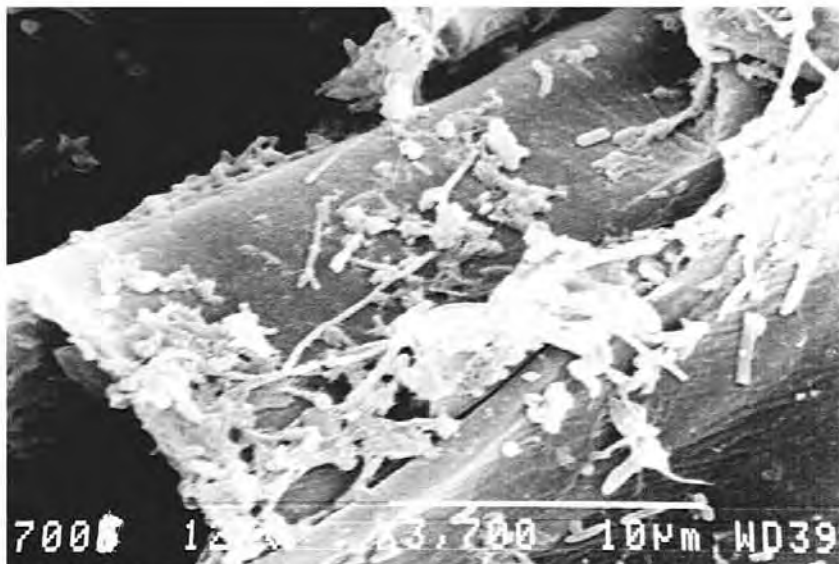


Figure 3.19 Time course of the percentage 2,4,6-TCP (◆), 2,4-DCP (■), 4-CP (▲) and phenol (X) concentrations detected in Reactor Two culture liquid relative to the inlet TCP concentrations perfused with 110 and 900 mg.L⁻¹ SO_4^{2-} .

A



B



C

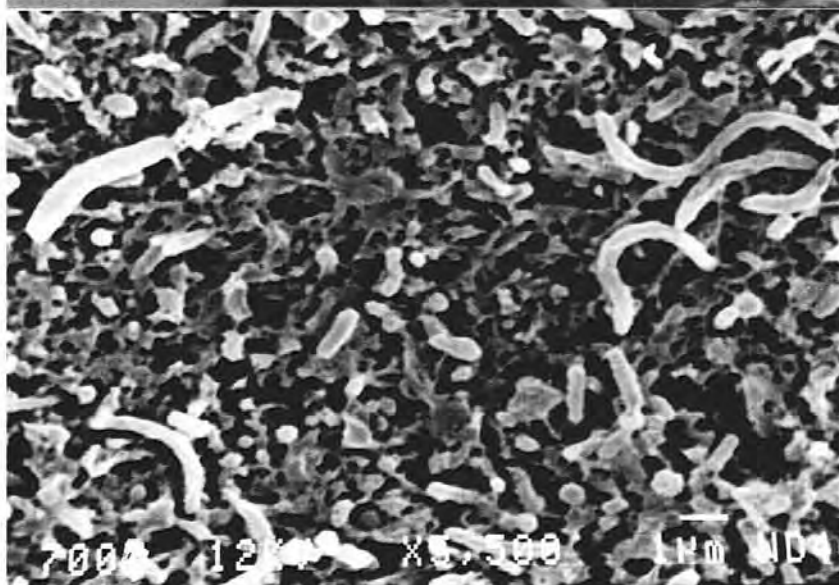


Figure 3.20 SEM micrographs of anaerobic consortium colonizing surfaces of pine chips (A; B) and culture liquid (C).

3.4 Discussion

Sulphate-reducing-dependent dechlorination was shown in this study. Transformation of 2,4,6-TCP to 2,4-DCP, 4-CP and phenol was accomplished by dechlororespiring bacteria which appeared to occur in a syntrophic relationship with SRB. Dehalogenation was dependent on the activity of SRB which, in turn, was affected by sulphate and lactate concentration.

Since the conditions in which sulphate was available in lower amount than that required to bring about complete consumption of lactate, it can be speculated that a combination of sulphate reduction and fermentation of lactate produce H_2 equivalents which was carried out by the SRB. The fermentation of yeast extract most likely contributed as well since it has been shown to support reductive dechlorination (Madsen and Aamand, 1992). SRB, for example *Desulfovibrio vulgaris*, *Desulfovibrio desulfuricans* and *Desulfovibrio gigas*, have been shown to metabolize substrates such as lactate with concomitant production of acetate, H_2 and CO_2 when grown in sulphate-deficient or depleted media (Bryant *et al.*, 1977; Cabirol *et al.*, 1998; Traore *et al.*, 1983). The dechlororespiring bacteria consume H_2 equivalents to drive the process. The high TCP to DCP dechlorination rate and 4-CP and phenol concentrations detected for sulphate-deficient conditions was, therefore, due to residual lactate which was fermentatively used with the release of H_2 equivalents. In contrast, when sulphate was available in concentration more than that needed to consume lactate, sulphate reduction was the dominant process and no lactate, or only small amounts of lactate were available for fermentative metabolism. Consequently, low H_2 equivalents would be produced with accompanying reduced dechlorination rates as was seen in the reactor.

The supposition was made on the premise that the chlororespiring bacteria are dependent on the fermentative activity of the SRB to produce H_2 equivalents. The dechlororespires are incapable of utilizing the lactate and/or yeast extract directly, or at least able to share the electron donor substrate(s) along with H_2 equivalents for dehalorespiration and growth, though competition for the substrate(s) occurred during sulphate-sufficient conditions. Molybdate was used to inhibit sulphate reduction in the batch experiment. With zero sulphate reduction (by using the competitive inhibitor), SO_4^{2-} was not limited to facilitate a switchover to fermentation thus no dechlorination occurred.

pH for sulphate-deficient feed culture fluids for both reactors was in the range 6.28 – 6.5 compared to 6.61 – 6.65 for sulphate-sufficient reactors (Figures 3.11, 3.12, 3.16 and 3.17). The suggested fermentative metabolism during sulphate-deficient conditions and associated production of H₂ equivalents would have effected a lower pH environment compared to sulphate reduction as the dominant process.

Total bacteria colony counts for the reactor effluent provided for further insight into the consortium composition at the two sulphate influent concentrations. Sulphate reduction was the principal process occurring under sulphate-sufficient conditions, therefore, SRB would be the predominant culture and hence the large total cell count. In comparison, under sulphate-deficient conditions lower cell counts (incomplete consumption of lactate) showed the dechlororespiring culture successfully functioning together with SRB.

Operating Reactor Two in recycling mode probably re-introduced low H₂ equivalent concentrations from sulphate-sufficient inhibited fermentation back into the reactor to drive dechlororespiration and consequently brought about the higher percentage TCP reduction.

Drzyzga and Gottschal (2002) and others (Cabirol *et al.*, 1998; Colberg, 1990; Drzyzga *et al.*, 1994; 2001; 2002; Mägli *et al.*, 1996; Traore *et al.*, 1983) have speculated about the possible role SRB play as hydrogen-providing organisms in anoxic environments contaminated with halogenated organic pollutants. The results from the current investigation supports the observations made by Drzyzga and Gottschal (2002) that bacterial dechlororespiration of chlorinated aliphatic compounds (PCE used in their study) and chlorinated aromatic compounds (TCP used in this study) may be indirectly stimulated by the fermentative activity of SRB under sulphate-limiting conditions. This has substantial potential for chlorinated organic-contaminated soil and aquifer remediation strategies since oxygen is frequently limited in these habitats and the electron donor:sulphate ratio employed should facilitate the switchover to fermentative metabolism by the SRB.

Löffler *et al.* (1999) speculated that chlororespiring bacteria may prevail in anaerobic environments, though the process of reductive dechlorination could be limited by the concentration or bioavailability of chlorinated organic compounds.

Although the sulphate-deficient flasks supported a higher TCP dechlorination rate, the process seemed to be less effective and slower in fed-batch operation compared to continuous immobilized reactor mode even though the flasks were incubated at constant temperature (28 °C). Various factors could have contributed to this. The consortium was not immobilized in the flasks which were operated as free-cell cultures. Fed-batch operation necessitated manual addition of electron donors and sulphate resupplementation, and the accumulation of sulphide in culture fluid has been shown to inhibit dechlororespiring bacteria (R.A. Sanford, pers. comm.). Armenante *et al.* (1992) concluded from free-cell batch vs. silica bead immobilized cell configurations that higher dechlorination rates were observed for immobilized bacteria plus they were able to withstand shock loads.

This study also clearly indicated the potential use of the sulphidogenic dechlororespiring microbial consortium to augment trichlorophenol-contaminated soil together with required carbon sources and SO_4^{2-} . TCP was mobilized and dechlorinated in both soil types when inoculated with the consortium. It did appear that inoculating with the exogenous sulphidogenic bacterial consortium, the immobilized TCP fraction was desorbed when compared to adsorption–desorption trends in the controls. The culture liquid pH was higher for the inoculated flasks for both soils. pH has been implicated in affecting the desorption behaviour of CP due to the fact that these compounds are ionizable organics and can exist as either protonated or deprotonated species (You and Liu, 1996). While it has been observed that the amount of CP desorption increases with increasing pH (deprotonated phenolate species), You and Liu (1996) found that specifically in the case of PCP, 2,4,6-TCP and 2,6-DCP, desorption did not increase when pH was greater than 7.0. Chang *et al.* (1996) observed more rapid dechlorination of PCP and 2,4,6-TCP in an alkaline soil (sandy loam, pH 7.3) compared to clayey loam soil (pH 6.6) and was attributed to less sorption and favourable conditions for microbial activity. They also established that dechlorination in the soil was the result of microbial activity and that the addition of a CP-adapted consortium facilitated the transformation of the pollutants.

Even though pH measurements were between 6.95 and > 8.0 for the inoculated flasks compared to between 6.3 and 7.0 for controls, mobilization of deprotonated TCP in the culture-augmented flasks was probably not further enhanced by the pH increase above 7 (You and Liu, 1996). Dechlorination in the sandy loam soil also was more rapid in the current investigation with high 2,4-DCP and 4-CP concentrations detected in the liquid fractions of the flasks which indicates the release of TCP before transformation and/or desorption of metabolites due to their lower hydrophobicity and concomitant dechlorination to lower chlorinated compounds in the supernatant.

The actual mechanisms by which microorganisms can utilize adsorbed compounds have not been actively investigated. Alexander (1999) proposed microbial-facilitated desorption and the direct utilization of sorbed compounds by microorganisms as possible mechanisms. The mechanism of facilitated desorption is still unresolved but it is suggested that it could involve the production of natural surfactants (biosurfactants) that release the compounds from the soil surface, cations that displace a charged compound, extracellular enzyme secretion, or a microbiologically-induced change in the pH at the surface of soils. The direct uptake and metabolization of sorbed compounds by a microorganisms-proposed mechanism is based on the fact that many bacteria attach themselves to surfaces. Frequently, most bacteria in ecosystems with clay and organic carbon particulate matter are associated with the solid fraction rather than the liquid (Alexander, 1999; Edgehill, 1999).

A study has shown that cells attached to solid surfaces promote desorption and biodegradation of immobilized 3-chlorodibenzofuran (Harms and Zehnder, 1995). Furthermore, microorganisms may come in direct contact with the sorbed compound which then penetrates into the cells when adhered to the same surface due to hydrophobic (solid surface, compound and microorganism cell surface) interactions. Since colonies were only detected in the soil fractions of the inoculated flasks, it is assumed that the cell densities were retained on the surfaces of the soils. This could have assisted in desorption and/or dechlorination of TCP to lesser-chlorinated phenols.

Linkfield *et al.* (1989) hypothesized that the acclimation period before anaerobic dechlorination begins is caused by the requirements of new enzymes, and may also be

effected by chemical structure and concentration of the chlorinated substrate. The successful enrichment for and establishment of a dechlorinating culture in this study serves as an indication of the existence of an inducible enzyme system for reductive dechlorination and given the right conditions, substantial potential exists for the acclimation to halogenated pollutants in anaerobic environments.

The reductive dechlorination of 2,4,6-TCP was an incomplete transformation process since degradation of phenol was not detected. The parental compound released by mobilization as was shown in the soil study and the dechlorinated products would have to undergo further treatment. A complimentary treatment stage would, therefore, be a necessity. Liquid would be pumped from the below-ground environment to an aerobic immobilized white-rot fungal reactor to mineralize the residual pollutant and its metabolites. The study indicated the impact of different sulphate concentrations on dechlororespiration rates. Though reduced or deficient sulphate levels increased the rate at which chlorines are removed from the ring compound structure due to the switchover to a fermentative metabolism and concomitant production of H_2 equivalents, excess sulphate concentrations did support reductive dechlorination of TCP. Implementing *in-situ* enhanced dechlororespiration would require the introduction of an electron donor, sulphate and, if necessary, a laboratory-adapted culture to the contaminated zone. The ratio of electron donor: SO_4^{2-} should ensure the exhaustion of sulphate and incomplete consumption of the carbon source to establish H_2 equivalent production. The ratio of lactate: SO_4^{2-} in the current investigation was $\approx 9:1$ where enhanced reductive dechlorination was detected. This is lower than the required 2:1 ratio for complete lactate consumption.

The dechlorination component of the bioprocess strategy could also be employed as a reactor unit in an anaerobic/aerobic bioreactor configuration for the treatment of organochlorine-contaminated industrial effluents. The transformation of halogenated pollutants to lower chlorinated metabolites followed by mineralization in the fungal reactor will reduce the chance of potentially toxic and endocrine disrupting metabolite production that may occur when the aerobic reactor is used as a free-standing wastewater treatment system.

Chapter Four details investigation of the linkage of the two reactor systems. The integrated bioprocess strategy was employed to eliminate TCP and the dechlorination metabolites produced during dechlororespiration.

Chapter Four

An Integrated Anaerobic/Aerobic Bioreactor Configuration and Degradation of Chlorinated Phenol

4. Introduction

The objective of this study was to assess the feasibility of an integrated anaerobic (sulphidogenic-affected reductive dechlorination) - aerobic (fungal-mediated mineralization) strategy to completely biodegrade a model chlorinated aromatic compound. The proposed dual stage strategy was separately studied in two independent compartments: an anaerobic fluidized packed-bed sulphidogenic-dependent dechlororespiration reactor (SDR) and aerobic immobilized white-rot fungal trickling packed-bed reactor (IFR). The two reactors were then linked to simulate the proposed degradation strategy for subsurface soil bioremediation where sulphidogenesis-assisted desorption would be followed by complete mineralization. The overall biodegradation efficiency was tested and performance of individual systems was monitored to assess the effect of one system on the other. Sulphate reduction produces an alkaline environment due to HS^- output whereas white-rot fungi favours an acidic environment to facilitate growth and extracellular enzyme secretion. It was thus anticipated that a complete sulphur cycle would operate in the background of the mineralization process with sulphate reduction (alkaline reaction) followed by sulphide oxidation (acidifying reaction).

4.2 Materials and Methods

4.2.1 The Linked Anaerobic/Aerobic Reactor Configuration

Pine chip-packed IFR and SDR were inoculated and started-up as previously outlined (2.2.2; 3.2.4). The reactors were independently fed with the specific growth medium (Appendix A) and 2,4,6-TCP supplemented in Postgate medium B (for SDR) only to a final concentration of 30 mg.L^{-1} . Oxygen-free N_2 was continuously purged into the feeding reservoir of the SDR at 30 ml.min^{-1} and into the headspace and sampling port S6 (10 cm) of the reactor to evacuate hydrogen sulphide to a zinc acetate (10.4 g.L^{-1})

gas trap twice daily. This was also done to maintain anaerobic conditions in the top section of the reactor due to the aerated stream introduced from the IFR → SDR feed loop (Figure 4.1). After a 20 d recycling period through an external loop to allow establishment of the anaerobic consortium in the SDR (recycle flow rate 35 ml.h⁻¹), the operation was changed to continuous mode for three culture volume changes before reactors were linked.

To allow fungal attachment to wood chips, reactor outlet was recycled through an external loop for up to 5 d (recycle flow rate 336 ml.h⁻¹). The IFR was continuously aerated with humidified air through an in-line membrane filter at 1.2 L.min⁻¹. A process flow diagram of the integrated system is presented in Figure 4.1. The reactors were linked via an anaerobic-to-aerobic loop from the bottom-to-top recycle loop of the SDR and the outlet was introduced into the top of the IFR. The aerobic-to-anaerobic return loop was connected to draw 15 cm from the bottom of the IFR. This was done to maintain the recirculation volume (RV) at 20% of void volume-level. The IFR-to-SDR loop was introduced into the top of the SDR.

The flow rate for the SDR and IFR feed loop, SDR recycling loop, SDR-to-IFR and IFR-to-SDR loops was kept constant at 35 ml.h⁻¹. The recycling flow rate for the IFR was 336 ml.h⁻¹. Retention times for the SDR and IFR were 102.6 and 20.2 h, respectively. The IFR was fed constantly with induction media (Appendix A), however, 2 g.L⁻¹ glucose and 0.8 g.L⁻¹ yeast extract was added to supply fungi with a continuous source of easy available carbon. Feed SO₄²⁻ concentration into the SDR was 900 mg.L⁻¹. Although previous results has shown sulphate-limiting conditions to enhance dechlororespiration activity, it was decided to subject the fungal reactor to higher sulphide loads (from SDR) to assess the potential inhibitory effects of the interaction. SDR overflow was collected via a drain located 3 cm from the top of the reactor.

The linked reactors were operated and monitoring was commenced for a period of 45 d and after three complete medium volume changes occurred steady-state conditions were assumed. About nine complete medium volume changes occurred during 45 d since total retention time for the linked system was 122.8 h. Figure 4.2 shows the linked reactor system operated in the laboratory.

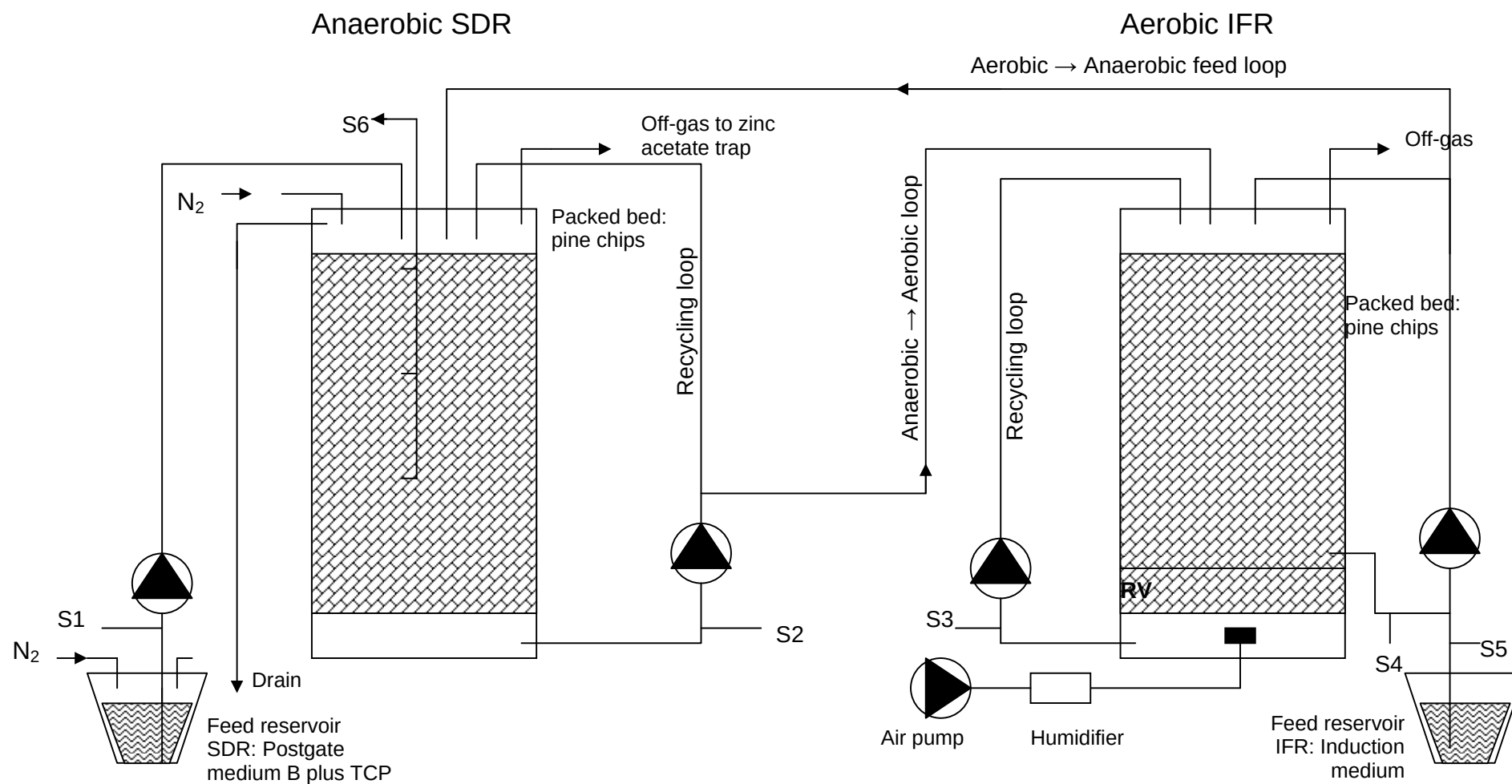


Figure 4.1 Process flow diagram for the laboratory bench-scale reactor system. Anaerobic fluidized packed-bed sulphidogenic-dependent dechlororespiration reactor, SDR; Aerobic immobilized white-rot fungal trickling packed-bed reactor, IFR; S1 – 6, sampling ports 1 – 6; RV, recirculation volume.

Samples were collected from sampling ports S2, S3 and S4 (Figure 4.1) for quantification of TCP and its metabolites (2,4-DCP, 4-CP and phenol), sulphate, sulphide, chemical oxygen demand (COD) and pH. From S1, samples were removed for influent TCP, sulphate and COD concentrations and from S5 IFR influent COD levels. Sampling port 6 was used to monitor pH at different levels inside the SDR (10, 20 and 40 cm).

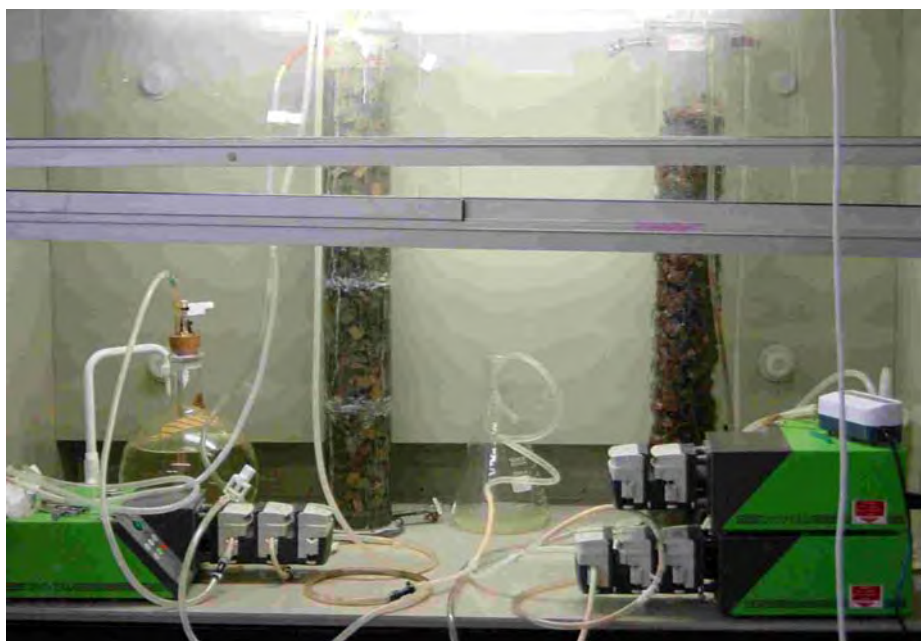


Figure 4.2 Linked anaerobic/ aerobic bioreactor configuration operated in a fume hood in the laboratory.

4.2.2 Analytical Methods

TCP 2,4-DCP, 4-CP and phenol, sulphate and sulphide analyses were performed according to methods outlined in Appendices B, D and E. The COD was determined using the Merck Spectroquant COD kit.

4.3 Results

2,4,6-TCP transformation with concomitant production of 2,4-DCP, 4-CP and phenol in the SDR is shown in Figure 4.3. Average TCP loading concentration for the 45-d operation was 28.0 mg.L^{-1} (measured at S1) with average outlet concentration 19.8

mg.L⁻¹ (Table 4.1). Samples were collected every 4 d (from S2) which equates to one HRT for the SDR. An average 29.2 % TCP reduction per HRT was calculated for the reactor which is a 10 % increase in dechlorination performance compared to average TCP outlet concentration for separate reactor operation (Reactor Two, see section 3.3.3). A decrease in removal was observed between days 8 and 12 (Figure 4.3) during the operation of the linked system, with only 6.6 – 9.6 % of TCP dechlorinated during that time. This together with sulphate reduction and sulphide production trends (Figures 4.5 and 4.6) indicated partial inhibition of the dechlorination process in the SDR.

Average sulphate reduction over the first 12 d was 39.8 %. If the total SDR SO₄²⁻ load from influent reservoir measured at S1 plus IFR outlet SO₄²⁻ concentration (measured at S4) is considered, an average 52.7 % reduction is calculated per four sampling times (Table 4.2). Sulphide production decreased during the same period (Figure 4.6) from 178.0 mg.L⁻¹ detected at the start of the monitoring programme to 46.9 mg.L⁻¹ at day 12. Average HS⁻ production for the period was 111.9 mg.L⁻¹ (Table 4.2).

The SDR culture fluid pH measured at sampling ports S2 and S6 (inside reactor) showed a decrease from 6.67 to 6.17 for S2 and 6.64 to 6.4 (S6) between days one and 12. It was assumed that the pH was affected by the outflow from the fungal reactor when introduced into the SDR. The IFR operated at pH 3.78 – 5.38. The lowered pH reduced sulphate reduction activity which, in turn, influenced TCP dechlorination since sufficient or excess SO₄²⁻ levels inhibit dechlororespiration (3.3.3). A pH range of 6.8 – 7.9 has been shown to be optimal for dechlorination with the rate increasing as the pH value increases, though pH dependency for a specific reactor application may vary from system to system (Ning *et al.*, 1998).

It was decided to apply pH correction to the reactor at this stage. 7.5 ml of a N₂-purged 1 M NaOH solution was introduced into the reactor via sampling port S6 at 10 cm. From there on, pH was measured on a daily basis and corrected when needed. This proved sufficient to increase pH to 6.6 – 6.71 (S6) and 6.69 – 6.83 measured at the outlet (S2). During the 33 d following pH correction, sulphate reduction increased to an average 229.6 mg.L⁻¹ outlet concentration and sulphide production 199.7 mg.L⁻¹ (Table 4.2). This,

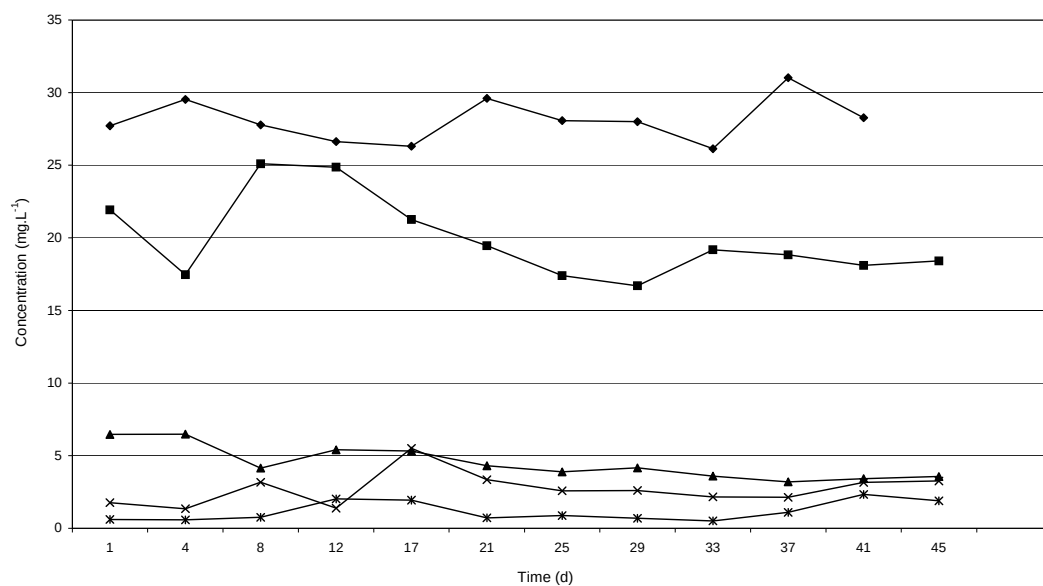


Figure 4.3 Influent (◆) (sampling port S1) and outlet (■) TCP, and 2,4-DCP (▲), 4-CP (X) and phenol (X) concentrations (sampling port S2) (mg.L⁻¹) detected for SDR during the 45 d linked operation period.

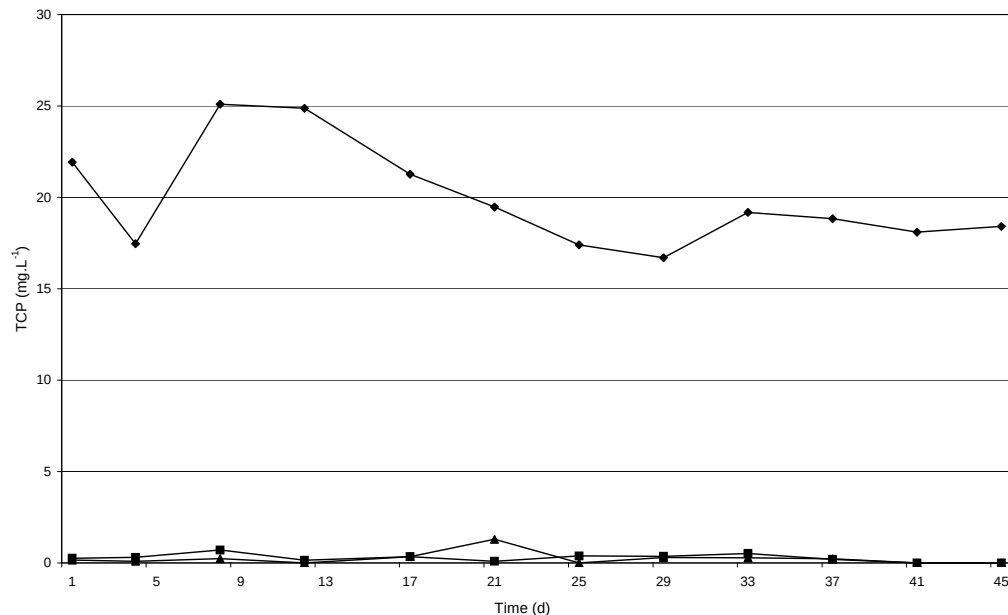


Figure 4.4 IFR influent (◆) TCP from SDR (sampling port S2) and outlet TCP concentrations (mg.L⁻¹) detected at sample ports S3 (■) and S4 (▲) for IFR during the 45 d linked operation period.

in turn, effected increased TCP dechlorination rates with 34.4 % of an average influent 27.9 mg.L⁻¹ dechlorinated over final 28 d (Figure 4.3).

Table 4.1. Average influent TCP and outlet TCP plus metabolites, 2,4-DCP, 4-CP and phenol, concentrations (mg.L⁻¹) for the linked system during the 45 d operation period

Average TCP in (S1) ^a (mg.L ⁻¹)	Average TCP out (S2) ^b (mg.L ⁻¹) (%) ^c	Average metabolite (S2) ^d (mg.L ⁻¹)			Average TCP out ^e (mg.L ⁻¹)		Total % ^g	Average metabolite (S4) ^h (mg.L ⁻¹)	
		2,4-DCP	4-CP	Phenol	S3	S4 (%) ^f		2,4-DCP	4-CP
28.0 ± 1.4	19.8 ± 2.8 (29.2)	4.4 ± 1.1	2.6 ± 1.1	1.1 ± 0.6	0.2 ± 0.2	0.2 ± 0.3 (98.9)	99.2	0.1 ± 0.1	0.1 ± 0.2

^aAverage SDR influent TCP concentration measured for triplicate samples at S1 ± standard deviation.

^bAverage SDR outlet TCP concentration measured for triplicate samples at S2 ± standard deviation.

^c%TCP reduction in SDR.

^dAverage SDR outlet 2,4-DCP, 4-CP and phenol concentrations measured for triplicate samples at S2 ± standard deviation.

^eAverage IFR outlet TCP concentration measured for triplicate samples sampling at S3 and S4 ± standard deviation.

^f%TCP reduction in IFR.

^gTotal % TCP eliminated from the linked system.

^hAverage IFR outlet 2,4-DCP and 4-CP concentrations measured for triplicate samples at S4 ± standard deviation.

The SDR outlet TCP plus 2,4-DCP, 4-CP and phenol was introduced into the IFR together with induction medium. The fungal reactor operated in flow through mode, though continuous recycling was applied as well. Samples were collected from the recycle loop (sampling point S3) and IFR to SDR loop (S4). Average TCP concentration detected in the outlets was 0.2 mg.L⁻¹ (S3 and S4) during the 45 d operation period (Figure 4.4, Table 4.1). This is a 98.9 % elimination of the average influent TCP concentration (19.8 mg.L⁻¹) from the SDR.

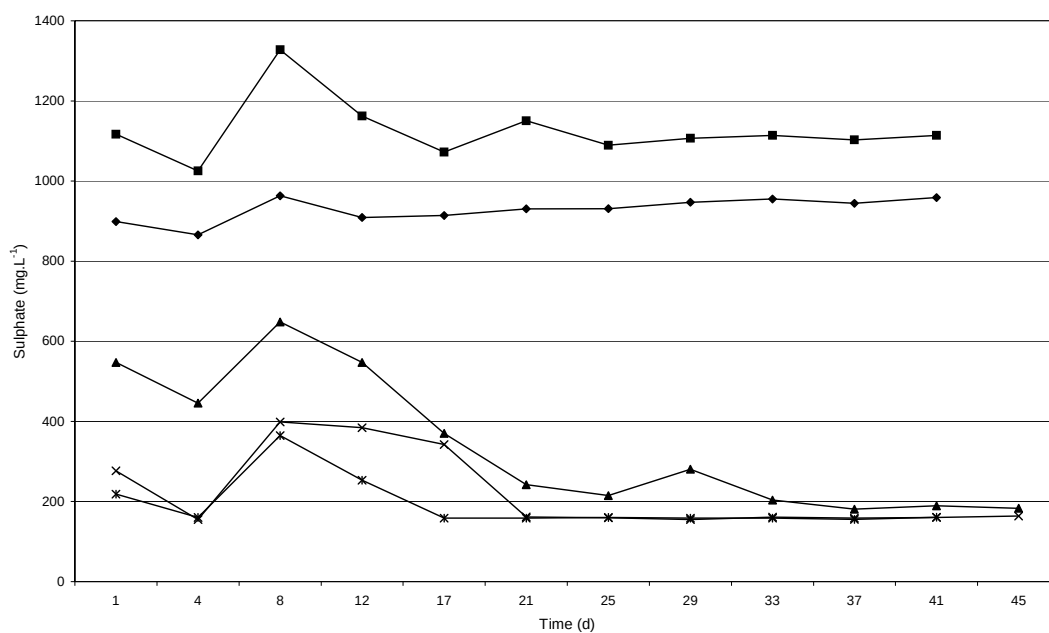


Figure 4.5 Influent (◆) (sample port S1), total (■) (sample port S1 plus IFR outlet, sample port S4) and outlet (▲) sulphate concentrations (mg.L⁻¹) for SDR; and outlet concentrations measured for IFR at sampling points S3 (X) and S4 (⋈) during the 45 d operation period.

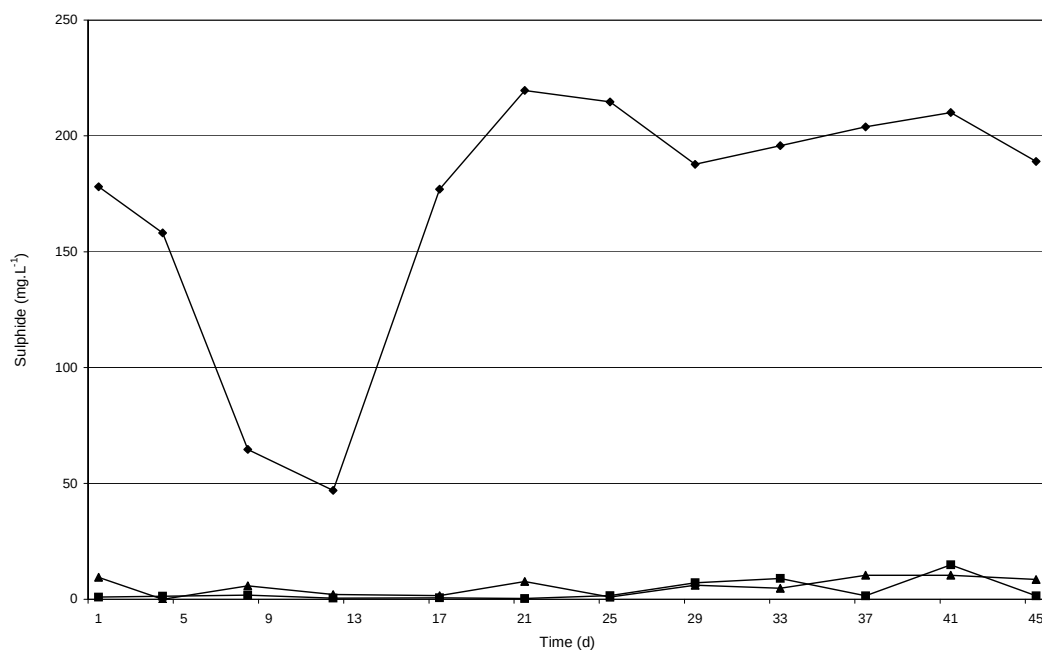


Figure 4.6 Sulphide concentrations (mg.L⁻¹) detected at sampling points S2 (◆) for the SDR; and S3 (■) and S4 (▲) for the IFR.

Table 4.2. Average influent and outlet sulphate and sulphide concentrations (mg.L⁻¹) detected for the linked reactor system during the 45 d operation period

Period ^a (d)	Average SO ₄ ²⁻ in (mg.L ⁻¹)		Average SO ₄ ²⁻ out (S2) (mg.L ⁻¹) (% S1 / S1+S4) ^d	Average HS ⁻ (mg.L ⁻¹)	Average HS ⁻ (mg.L ⁻¹)	
	S1 ^b	S1 + S4 ^c			S3 ^f	S4 ^f (%) ^g
0 – 12	909.1	1158.2	547.0 (39.8 / 52.7)	111.9	1.1	4.3 (3.8)
> 12	940.4	1104.7	229.6 (75.5 / 79.2)	199.7	4.5	6.2 (3.1)

^aPeriod divided into time before pH correction to SDR was made (0 – 12 d) and after (> 12 d).

^bAverage SDR influent SO₄²⁻ concentration measured at sample point S2.

^cSDR total influent SO₄²⁻ concentration calculated from sum [SO₄²⁻] detected at sample points S2 and S4 (IFR outlet).

^d% SO₄²⁻ reduction calculated from average outlet concentration detected at sample point S2 to SDR influent [SO₄²⁻] (S1) and total influent [SO₄²⁻] (S1 plus S4).

^eAverage HS⁻ concentration measured at sampling point S2 (SDR).

^fAverage HS⁻ concentrations measured at sampling points S3 and S4 (IFR).

^g% HS⁻ measured in IFR outlet (S4) to SDR outlet (S2).

It is expected that stripping of the CP contributed to the IFR performance, however, this was shown to play a marginal role with respect to biodegradation (2.3.2). An average total TCP reduction of 99 % for a single total retention time was shown since samples from S4 were collected ± 24 h after collection from S2. IFR outlet (S4) 2,4-DCP and 4-CP concentrations were 0.1 and 0.1 mg.L⁻¹. No phenol was detected. IFR operated at pH between 4.94 and 6.07 despite the average sulphide load of 199.7 mg.L⁻¹ from the SDR after pH correction. Sulphide oxidation was rapidly induced due to aeration of the IFR and outlet concentrations were 4.5 mg.L⁻¹ (S3) and 6.2 mg.L⁻¹ (S4) (Figure 4.6). Comparing sulphide concentrations in IFR outlet (S4) before and after pH correction was made to SDR, a similar percentage HS⁻ removal was shown (Table 4.2). This indicates that no significant sulphide accumulation occurred over the operational period.

The average influent COD of the growth medium (from sample port S1) and total average influent COD (from S1 plus COD detected from IFR outlet) were 3 032.9 and

3 682.5 mg.L⁻¹ for the SDR, respectively. COD was reduced by an average 58.2 %, or 65.6 % for total COD, per single HRT in the SDR (Table 4.3). Total average influent COD was reduced by 94.5 % in the IFR. COD influent and outlet concentrations for the operational period is presented in Figure 4.7.

Table 4.3. Average influent and outlet COD (mg.L⁻¹) measured for the linked reactor system during the 45 d operation period

Average COD in (SDR) (mg.L ⁻¹)		Average COD out (S2) (mg.L ⁻¹)	Average COD out (mg.L ⁻¹)		Average COD in (IFR) (mg.L ⁻¹)	
S1 ^a	S1 + S4 ^b	(% S1 / S1 + S4) ^c	P3 ^d	P4 ^d (% S5 / S5 + S2) ^e	P5 ^f	P5 + P2 ^g
3 032.9	3 682.5	1 265.0 (58.2 / 65.6)	851.1	649.5 (93.8 / 94.5)	10 588.9	11 853.9

^aAverage SDR influent COD measured at sampling point S1.

^bSDR total influent COD calculated from sum COD measured at S1 and S4 (IFR outlet).

^c% COD reduction in SDR calculated from average outlet COD detected at S2 to SDR influent (S1) and total influent (S1 plus S4).

^dAverage COD measured at S3 and S4 (IFR).

^e% COD reduction in IFR calculated from average outlet COD detected at sample point S4 to IFR influent (S5) and total influent (S5 plus S2).

^fAverage IFR influent COD measured at S5.

^gIFR total influent COD calculated from sum COD measured at S5 and S2 (SDR outlet).

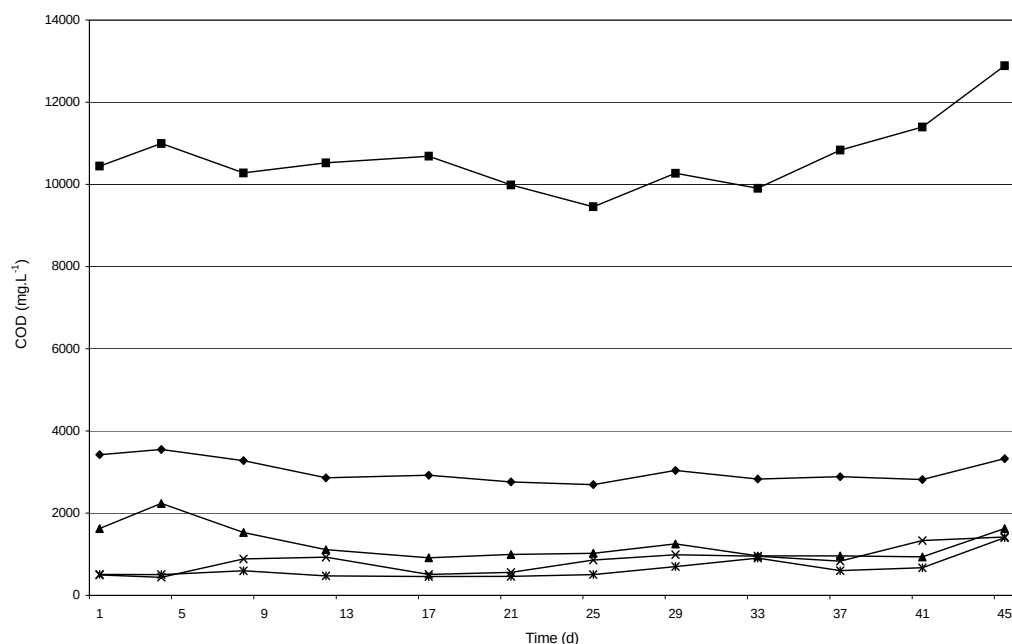


Figure 4.7 Influent COD (mg.L^{-1}) detected for SDR (♦) (sampling port S1) and IFR (■) (S5) and outlet COD detected at sampling ports S2 (▲) for SDR; and S3 (X) and S4 (⋈) for IFR.

4.4 Discussion

The integrated process effectively biodegraded 2,4,6-TCP, with > 99 % eliminated from the influent feed by reductive dechlorination to lower CP followed by mineralization in the IFR. The removal efficiency was observed over the total retention time of the system and averaged for the operating period. It was necessary to correct the pH in the SDR since the acidic influent stream from the fungal reactor outlet effected reduced pH conditions which, in turn, inhibited sulphate reduction and dechlororespiration. The sulphide in SDR culture fluid did not seem to suppress fungal activity since rapid sulphide oxidation occurred.

Two studies operating integrated methanogenic-dependent reductive dechlorination and aerobic bacterial degradation reactor systems for the mineralization of CP showed contrasting performance. Armenante *et al.* (1992) employed a linked anaerobic/aerobic process for the biodegradation of 2,4,6-TCP. The anaerobic stage was conducted in a down-flow porous silica bead-packed reactor (0.4, void fraction; 2.1 L, liquid volume).

Complete dechlorination of 2,4,6-TCP ($\pm 25 \text{ mg.L}^{-1}$) to 4-CP was observed with transient accumulation of 2,4-DCP during a single retention time. The anaerobic effluent was introduced into the aerobic component (2.15 L, liquid volume) of the system at flow rate 20 ml.h^{-1} . Both the system components were inoculated with laboratory-adapted bacterial consortia enriched from anaerobic digester sludge of sewage treatment facilities. Complete degradation of 4-CP was consistently observed in the aerobic reactor during a 3 week operation. A two-biofilm reactor system packed with motor tyre chips was used to investigate removal of 2,4-DCP (Shin *et al.*, 1999). The anaerobic component (0.47, void fraction; 1.4 L, working volume) completely dechlorinated $20 - 40 \text{ mg.L}^{-1}$ 2,4-DCP to less than stoichiometric 4-CP concentrations in 10 d. DCP and CP adsorption by the tyre chips was negligible. 70 – 98 % 4-CP degradation in the aerobic reactor (0.52, void fraction; 1.4 L, working volume) was observed depending on influent concentration from the anaerobic biofilm reactor.

Though comparable degradation performance between the studies of Armenante *et al.* (1992) and Shin *et al.* (1999) and the current investigation is shown, the sulphidogenic dechlororespiration–fungal mineralization system resulted in almost complete removal of TCP in 122.8 h whereas Armenante *et al.* (1992) indicated a total retention time of 202.7 h for the mineralization of TCP. The rapid fungal degradation of the compounds played a substantial role in reducing retention time in the current study. A 97.7 h HRT for the aerobic bacterial reactor was employed by Armenante *et al.* (1992) compared to 20.2 h for the immobilized fungal reactor.

This study showed the potential for employing an integrated anaerobic/aerobic bioprocess environment for the elimination of chlorinated pollutants from subsurface environment by reductive dechlorination followed by mineralization in an immobilized white-rot fungal reactor configuration. The implementation of *in-situ* enhanced reductive dechlorination in soil/groundwater would require the introduction of a carbon source, sulphate and augmentation with the laboratory-acclimated culture. As was previously discussed, careful manipulation of the carbon: SO_4^{2-} ratio is essential to support both sulphate reduction and fermentative production of H_2 equivalents for reductive dechlorination to occur. Mobilized pollutant and dechlorination products will have to be pumped to a wood chip-immobilized white-rot fungal reactor. Nutrient requirement necessary to sustain fungal activity will have to be determined by operational

experience. Additional nutrient supplements may be required which could also prolong packing life. Following mineralization of the compounds, the liquid may be returned to the subsurface environment supplemented with carbon source and SO_4^{2-} to further stimulate reductive dechlorination and pollutant mobilization in soil. The potential inhibitory effect of acidifying sulphide oxidation, in the fungal reactor, on the alkaline sulphidogenic environment needs to be addressed by applying pH correction (e.g. addition of lime) to the returned liquid. This was, however, a preliminary investigation and for scale-up application it will be necessary to further understand the process by investigating the following aspects:

- Reducing the retention time for the integrated system by employing increased flow rates so as to assess especially the effect on the reductive dechlorination stage of the process. Increased flow rates will eventually lead to the wash out of cells when growth doubling times surpass reactor retention time. The threshold will have to be established for optimum reductive dechlorination at the shortest possible retention time.
- Identifying maximum threshold chlorinated phenol concentrations that will be tolerated by the microbial consortium.
- The reductive dechlorination component of the anaerobic/aerobic bioprocess was studied only in laboratory bench-scale systems under reasonably controlled conditions. For application purposes the process needs to be investigated at pilot- or field-scale since site-specific characteristics and environmental factors such as temperature, pH, nutrient status, availability/levels of sulphate, competition with indigenous microorganisms when augmenting the contaminated site, pollutant specific degradability, and concentration and availability of pollutant(s) play major roles in *in-situ* remediation efficiency.

Chapter Five

Intermediate Scale-Up of the Aerobic Component of the Dual-Stage System in the Treatment of a Waste Oil Hydrocarbon Effluent Stream

5. Introduction

While the potential application of the linkage of the anaerobic and aerobic reactors in the integrated unit operation shown here strongly indicated a need to evaluate the system at large scale, it proved difficult to locate an appropriate CP soil contaminated site to set-up evaluation of the anaerobic component operation. An opportunity to evaluate the aerobic fungal reactor occurred on the site of FFS Refiners (Pty) Ltd. who recycle waste oils at their Pietermaritzburg operation to produce low-grade furnace oils. An effluent is produced during the waste oil processing in which water is drained from oil and oil slops from tankers and stripped off during the drying process in an evaporator (Figure 5.1). Water that is drained periodically from the bottom of storage tanks and factory run-off from washing also forms part of the effluent make-up. The plant generates between 20 and 30 m³ effluent daily. The company disposes of the effluent via the municipal sewer system but since the COD of the effluent varies between 12 and 55 g.L⁻¹ and the Umgeni Water Board prescribes a maximum discharge COD of 350 mg.L⁻¹ without a penalty adjunction, FFS Refiners investigated the employment of a treatment step before discharging the effluent so as to minimise future penalties. FFS Refiners decided to investigate the application of the aerobic component of the anaerobic/aerobic bioprocess strategy to treat the effluent and reduce COD to more acceptable levels.

The direct scale-up of the bench reactor (0.006 m³) to pilot-scale (14 m³) was not considered desirable and thus an intermediate-sized reactor (0.123 m³) was used to facilitate the transition from small to field application. Thus, the scale-up procedure was roughly 20-fold from the preliminary bench-scale to the intermediate-scale and 114-fold from intermediate-scale to pilot plant. Due to time constraints, the anaerobic stage of the linked system was not applied to an actual pollution situation.

The aim of the study was, therefore, to assess immobilized white-rot fungal degradation of the petroleum hydrocarbon effluent by monitoring COD and specific effluent constituent reduction operated in the trickling packed-bed reactor in SBR mode on bench-scale and intermediate-scale. Although CP were not present in the effluent produced by FFS Refiners, 2,4,6-TCP breakdown was assessed by spiking the effluent with TCP in the intermediate-scale study. FFS Refiners treats a wide range of hydrocarbon wastes at different sites in South Africa and would move onto treating CP contaminated wastes should the system prove effective.



Figure 5.1 Water stripped off waste oils at FFS Refiners (Pty) Ltd. in the evaporator prior to cracking and recycling as furnace oils.

5.2 Materials and Methods

5.2.1 Effluent Collection and Reactor Operation

Samples were collected from the evaporator effluent over a 12 h period on several occasions. The refinery effluent was stored at 4 °C before and during the study. The effluent was initially diluted (1:2 v/v) with the growth medium (Appendix A) and fed into a pine chip-packed reactor (0.006 m³) inoculated and operated under identical conditions

as outlined above (2.2.2). After allowing an acclimation period (five batch cycles, 24 – 30 h each), 1:1 (v/v) diluted samples supplemented with glucose (2 g.L^{-1}) and yeast extract (0.8 g.L^{-1}) were introduced for a further three batch cycle. Thereafter, 1:1 (v/v) diluted samples with no supplements were introduced. During three consecutive batches samples were collected at specific time intervals and analysed for aromatic and aliphatic hydrocarbon content, as well as COD and pH. It was decided to use the wood chip packing due to the scale-up prospects. Pine chips from the forest products industry were freely availability in close proximity to the FFS Refiners Pietermaritzburg operation.

An intermediate-scale reactor (0.123 m^3) was operated in the laboratory (Figure 5.2). The reactor was constructed using a PVC pipe (1 740 mm, height; 0.3 m, ID) and it was packed to a height of 1 400 mm with pine chips of similar size previously used. A grid for wood chips suspension was mounted (174 mm) to allow for 10 % reactor volume below grid. The packed volume was 0.09 m^3 .

Growth medium and mycelial suspension (10 % v/v) was introduced at flow rate 15.7 L.h^{-1} into the top of the reactor followed by recirculating through a bottom-to-top loop (7.8 L.h^{-1}). Continuous recycling at constant flow rate was allowed for 3-5 d whereafter the liquid was discharged. The inoculation procedure was repeated twice. Due to the packing material, a void volume of 0.07 m^3 was calculated by computing the ratio of void volume determined in the bench-scale reactor ($3\,540 \text{ cm}^3$) to total bench-scale reactor volume ($6\,300 \text{ cm}^3$) since identical chip sizes were used. The void volume represents empty volume (below grid and above packing) plus wood chip packed volume. The intermediate-scale reactor was operated in sequencing batch format and the vessel was filled with 14 L (20 % of void volume, recirculation volume) to 260 mm. The HRT was 1.8 h.

Humidified air was introduced from a dry air gas cylinder (Afrox) at 190 L.h^{-1} controlled by a regulator into the bottom through air eductors to facilitate adequate distribution and dispersion of air into the packed bed.

After the inoculation procedure the intermediate reactor was fed with an effluent sample (diluted 1:2 v/v with growth medium). The acclimation protocol was identical to the one described above, however, three consecutive batch cycles of diluted (1:1 v/v), 2,4,6-

TCP-spiked (to final concentration of 50 mg.L^{-1}), as well as undiluted effluent were treated.

To determine the role stripping of the volatile compounds play in the removal of effluent components, off-gas was passed through three in-series water absorption flask gas traps during one batch cycle treatment with 1:1 (v/v) diluted effluent. Samples were collected from the flasks and analysed for compound content.



Figure 5.2 0.123 m^3 trickling packed-bed reactor operated in the laboratory. Picture on the right shows the wood chip packing.

5.2.2 Analytical Methods

5.2.2.1 Solvent extraction and gas chromatography

To characterize fresh effluent and residual compounds present in the reactor liquid after a recycling period, effluent was extracted into an organic solvent for analysis by gas chromatography.

The extraction ratio of effluent : dichloromethane was 5:1. The sample was placed in a separating funnel and shaken vigorously while venting the contents frequently (repeated twice for the liquid phase). Due to the nature of the injection syringe and instrument's splitless injector, a large variation in integrated peak areas was found (up to 60 %) between injections. An internal standard was required to scale respective areas of peaks per injection for the purpose of comparison. Values for specific effluent component compounds were expressed as peak area ratios (in relation to the internal standard) to show removal trends. 4-CP (0.2 g.L^{-1} in dichloromethane) was used as the internal standard. The extracts were then dried by filtering through anhydrous sodium sulphate (0.5 g) before injection into the gas chromatograph (GC).

A Hewlett-Packard 5890 GC with a flame-ionization detector (FID) was used. Helium was used as the carrier gas at flow rate 2 ml.min^{-1} . Make up gas (hydrogen) flow rate was 30 ml.min^{-1} and a head pressure of 100 kPa was maintained. The temperature programme was as follows: 40°C for 1 min followed by a 4°C.min^{-1} ramp to final temperature of 250°C and held at this temperature for 12 minutes. The injector was set at 225°C and FID, 250°C . The temperature programme was found to provide adequate separation.

A J&W DB-1 fused silica capillary column (100% Dimethylpolysiloxane, 30 m length x 0.247 mm ID x $0.25 \mu\text{m}$ film thickness) was used and GC trace integration was carried out with Beckman Coulter, Inc. 32 Karat Software using a Scientific Software, Inc. TP420-Rev B interface board. Undiluted effluent was fingerprinted and compounds were identified using pure standards and standard mixtures (Supelco BTEX Mix, UST Modified Range Organics and ASTM D2887 Calibration Mix).

A $1 \mu\text{l}$ injection syringe was used, and $1 \mu\text{l}$ sample injected for all runs.

5.2.2.2 COD

The COD of the refinery effluent and reactor outflow was determined using the Merck Spectroquant COD kit.

5.3 Results

5.3.1 Preliminary Bench-Scale Reactor

Figure 5.3 shows a GC chromatogram of the effluent. Peaks identified in fresh effluent sample include monoaromatic hydrocarbons belonging to the BTEX (benzene, toluene, ethylbenzene, *o*-, *m*-, and *p*-xylene) group of organopollutants. Benzene was not detected as it co-eluted with the solvent peak. The other constituents were aliphatic hydrocarbons. As used and waste lubricating oils were the principle source for purification at this recycling plant, straight-chain alkanes or paraffin hydrocarbons were a major component of the effluent make-up rather than aromatic compounds.

Although the majority of the peaks identified were *n*-alkanes (linear), branched alkanes also referred to as isoprenoid alkanes were detected including farnesane, norpristane and pristane. Due to the complexity of the effluent make-up and compositional variance at source between samples collected at various times, differences in GC peak area profiles were observed. Also, transportation and storage would have influenced effluent composition and component levels. Table 5.1 presents averaged analytical effluent data for a nine-month period.

Table 5.1. Average analytical data for the oil recycling effluent for a nine-month period of plant operation

pH	6.2
Conductivity	129 mS.m ⁻¹
Suspended solids	104.6 mg.L ⁻¹
Copper	< 0.05 mg.L ⁻¹
Zinc	2.63 mg.L ⁻¹
Lead	937 µg.L ⁻¹
Chromium	< 170 µg.L ⁻¹
Nickel	< 100 µg.L ⁻¹
COD	12 362 – 54 425 mg.L ⁻¹ O ₂
Soap, oil and grease	111.7 mg.L ⁻¹

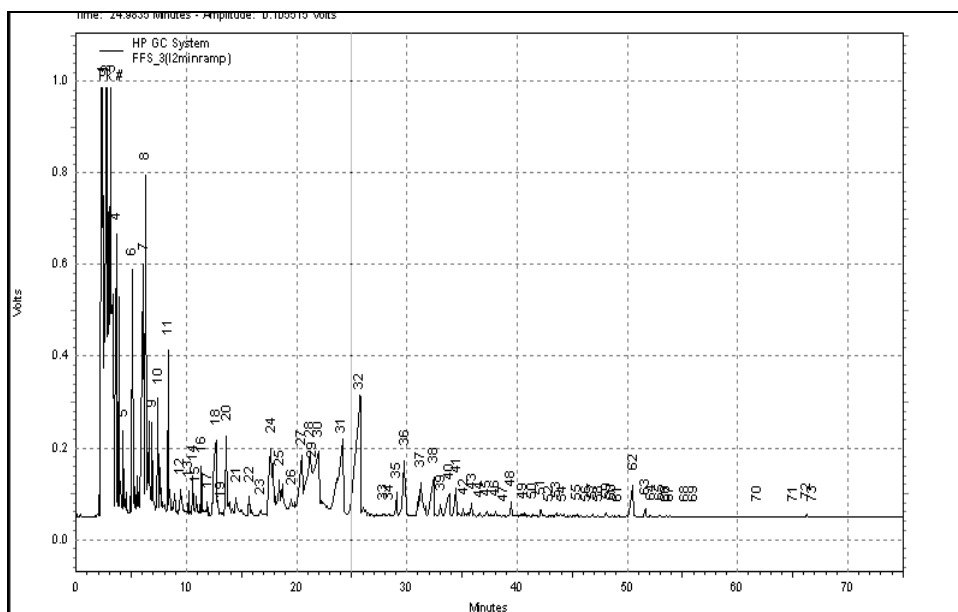


Figure 5.3 GC chromatogram of fresh waste oil recycling effluent sample. Effluent constituents were identified as: Peak 4, toluene; Peak 6, C8; Peak 8, ethylbenzene; Peak 9, *p*-/*m*-xylene; Peak 10, *o*-xylene; Peak 17, C10; Peak 22, C11; Peak 26, C12; Peak 32, farnesane; Peak 34, C16-isoprenoid; Peak 36, C15; Peak 38, C16; Peak 40, norpristane; Peak 42, C17; Peak 43, pristane; Peak 46, C18; Peak 49, C19; Peak 52, C20; Peak 54, benzidine; Peak 55, C21; Peak 59, C22; Peak 64, C24; Peak 67, C25; Peak 69, C26.

Initially the effluent was diluted 1:2 (v/v), followed by 1:1 (v/v) and after several successive introductions during the acclimation period, the reactor was fed with 1:1 (v/v) diluted effluent samples without any supplements for three batch cycles.

Figure 5.4 shows the GC trace of a 1:1 (v/v) diluted sample before introduction into the reactor and Figures 5.5 and 5.6 after a 6 and 24 h recycling period. As can be seen by the disappearance of peaks and reduction of the FID response to the effluent's constituents after the recycling periods, overall elimination of aromatic and aliphatic compounds occurred. The influent COD was 22.5 g.L^{-1} with 53.3 and 82 % reduction after 6 and 24h.

GC peak area ratios (in relation to the internal standard) indicated a substantial removal of effluent component compounds. It was decided to concentrate on five compounds for the purpose of comparison between batch cycles. The compounds were the straight

chain alkanes: C8 (*n*-octane), C10 (*n*-decane), C11 (*n*-undecane) and C12 (*n*-dodecane) and aromatic, *o*-xylene.

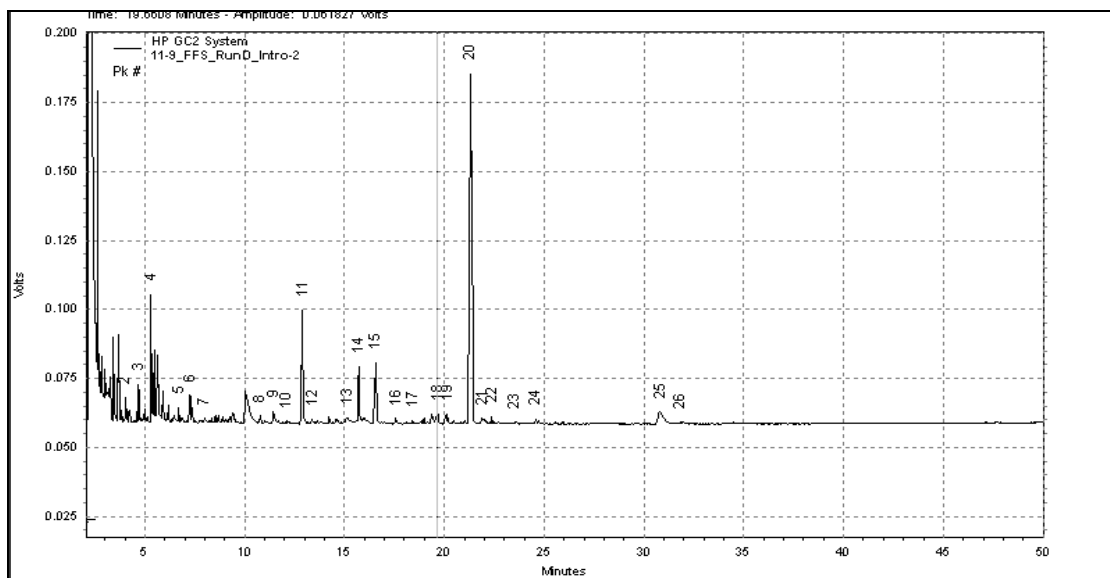


Figure 5.4 GC chromatogram of effluent (1:1 v/v) before introduction into pine chip-packed reactor. Peaks identified were: Peak 2, toluene; Peak 3, C8; Peak 5, *p*-*m*-xylene; Peak 6, *o*-xylene; Peak 7, C9; Peak 10, C10; Peak 14, C11; Peak 18, C12; Peak 20, 4-CP (internal standard).

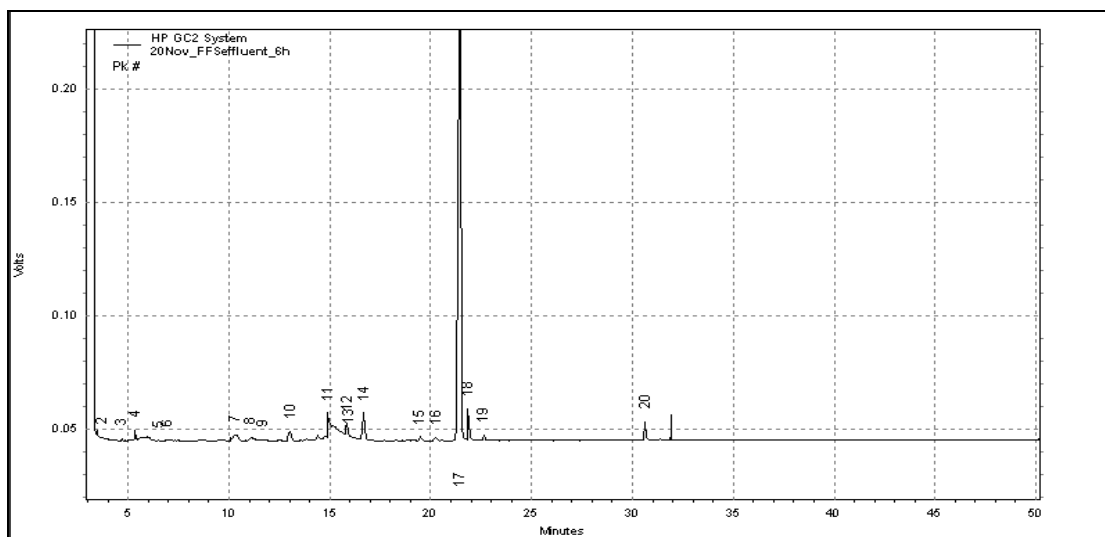


Figure 5.5 GC chromatogram of effluent after recycling for 6h. Peaks identified were: Peak 2, toluene; Peak 3, C8; Peak 5, *p*-*m*-xylene; Peak 6, *o*-xylene; Peak 9, C10; Peak 12, C11; Peak 15, C12; Peak 17, 4-CP (internal standard).

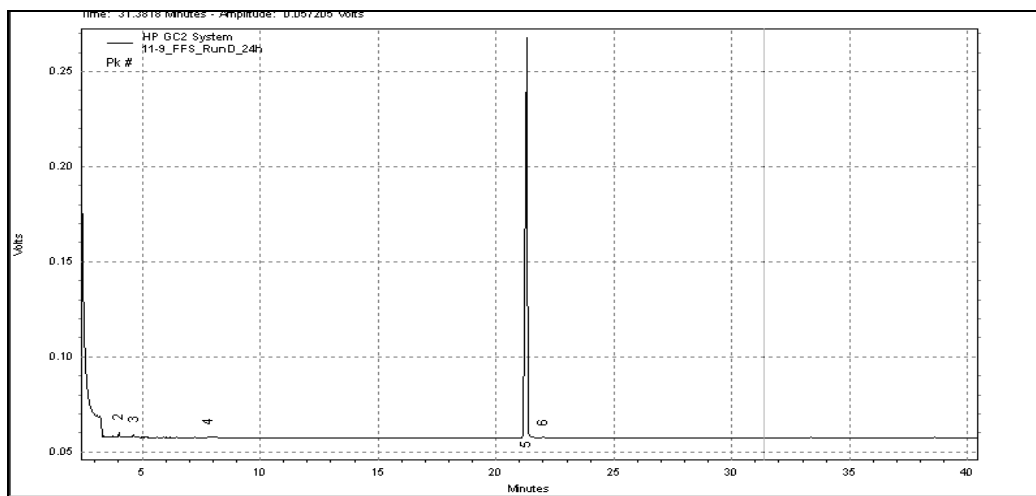


Figure 5.6 GC chromatogram of effluent after recycling for 24 h. Peaks identified were: Peak 2, toluene; Peak 3, C8; Peak 4, C9; Peak 5, 4-CP (internal standard).

Figure 5.7 shows three consecutive batch cycles (48 h each) of 1:1 (v/v) diluted effluent treated in the bench-scale reactor.

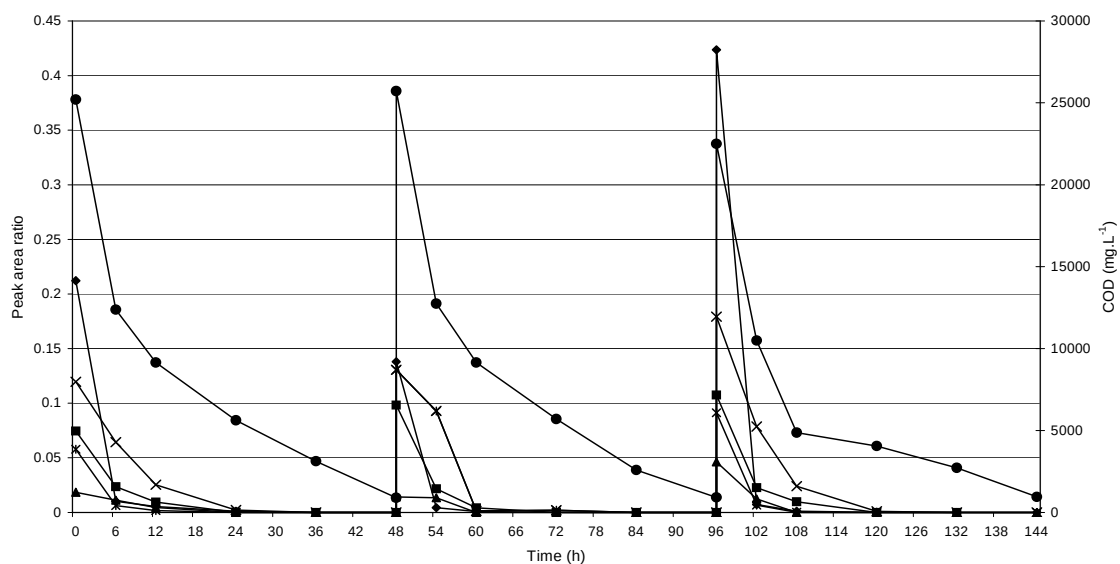


Figure 5.7 Three batch cycles of 1:1 (v/v) diluted effluent treated in preliminary bench-scale pine chip-packed reactor showing C8 (□), o-xylene (■), C10 (▲), C11 (x) and C12 (X) effluent constituent peak area ratios and COD (●) (mg.L⁻¹).

Peak area ratios indicate a 100 % removal of the targeted compound over a 24 h period with average COD reduction of 79 % for the three cycles (influent COD average 24.4 g.L⁻¹). During the second 24 h of the batch cycles, COD was reduced by another 17.2 % indicating that the larger fraction of metabolizable carbon is utilized in the first half of the batch cycle. Average COD upon discharge was 919.3 mg.L⁻¹ (Table 5.2). Rate of COD reduction during first 6 h was 2 114.2 mg.L⁻¹ COD.h⁻¹, followed by 241.0 mg.L⁻¹ COD.h⁻¹ in remaining 42 h. A reproducible trend was noted for three batches and with no significant differences in COD levels in the outlet for the treatment cycles, steady state conditions were assumed.

Table 5.2. Average COD introduced and discharged (mg.L⁻¹), average COD levels (mg.L⁻¹) at 1.8 h and 6 h time intervals and COD reduction rate (mg.L⁻¹.h⁻¹) for three batch cycles in preliminary bench and intermediate-scale reactors

Scale ^a	Dilution (v/v)	Average COD in (mg.L ⁻¹)	Average COD out (mg.L ⁻¹) (%) ^b	Average COD at 1.8 / 6 h (mg.L ⁻¹) (%) ^b	Average COD reduction rate (mg.L ⁻¹ .h ⁻¹)
B	1:1	24 477.3	919.3 (96.2)	11 791.6 (51.8) 6h	2 114.2 (0-6 h) / 241.0 (6-48 h)
I	1:1	24 971.9	514.4 (97.9)	5 109.7 (79.5) 1.8 h / 3 233.4 (87) 6 h	11 221.5 (0-1.8 h) / 85.3 (1.8-48 h)
I	1:1 plus TCP	23 792.3	902.2 (96.2)	5 915.2 (75.1) 1.8 h / 1 907.3 (91.9) 6 h	10 100 (0-1.8 h) / 72.3 (1.8-48 h)
I	-	37 065	665.1 (98.2)	11 719.4 (68.3) 1.8 h / 6 757.1 (81.7) 6 h	14 319.7 (0-1.8 h) / 159.2 (1.8-60 h)

^aB = bench-scale; I = intermediate-scale.

^b% reduction.

5.3.2 Intermediate-Scale Reactor

Figures 5.8, 5.9 and 5.10 show C8, C10, C11, C12 and *o*-xylene plus COD removal for three consecutive batch cycles for diluted (1:1 v/v) effluent, diluted (1:1 v/v) effluent spiked with 2,4,6-TCP and undiluted effluent, respectively. It was interesting to note that degradation was less effective for the shorter chained *n*-octane than C10 – C12. *n*-decane, *n*-undecane and *n*-dodecane were completely mineralized after 6 h with residual *n*-octane detected between 24 and 30 h for diluted effluent. With an average COD load of 24.9 g.L⁻¹, 79.5 % was eliminated after the first HRT (Table 5.2).

Average COD upon discharge was 514.4 mg.L⁻¹ which corresponds to a 97.9 % reduction after 48 h (Figure 5.8). In similar fashion to the bench-scale reactor, accelerated COD reduction was observed over the first 6 h of operation. With a 51.8 % compared to 87 % reduction in the intermediate-scale reactor (11 221.5 mg.L⁻¹ COD.h⁻¹ during first HRT; followed by 85.3 mg.L⁻¹ COD.h⁻¹) higher COD reduction was monitored in the intermediate reactor during initial hydraulic retention times, especially the first 108 min.

Accelerated COD reduction during the first HRT was also detected in the TCP-spiked effluent and undiluted effluent treatment cycles (Figures 5.9 and 5.10). Average COD levels were reduced to 902.2 mg.L⁻¹ (3.8 % of influent COD) and 665.1 mg.L⁻¹ (1.8 %) for TCP-spiked and undiluted effluent, respectively (Table 5.2). The undiluted effluent was treated over a 60 h batch cycle period though COD decreased by only a further 93.9 mg.L⁻¹ during the 12 h following 48 h. C10 – C12 was mineralized during the first 48 h and C8 and *o*-xylene in 54 h as depicted by peak area ratio reduction (Figure 5.10). 100 % TCP reduction was detected in 48 h for the first two batch cycles but residual compound (0.5 %) was measured after 48 h during the 3rd cycle (Figure 5.9).

To assess the contribution of stripping of the effluent's volatile compounds on measured degradation, the off-gas was captured in water traps during one batch cycle. Samples were collected from the traps at specific time intervals and analysed for C8, C10-C12 and *o*-xylene content to express peak area percentage loss due to volatilization that contributed to the monitored removal.

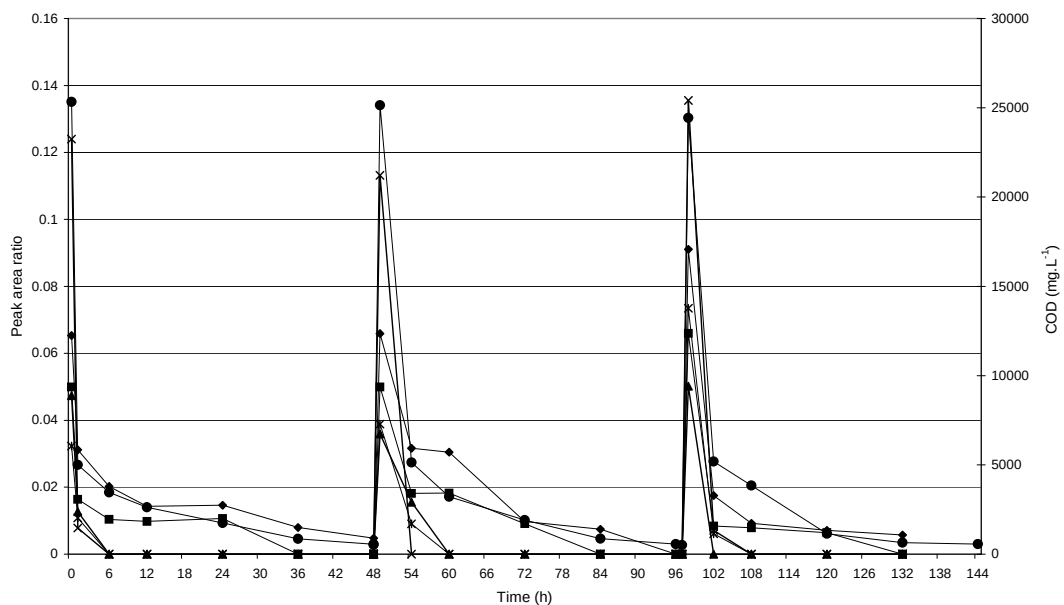


Figure 5.8 Three batch cycles of 1:1 (v/v) diluted effluent treated in intermediate-scale reactor showing C8 (□), o-Xylene (■), C10 (▲), C11 (x) and C12 (✕) effluent constituent peak area ratios and COD (●) (mg.L^{-1}).

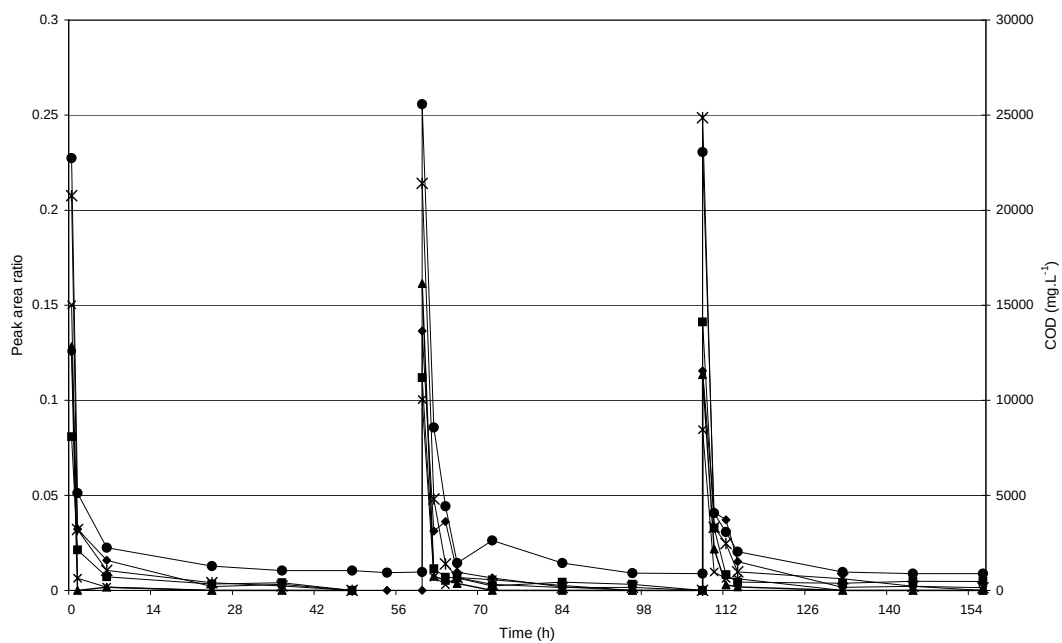


Figure 5.9 Three batch cycles of 1:1 (v/v) diluted effluent spiked with TCP treated in intermediate-scale reactor showing C8 (□), o-Xylene (■), C11 (▲) and C12 (x) effluent constituents peak area ratios, COD (●) (mg.L^{-1}) and 2,4,6-TCP (✕) peak area ratio.

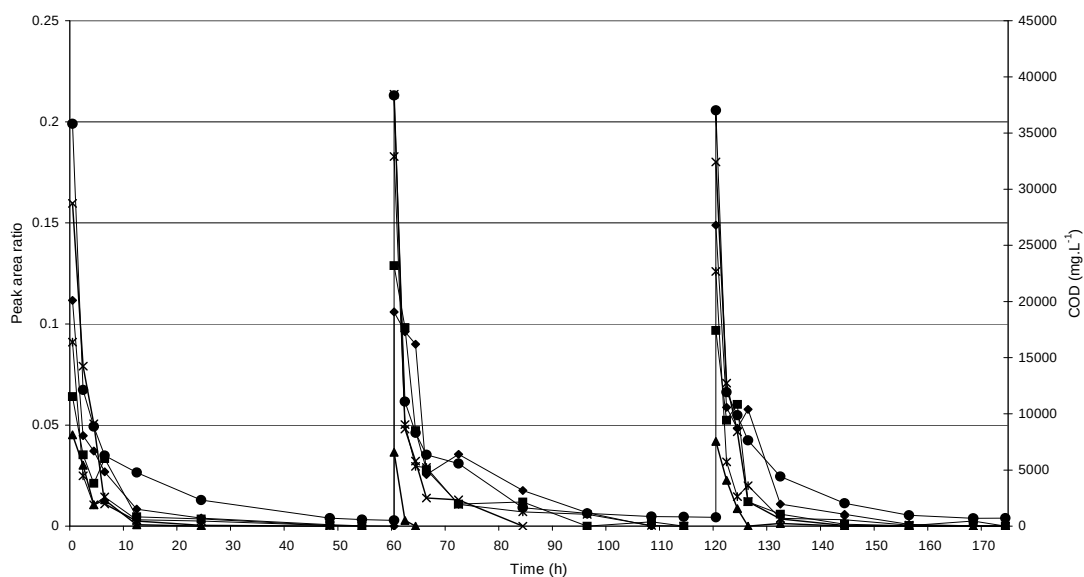


Figure 5.10 Three batch cycles of undiluted effluent treated in intermediate-scale reactor showing C8 ($\square$), o-xylene ($\blacksquare$), C10 ($\blacktriangle$), C11 (x) and C12 ($\star$) effluent constituent peak area ratios and COD ($\bullet$) (mg.L^{-1}).

After one HRT, C8, C10, C12 and o-xylene and also toluene, ethylbenzene and *p*-/*m*-xylene were detected in the traps. For *n*-octane, 1.5 % was found to contribute to the 44.8 % reduction according to the integrated GC peak area sizes. 1.0 %, 0.4 % and 0.5 % of 41.6 %, 69.0 % and 61.2 % reduction of o-xylene, C10 and C12 was detected in the gas traps, respectively.

After an additional retention time (sampling at 4 h from the start of the treatment cycle), 0.3 % C8 was detected in the traps. This represents a 67.5 % loss during 2.2 h. No o-xylene/C10/C12 was detected after the 1.8 h sampling point and no C8 was detected after 4 h.

5.4 Discussion

The investigation demonstrated substantial removal and reduction of a waste oil recycling plant effluent's hydrocarbon compounds and COD by immobilized fungi in a trickling packed-bed reactor.

COD removal was > 96 % in 48 h in the bench and intermediate-scale reactors operated in sequencing batch format. Undiluted effluent (COD > 37 g.L⁻¹) did not pose any inhibitory effect on fungal metabolism. Stripping of compounds in the effluent contributed negligibly to removal. It is suggested that after volatilization hyphal-associated processes mineralize the stripped pollutants as the vapour migrates through the packed bed.

The results compare favourably to other studies investigating complex effluent treatment using fungi or bacteria including oil recycling wastewater. Jou and Huang (2002) employed a pilot fixed-film bioreactor for the treatment of an oil recycling effluent. The aerobic bacterial-mediated system facilitated the removal of 85 – 90 % COD (influent 510 mg.L⁻¹) in 8 h HRT with bacterial cells immobilized on a mix of polyurethane foam slabs and polypropylene. Huddleston *et al* (2000) used a constructed wetland for treatment of a waste oil recycling effluent. They monitored an average BOD₅ removal of 80 % during a 48 h HRT, as well as 95 % of ammonia and concomitant reduction in toxicity. D'Annibale *et al.* (1998) observed between 73 and 88 % removal in TOC content and a reduction in total phenolic content to 0.7 – 0.78 g.L⁻¹ (3.1 g.L⁻¹ total phenols for untreated material) for olive-mill wastewater (OMW) treated with polyurethane sponge-immobilized *L. edodes* in fermentation studies. New isolated strains of *Pleorotus ostreatus* was applied to OMW and an 80 % reduction in phenolic content was shown in batch flasks in 7 d (Iconomou and Zanganas, 2001). Kapdan *et al.* (2000) showed a 50 – 97 % decolorization and 80 % TOC removal efficiency employing metal mesh-immobilized *T. versicolor* in repeated batch-mode shake flasks treating 50 – 200 mg.L⁻¹ textile dyestuff in 7 d.

The transition from preliminary bench to intermediate-scale did not affect biodegradation performance and TCP in the spiked effluent was favourably degraded which suggest the potential of a full-scale immobilized white-rot fungal reactor to metabolize complex mixtures of hydrocarbons (free-standing operation) and chlorinated pollutants (as component of the integrated anaerobic/aerobic strategy).

Chapter Six

Full-scale Evaluation of the Immobilized White-Rot Fungal Reactor in the Treatment of a Waste Oil Hydrocarbon Effluent Stream

Based on the above results FFS Refiners (Pty) Ltd. requested a Process Design for the scale-up evaluation of the immobilized fungal reactor in a pilot plant (14 m³) erected on-site at the company's Pietermaritzburg operation. A process scale-up of 114-fold from intermediate-scale to pilot plant was involved in this exercise.

6. Process Design

A Process Design was formulated on the basis of the bench and intermediate-scale reactor studies, and detailed the process flow diagram, component specifications, operating protocol and process monitoring specifications. This was approved by FFS Refiners and construction of the pilot plant was undertaken at the FFS Refiners Pietermaritzburg site.

6.1.1 Plant Component Specifications

Photographs of the reactor are shown in Figures 6.1 and 6.2 and the Process Flow Diagram is presented in Figure 6.3.

6.1.1.1 Sequencing batch reactor

The reactor vessel volume was 14 m³ (5.53 m, height; 1.82 m, diameter). Untreated pinewood chips (3 x 3 cm) was used as packing/support material. Packing height was 4 420 mm (80 % reactor volume). The liquid level was located at 30 % of reactor volume (RV, recirculation volume; 1 660 mm) in stationary phase. A grid for wood chips suspension was mounted to allow a 15 % reactor volume (840 mm) below the grid. The recirculation exit was located ½-way between grid and reactor bottom. A heat exchanger was employed in-line in recirculation feed. An off-gas pipe was positioned well above ground level for gas discharge and sample collection. A sight glass was used to monitor liquid levels and to manage the reactor feeding operation.

6.1.1.2 Feed tanks

Two reservoir tanks were constructed (14 m³ each; 5.53 m, height; 1.82 m, diameter). The holding tank received evaporator effluent on a continuous throughput basis whereas the feed tank was filled via the holding tank where make-up feed stream was made.

6.1.1.3 Pumps

Orbit AO901 (1430 rpm, 4 kW, inverter drive) pumps were used. Pump delivery was recommended between 2 – 6 m³.h⁻¹ calculated on a basis of delivery requirements between 0.5 and 1.5 vol.h⁻¹.

Aeration of the reactor was accomplished from the bottom by employing an air pump fitted with a rotameter. Air eductors were fitted in the bottom of the reactor to facilitate adequate entry and distribution of air into the packed-bed. Appropriate aeration rates were to be identified based on operational experience.

6.1.1.4 Settling tank

A cylindrical vessel (10 m³) with a conical bottom was used as a clarifying tank for discharged effluent. Sludge was collected and recycled back to the reactor upon start of a new batch.

6.1.1.5 Heat exchanger

Temperature adjustment was made to the recirculation feed by employing a 1 m² heat exchanger set to maintain temperature at 25 - 32 °C (max. 37 °C).

6.1.2 Controls

The SBR was operated manually. During a single batch cycle the reactor was filled to 30 % (1 660 mm) via the feed tank plus settled material from the clarifying tank. Recirculation commenced for 48 – 72 h, whereafter a period was allowed for settling of effluent before

discharge to the clarifier. The settling tank was emptied manually and sludge recycled to the reactor at the start of a new cycle.



Figure 6.1 The reactor was packed with the wood chips by employing a loading bag and crane.



Figure 6.2 The plant upon completion before commissioning.

6.1.3 Process Monitoring

COD, pO_2 , LEL (lower explosives limit) and pH parameters were used to monitor plant performance. Samples for COD and pH measurements were taken from the feed tank and recirculation line at predetermined time intervals. Oxygen and LEL levels were measured at the off-gas exit.

6.2 Construction, Packing and Commissioning of Plant

The operation of the field plant required development of an appropriate fungal inoculum. It was decided to employ culture development in the laboratory and on-site in Pietermaritzburg. The pine chips from the intermediate-scale reactor (5.2.1) were used as the laboratory-prepared inoculum for pilot-scale operation. A bioremediation site was established at the Pietermaritzburg plant where effluent and oil-contaminated soil was deposited (Figures 6.4). A new biopile was made with loosely packed contaminated soil and wood chips, inoculated with fungi from current pile, watered and turned to allow for fungal growth.

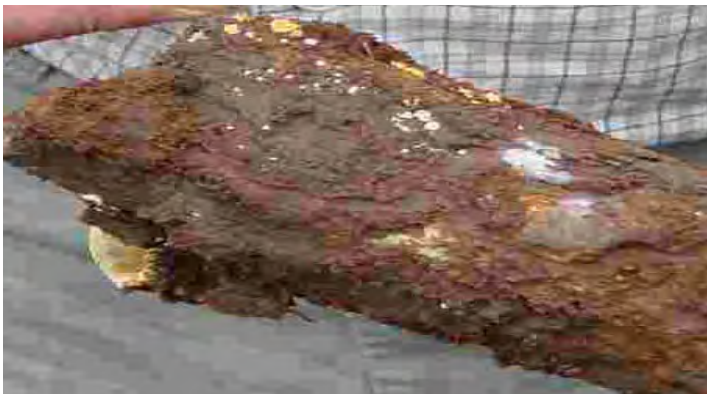


Figure 6.4 Wood material from the FFS Refiners Pietermaritzburg bioremediation site colonized by fungi.

The steel reactor vessels were sourced from previously used underground fuel storage tanks. Untreated pinewood chips were sourced from Sappi in Karkloof, KwaZulu-Natal. Chip sizes were smaller than the prescribed size (3 x 3 cm) used in laboratory studies. Sizes varied between 9 x 21 x 3 mm for smallest, 20 x 55 x 8 mm (medium) to 40 x 60 x 8 mm for largest. The wood was randomly packed into the reactor vessel to a height of 4 299 mm interspersed by inoculated chips from the culture development operations in the laboratory and Pietermaritzburg by employing a canvas loading bag and crane (Figure 6.1). Liquid discharge of two batch cycles from the intermediate-scale reactor operated in the laboratory was also introduced.

The percentages of treated to untreated material were as follows: wood chips, 9 m³ untreated wood; 0.075 m³ inoculated wood (0.8 %); and liquid, 2.6 m³ fresh effluent; treated liquid 0.04 m³ (1.5 %). ± 2 m³ effluent filled reactor to the 1 660 mm level due to wood chip volume displacement. 23.7 % of bed volume was submerged. Figures 6.5, 6.6 and 6.7 show components and the plant upon completion of construction.

It was decided to limit the influent COD to a max. 20 g.L⁻¹. This decision was based on operational experience with the laboratory investigation: COD removal of 85 – 90 % was expected over 48 h as observed in the laboratory, and ensured high concentration of easily available metabolizable carbon which does not constitute a possible toxic or inhibitory factor during the start-up procedure. The high carbon dosage was deemed necessary to facilitate proliferation and distribution of fungi throughout the packed bed. The COD of evaporator effluent was checked and adjusted with tap water in the feed tank to produce a final COD of ± 20 g.L⁻¹.



Figure 6.5 The air eductors in the bottom of the reactor vessel.



Figure 6.6 Heat exchanger unit located in the recirculation line and feed pump between clarifying tank (on the left) and reactor vessel.



Figure 6.7 The air pump located at the base of the reactor vessel.

6.3 Results

Recirculation and discharge delivery was set at a fixed rate ($4 \text{ m}^3 \cdot \text{h}^{-1}$). The reactor filling rate from feed the tank was $8 \text{ m}^3 \cdot \text{h}^{-1}$. Air delivery was $8331 \text{ L} \cdot \text{min}^{-1}$ ($49 \text{ m}^3 \cdot \text{h}^{-1}$; 5041 rpm; 25 kPa).

Regular effluent feeds were administered during every 36 – 48 h for 10 d after the plant was commissioned. During this period the spray nozzle, which ensured adequate distribution of influent liquid across the packed-bed, was replaced with a spray plate. Due to small wood chip fragments in the recycle feed, the nozzle was obstructed, but as the bed settled the nozzle was reconnected. Also, absorption of the effluent by the dry wood chips was observed and levels decreased to $< 1\,000 \text{ mm}$. Fresh effluent was introduced to top the level up to $1\,660 \text{ mm}$. Levels eventually stabilized during the start-up period.

COD measurement commenced after this period. It was observed that reactor influent COD from the feed tank was $< 10\,000 \text{ mg} \cdot \text{L}^{-1}$. Since $\pm 2 \text{ m}^3$ liquid was introduced per single batch cycle, effluent was allowed to occupy the feed tanks for days which resulted in the settling of suspended solids and stripping of volatile effluent components due to elevated ambient

temperatures ($> 32\text{ }^{\circ}\text{C}$). This effected reduced COD influent concentrations. Therefore, it was decided to feed fresh evaporator effluent into the feed tank and dilute with tap water, if necessary, immediately before a new batch cycle started. The COD feed was maintained at $\pm 20\,000\text{ mg.L}^{-1}$.

COD influent levels during 28 d following the start of measurements (25/11/2002 – 20/12/2002) averaged $8\,887\text{ mg.L}^{-1}$. Batch cycles duration varied between 48 and 120 h and discharged COD between $1\,310$ and $3\,680\text{ mg.L}^{-1}$. Raw data for the start-up period is presented in Table 1 (Appendix F). Figure 6.8 shows running influent vs. outlet COD concentrations. After 24 December 2002, stable COD feeds were administered and five batch cycles were allowed. Batch cycles started on 30/12/2002, 06/01/2003 and 14/01/2003 were 168, 192 and 144 h, respectively. Running COD concentrations were determined. During these prolonged cycles, COD was reduced to $2\,990$, $1\,580$ and $1\,730\text{ mg.L}^{-1}$ from $19\,050$, $14\,700$ and $15\,840\text{ mg.L}^{-1}$ upon discharge.

Following the five cycles multiple 48 h cycles were operated. This was done to assess whether the plant had entered stable steady-state conditions, to compare COD removal efficiency between repeated cycles during this period, and to evaluate performance over reduced cycle time. Ultimately, it was hoped that a 48 h (or shorter) batch cycle would be sufficient to reduce COD concentration to acceptable municipal sewer discharge levels, or to achieve maximum potential reduction over this period. During the 48 h trial, pH, pO_2 and LEL were measured regularly. Figure 6.9 shows the data acquired during repeated cycles. Raw data is presented in Table 2 (Appendix F).

During the first two 48 h cycles, it was observed that COD load ($18\,300$ and $19\,420\text{ mg.L}^{-1}$) was reduced by $\pm 50\%$ in the initial 4 h after feed. It was, therefore, decided to collect samples on an hourly basis for the first 4 h and thereafter every 4 to 8 h. COD reduction for the initial cycles was 78.7 and 61.9% for 1.76 and 1.93 m^3 effluent treated (recycled material from the clarifying tank constituted 14.7 and 9.8% of feed). The percentage oxygen detected in the off-gas outlet showed a 0.6% reduction during the first 48-h cycle (ambient O_2 20.8%). 19.3% followed by 15.5% O_2 was detected during the second cycle.

COD reduction for the first hour varied with 22% eliminated for cycles operated between 27/01/2003 to 31/01/2003 followed by 11.7 , 23.1 and 14.7% during the subsequent three

cycles. It was expected that a dilution factor contributed to concentrations detected since an average 3.6 % of influent feed make up, albeit small fractions, was recycled sludge material from the settling tank ($0.076 - 0.09 \text{ m}^3$).

During the 48 h batches following the initial two cycles, a reduction in COD removal efficiency was observed. An average removal of 62.8 and 70.3 % was calculated for 24 h and 48 h, respectively (batches 20/01/2003 – 24/01/2003). This compares to 37.4 and 51 % for the subsequent six batches. It was suggested that the reduced efficiency could be due to restricted oxygen transfer possibly exacerbated by effluent circuiting through the wood chips and plugging of the packed-bed by excessive fungal growth. It is also clear that the larger percentage of COD reduction occurred during the first 24 h of each batch and with an average residual $8\,020 \text{ mg.L}^{-1}$ COD upon discharge, it was decided to increase batch cycle duration to 72 h.

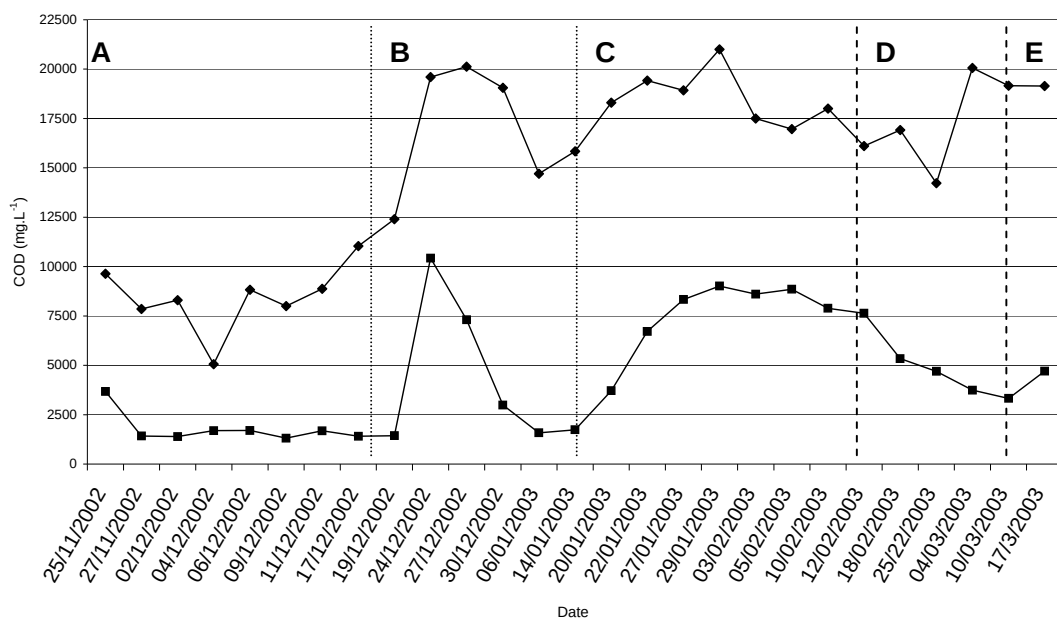


Figure 6.8 Running influent (♦) and outlet (■) COD (mg.L^{-1}) detected during the operation of the plant: start-up (A), $\text{COD} \pm 20 \text{ g.L}^{-1}$ (B), 48 h trial (C), 72 h trial (D), pH correction trial (E).

pH levels increased incrementally from ± 4.5 to 6.6 during a treatment cycle (Figure 6.9). LEL levels, which is the percentage of volatile organic compounds (VOC) present in the air,

decreased from an average 11.8 to 5.6 % during the first hour, thereafter, it declined to between 2 and 4 % towards the end of a treatment cycle.

To investigate the contribution that stripping of VOC had on the performance of the plant, 0.2 m³ raw effluent was aerated continuously in a 200 L drum. The plant air flow was split to accomplish aeration of the drum. This was done for a 48 h period and repeated. COD was reduced by ± 7.4 % and pH remained ≤ 4.9 during the period (Table 2 Appendix F).

72 h cycles were started on 18 February 2003. Air delivery was increased to 65 m³.h⁻¹ without changing pump delivery near the end of the 48 h trials, thereafter, the delivery fluctuated between 55 and 60 m³.h⁻¹ during the 72 h trial. It was suggested that the bed opened up and the air followed the path of least resistance (air channelling). Wood chip samples were collected from the top at ± 40 cm into the packed-bed and subjected to light microscopy investigation. Slimy biofilms were observed on the chips. Figures 6.10, 6.11 and 6.12 show photographs taken from microscopy preparations of biofilm sections and reactor liquid.

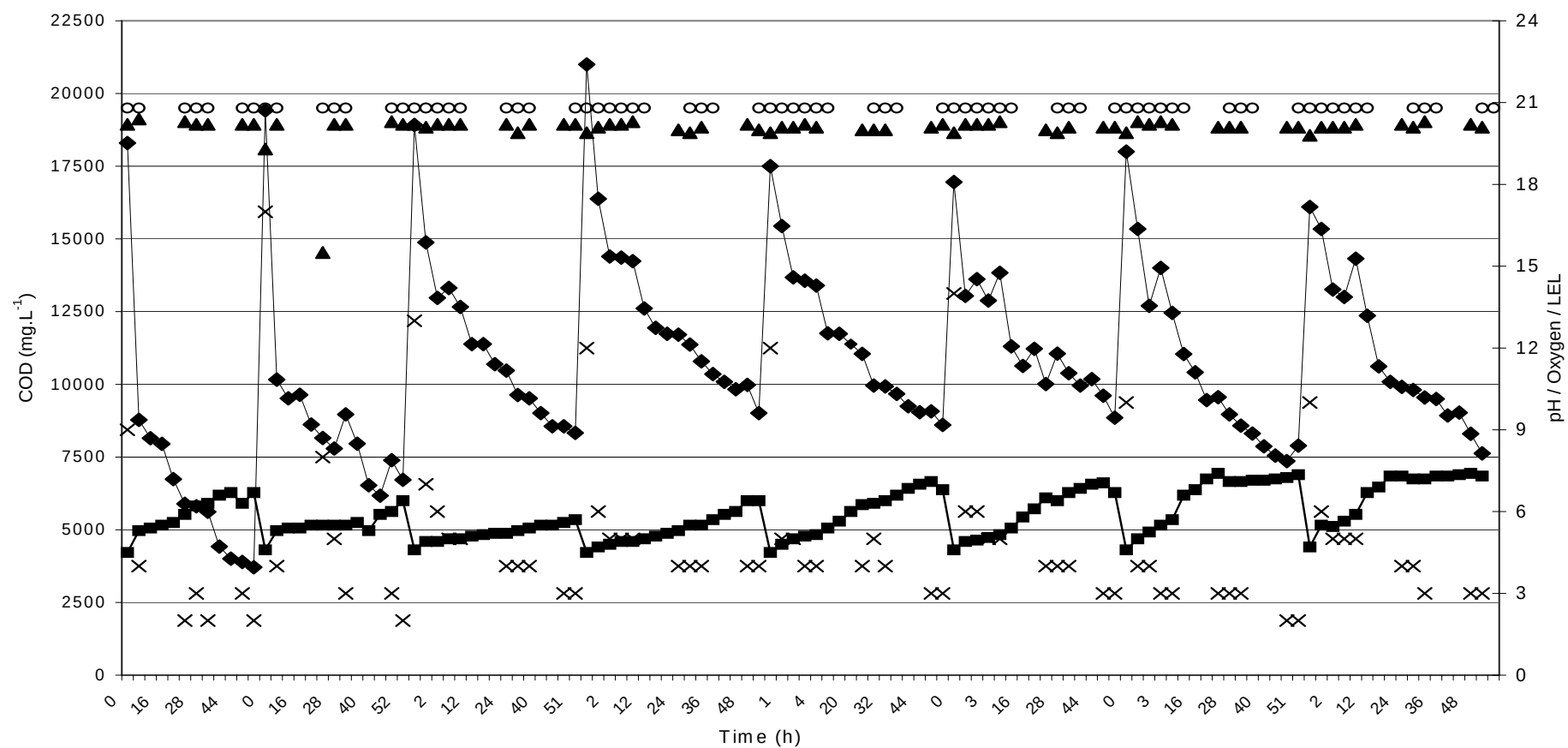


Figure 6.9 Eight 48 h batch cycles of effluent treated in full-scale reactor showing COD (♦) (mg.L⁻¹), pH (■), LEL (%) (X) and O₂ (%) (▲) of 20.8 % ambient O₂ level (○).

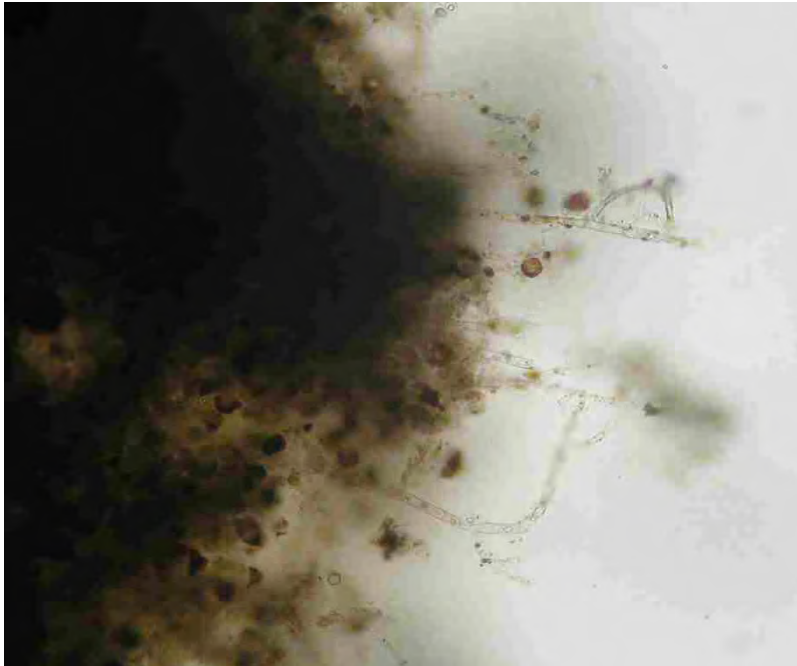


Figure 6.10 Section of the biofilm on pine chips sampled from the plant examined under the light microscope showing fungal hyphae attached to a wood chip and penetrating the surrounding environment.



Figure 6.11 A section of a fungal hyphae clearly shows septa.



Figure 6.12 Photograph of a nematode in liquid collected from the reactor.

Average 24, 48 and 72 h % COD reduction for four 72 h cycles were 50.3, 64.7 and 74.8 %, respectively. There did seem to be an improvement in performance towards the last two cycles: residual COD after 24 and 48 h were 8 815 and 5 485 mg.L⁻¹ (Table 3 Appendix F). This was an increase of 37.3 and 20.9 % up on the average for six 48 h cycles for the same periods. COD was reduced by ≈ 10 % in the additional 24 h, with average discharged concentration for the last two cycles 3 530 mg.L⁻¹. Average pO₂ detected was 19.8 %. Figure 6.13 shows COD, pH, % LEL and % O₂ measured during the 72 h trial.

The increased air delivery may have assisted in higher O₂ transfer into the packed-bed, thus effecting better COD consumption. Trends have shown a pH increase between the start of the 48 h trial and during the 72 h trial. Starting cycle pH was a constant 4.5, thereafter it increased incrementally to 5.2 – 5.9 after 24 h during the first 48 h batches. The pH increased to 6.25 – 7.5 in the final 48 h cycles and 72 h trial (Tables 2 and 3 Appendix F). It was suggested that the higher pH measured could be affecting fungal activity and may explain the moderate COD consumption following the initial accelerated elimination of effluent components.

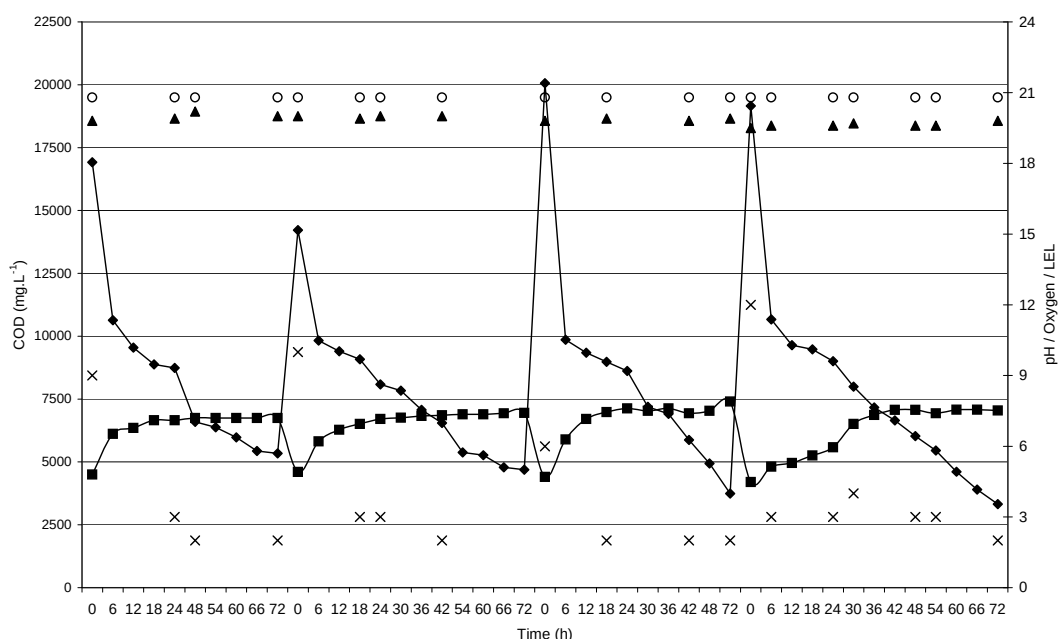


Figure 6.13 Four 72 h batch cycles of effluent treated in full-scale reactor showing COD (♦) (mg.L^{-1}), pH (■), LEL (%) (X) and O_2 (%) (▲) of 20.8 % ambient O_2 level (○).

Following the 72 h trial, pH correction was applied. 3 L of a 4 % phosphoric acid solution was added every 24 h to two batch cycles. This effected only a slight reduction in pH increase during a treatment cycle with pH > 6.4 after 48 h though pH was 5.2 and 4.9 at 24 h for the two cycles (Table 4 Appendix F). Average COD removal for the two cycles with pH correction at 24, 48 and 72 h were similar to the performance detected during the 72 h trials (with no pH correction). A COD of $25\,460\text{ mg.L}^{-1}$ was introduced into the reactor during the second pH corrected batch cycle started on 25/03/2003 (Table 4 Appendix F). Although this was $\pm 5\,000\text{ mg.L}^{-1}$ COD more than the target influent level, 56.4 % was eliminated after 48 h and additional 10 % during the subsequent 24 h. It was decided to increase the phosphoric acid dosage to maintain pH below 5.5 throughout a cycle.

6.4 Discussion

A full-scale immobilized fungal reactor was commissioned on 18 November 2002 and operated for 20 weeks. During the start-up period the reactor was regularly fed with the oil recycling effluent on a continuous basis to facilitate fungal growth and culture distribution throughout the packed-bed. Influent COD concentration was stabilized during the initial stages of the monitoring programme. 48 h followed by 72 h batch cycle period monitoring trials were undertaken. A reproducible COD removal trend was observed during the trials and a similar percentage COD reduction was detected, therefore, steady-state conditions were assumed. Comparable average % COD reduction for 48 and 72 h trials were detected: 50.1 % (at 24 h, 48 h trial), 50.3 % (24 h, 72 h trial), 60.6 % (48 h, 48 h trial) and 64.7 % (48 h, 72 h trial). An additional ≈ 10 % COD was removed during the final 24 h of the 72 h trial. Average COD upon discharge for four 72 h batch cycles was $4\,272.5\text{ mg}\cdot\text{L}^{-1}$. $p\text{O}_2$ measurement in the gas-venting exit during the cycles indicated that between 0.6 – 1.5 % O_2 was consumed by the fungi. Increased % O_2 consumption was detected during the initial hours of the batch cycles of the last 48 h trial cycles and 72 h trial. This together with the accelerated COD reduction measured for the same period indicated increased fungal activity and rapid elimination of effluent components.

A study where effluent was aerated in a drum in the absence of the fungal inoculum showed minimal COD loss thereby indicating that volatilization of VOC effluent components marginally contributed to COD reduction. This was, however, a preliminary investigation and reactor headspace gas samples will have to be collected and analysed to assess and quantify the effluent components stripped and released into the vapour phase.

Light microscopy examination of the biofilm on the wood chips showed fungal hyphae attached to the chips. This indicates that fungi colonized and penetrated the wood chips to extract nutrients and were growing into the surrounding reactor environment where fungal mineralization processes degrade the effluent components. A slimy biofilm was observed throughout the top 30 to 40 cm of the packed-bed. Nematodes were noted in the reactor liquid and biofilm preparations. It was suggested that the nematodes crop or harvest the biofilm so as to minimize excessive microbial growth.

Though a $\pm 50\%$ COD reduction was monitored for the initial 24 h of the batch cycles, only a further 20 to 24 % COD was reduced in the subsequent 48 h. The increase in the pH during treatment cycles might have affected fungal activity. By correcting the pH and maintaining an acidic environment ($\text{pH} < 5.5$) to support fungal activity throughout a batch cycle, an increase in the % COD reduction was anticipated.

Although CP were not present in the waste oil recycling effluent, 2,4,6-TCP mineralization was shown by spiking the effluent with TCP in the intermediate-scale laboratory study. A reasonable assumption can be made that the outcome of the spiked effluent laboratory study also applies to the pilot plant.

This is an ongoing study and the following parameters and supplements to the reactor would need to be investigated to ultimately optimize plant performance and increase % COD reduction:

- pH correction by adding an increased phosphoric acid dosage to the reactor vessel.
- The addition of yeast extract as an auxiliary nutrient source. Adding an additional carbon source may extend the life span of the packing and the consumption of the wood tissue would be delayed.
- The measurement of effluent dissolved oxygen (DO) concentration during batch cycle treatments combined with the increase in air delivery to identify the optimum air flow rate at which an ideal DO concentration and increase performance is achieved.
- Measurement and quantification of volatilized effluent components in reactor head space.
- Packing the reactor with larger wood chips. The pine chips used in the reactor were smaller than the 3 x 3 cm prescribed originally in the Process Design. A larger wood chip might reduce air channelling by minimizing the plugging of the packed bed by excessive microbial growth.
- Since a fungal/bacterial consortium should have established, investigation of the microbial ecology of the reactor would provide for an understanding of the degradation mechanisms. This could lead to further parameter changing and optimization studies (for e.g. addition of specific nutrients).

Chapter Seven

Conclusions

This study showed successful operation of an integrated anaerobic/aerobic system to dechlorinate and mineralize a chlorinated aromatic pollutant, as well as a complex hydrocarbon effluent stream in the aerobic component of the proposed bioprocess environment. The following aspects of the system were investigated:

- White-rot fungal cultures were immobilized in a SBR configuration and mineralization of phenol and 2,4,6-TCP-contaminated liquid shown.
- Mobilization and dechlororespiration of 2,4,6-TCP in a sulphate-reducing environment and the potential of the system for groundwater and soil remediation was shown.
- The anaerobic and aerobic reactor units were evaluated as linked unit operations. The dechlorination, and mineralization of TCP and its dechlorination products, was shown.
- Scale-up implication of the SBR immobilized white-rot fungal component in the treatment of a real hydrocarbon waste stream, and the potential application of the reactor as a free-standing treatment technology, was also shown.

Implementing the dual-stage bioprocess system at an actual deep subsurface CP-contaminated site would require the administration of the correct carbon:SO₄²⁻ ratio to facilitate sulphate-reducing-affected dechlororespiration. This is necessary to effect sulphate reduction and the fermentation of the carbon source to optimize the dechlorination rate. The supplementation of the contaminated soil/aquifer zone with sulphate presents an opportunity to induce active pollutant transformation without further jeopardizing environmental quality such as nitrate supplements would do by introducing potentially toxic effects. It is a fairly prevalent electron acceptor in subsurface and marine habitats. In environments where negligible to zero sulphate concentrations are available for sulphidogenic-affected reductive dechlorination reactions, sulphate supplements can easily be made since sulphate is the least problematic electron acceptor and it is relatively easy to control input SO₄²⁻ concentrations.

Sulphidogenic-dependent reductive dechlorination was shown to promote mobilization of soil adsorbed TCP either through mobilization of the compound before dechlorination and/or desorption of metabolites due to their lower hydrophobicity and concomitant dechlorination to lower chlorinated compounds in the aqueous environment. The application of the SRB-dechlororespiring co-culture should promote mobilization and transformation of adsorbed CP through increased dechlororespiration rates in the contaminated subsurface environment. The mechanisms of facilitated desorption is still unresolved and the application of microbially-induced desorption and the utilization of adsorbed compounds by microorganisms have not been actively investigated. This study indicated the possible application of the co-culture in soil remediation applications and, according to the researcher's knowledge, was the first attempt to demonstrate pollutant desorption during sulphate-reducing-affected dechlororespiring reactions. Though pH has been implicated in affecting the desorption behaviour of CP (You and Liu, 1996), Chang *et al.* (1996) established that transformation of PCP and TCP in alkaline and acidic soils was the result of the activity of a CP-adapted bacterial consortium. Enhanced *in-situ* reductive dechlorination, however, needs to be investigated at pilot- or field-scale to address site-specific characteristics that may affect the efficiency of desorption and dechlorination reactions.

Following the rapid mineralization of the pollutant and its dechlorination products in the aerobic compartment, the return of the liquid to the subterranean environment would require pH correction since the acidic water was shown to inhibit sulphate reduction and reductive dechlorination.

This research programme constitutes a novel approach to remediate chlorinated aromatic compound-contaminated subterranean soil/aquifer environments. Dual or subsequent anaerobic/aerobic bioprocesses have been considered to clean-up contaminated soil/aquifer/surface water zones, however, these integrated biodegradative processes relied on reductive dechlorination in methanogenic environments and gradual aerobic bacterial degradation. Integrating a SRB-dechlororespiration co-culture compartment with rapid elimination of the parental CP compound and its metabolites by employing white-rot fungal mineralization activity is to our knowledge a new and unique approach to clean-up halogenated pollutant contamination.

Scale-up opportunity was only available for the fungal SBR compartment of the integrated anaerobic/aerobic system. Subsurface soil contaminated with CP was not located due to time constraints to initiate a pilot investigation of the anaerobic compartment of the dual-stage system. However, the mineralization of TCP-spiked waste oil recycling effluent treated in the laboratory fungal intermediate-scale reactor showed the potential application of the aerobic compartment as part of the integrated process. Nevertheless, given the opportunity presented the fungal SBR component was successfully scaled-up for technical-scale investigation. Clearly further work will need to investigate the scale-up of the sulphidogenic dehalogenation reactions in *in-situ* soil bioremediation. The potential application of the dual-stage system in halogenated pollutant containing wastewater treatment when operated as linked bioreactor units also validates a scale-up evaluation of the system. Transformation of these pollutants to lower chlorinated metabolites followed by fungal mineralization will reduce the chance of potentially toxic and endocrine inhibiting compound production that may occur when the aerobic reactor is used as a free-standing wastewater bioremediation system. FFS Refiners (Pty) Ltd. has the capacity to investigate the remediation of chlorinated compound-contaminated effluent and would pursue technical-scale assessment of the dual-stage system once optimisation of the scaled-up pilot plant is concluded.

Nevertheless, considerable progress has been made in the demonstration and development of a novel approach. Among the issues that require further investigation are the following:

- The sustainability of the physical integrity of wood tissue as supporting medium and the admission of externally added nutrient supplements to extend material life span in the fungal reactor.
- Evaluating the impact of an auxiliary carbon source in alternating growth/induction cycles on LiP and MnP secretion and activity in relation to biodegradation rates.
- The optimization of operating conditions such as retention times, carbon to sulphate ratios, pollutant concentrations and air delivery.
- The dechlorination reaction specificity in relation to the co-culture and other chlorinated pollutants.

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Appendix A

Growth Medium Recipes

1. Fungal Growth Medium (g.L⁻¹)

Glucose, 10

Yeast extract, 0.8

KH₂PO₄, 5

MgSO₄·7H₂O, 0.3

CaCl₂, 0.7

NH₄Cl, 10

Final medium pH was $\pm$ 4.5. Medium was autoclaved at 121 °C for 20 min.

2. Induction Medium (g.L⁻¹)

KH₂PO₄, 5

MgSO₄·7H₂O, 0.3

CaCl₂, 0.7

Glucose, 2 (foam glass bead-packed reactor only)

3. Postgate Medium B (g.L⁻¹)

Sodium lactate, 1.1 (10 mM lactate)

NH₄Cl, 1

Na₂SO₄, 1.3

K₂HPO₄, 0.5

CaCl₂·6H₂O*, 0.1

MgSO₄*, 2

Yeast extract, 1

Ascorbic acid[‡], 0.1

Sodium thioglycolate[‡], 0.1

Final medium pH was adjusted to 7.5 using 1 M NaOH

*[‡]Prepared and autoclaved separately and 10 ml each added to medium

Table 1 for Dechlorpaper. Postgate medium B composition

Sodium lactate	1.1 g.L ⁻¹ (10 mM lactate)
NH ₄ Cl	1
Na ₂ SO ₄	1.3
K ₂ HPO ₄	0.5
CaCl ₂ .6H ₂ O [*]	0.1
MgSO ₄ [*]	2
Yeast extract	1
Ascorbic acid [‡]	0.1
Sodium thioglycolate [‡]	0.1

Final medium pH was adjusted to 7.5 using 1 M NaOH

^{*‡}Prepared and autoclaved separately and 10 ml each added to medium

Appendix B

Analytical Methods

1. Extraction Procedure

A sample (5 ml), 9 ml ethyl acetate and 1 ml of a 1 mg.L⁻¹ solution of 4-bromophenol (internal standard) was combined. The mixture was added to a separating funnel and shaken vigorously while venting the contents frequently. The procedure was repeated with the liquid phase and extracted organic solvent phases combined and evaporated under reducing pressure using a rotary evaporator (Büchi). The extractives were resuspended in the HPLC mobile phase (10 ml) and filtered through a 0.45 µm nylon filter (Osmonics) before injection into the instrument. The extraction efficiency measured was ≥ 92 %.

2. HPLC Operation

Analyses were carried out using a Beckman HPLC (Solvent Module 126) equipped with a UV detector set at 280 nm and a Nova-Pak® C₁₈ column (4.6 x 250 mm) (Waters). Acetonitrile (methyl cyanide) – MilliQ distilled water– acetic acid (79:20:1) was used as eluent at 1 ml.min⁻¹. Calibration standards were run for quantification purposes. Peak area integration was done with Beckman Coulter, Inc. 32 Karat software. The sample injection volume was 50 µl.

Appendix C

Electron Microscopy Protocols

1. Transmission Electron Microscopy

Wood tissues were flooded with cold buffered glutaraldehyde (2.5 % in 0.1 M phosphate buffer consisting of 80 parts Solution 1: 35.8 g.L⁻¹ Na₂HPO₄.12H₂O and 20 parts Solution 2: 13.6 g.L⁻¹ KH₂PO₄) and dissected into small cubes. The tissue pieces were transferred to a petri dish containing cold buffered glutaraldehyde and cut into smaller cubes (no larger than 1 mm³). Thereafter, the pieces were placed in specimen tubes containing cold buffered glutaraldehyde and allowed to fix for a minimum period of 12 h at 4 °C.

During the following secondary fixation, dehydration and embedding procedure, the glutaraldehyde was decanted and tubes washed twice with phosphate buffer at 10 min each wash.

Thereafter, the buffer was decanted and 1 % buffered osmium tetroxide solution (0.5 g osmium tetroxide, 45 ml Solution D and 5 ml Solution C; Solution D = 41.5 ml Solution A: 2.26 % NaH₂PO₄.H₂O, 2.25 %, NaHPO₄.2H₂O and 8.5 ml Solution B: 2.52 % NaOH; Solution C: 5.4 % glucose) was added to cover tissue samples. After 90 minutes the osmium tetroxide was decanted off and washed twice with buffer (10 min each wash).

The tubes were refilled with 30 % ethanol and left for 5 min whereafter the procedure was repeated for 50, 70, 80, 90 % and absolute ethanol. The dehydrating procedure for all ethanol concentrations used two changes of absolute ethanol.

Absolute ethanol was decanted and refilled twice with propylene oxide and left 15 min in each case. Propylene oxide : embedding mixture (75:25) was added to tubes and allowed to infiltrate for 90 minutes. The procedure was repeated for 50:50, 25:75 and pure embedding media and allowed to infiltrate overnight.

The embedding mixture consisted of: Mixture A (Epon 812, 6.8 g; DDSA, 8.3g) and Mixture B (Epon 812, 10.3 g; NMA, 9.1g). Final mixture comprised of Mixture A (12.1 g),

Mixture B (17.0 g) and DMP 30 (0.8 g). The mixture was stirred for 20 min and poured in McCartney bottles and allowed to mix at 1 rpm on a shaker until use.

Flat specimen molds were filled to about $\frac{2}{3}$ full with pure embedding media and samples from the tubes were transferred singly to the capsules. The molds were placed in an oven (set at 60 °C) to allow embedding media to polymerize for 48 h. Thereafter, the molds were cooled and polymerized blocks removed and stored in labelled specimen tubes. Blocks were trimmed and ultra thin slices cut using glass knives and a RMC MT7 ultramicrotome.

Tissue slices were seized onto grids and heavy metal staining using uranyl acetate and lead citrate concluded the procedure. The grids were examined in a JEOL 1210 TEM.

2. Scanning Electron Microscopy

The samples were fixed in 2.5 % gluteraldehyde solution in 0.1 M phosphate buffer (pH 7.0) overnight. The next day the samples were washed twice in 0.1 M phosphate buffer for 10 min. The samples were then passed through an alcohol dehydration series: 30, 50, 60, 80, 90 and 100 % ethanol for 10 min, followed by two 100 % ethanol washes for 10 min. Following the alcohol dehydration, samples were critical point dried using liquid CO₂ in a Polaron E3000 Critical Drying Apparatus, and sputter coated in gold with Polaron E5100 Sputter Coating Unit. The samples were then observed in a JEOL JSM 840 SEM.

Appendix D

Sulphate Determination

1. HPLC Anion Exchange Chromatography

Sulphate was determined using a Waters 501 HPLC pump, a Hamilton PRP-X100 anion exchange column (150 x 4.1 mm, dimension; 10 μm , particle size), a Waters conductivity detector (Model 430) and JMBS Heculite chromatography interface. Analysis was carried out using Borwin chromatography software. *p*-Hydroxybenzoic acid mobile phase (4 mM *p*-hydroxybenzoic acid in deionized distilled water, 2.5 % v/v methanol, pH 8.5) was used as eluent at a flow rate 2 $\text{ml}\cdot\text{min}^{-1}$. Calibration standards (tested for reproducibility and linear response in the range 0 – 200 $\text{mg}\cdot\text{L}^{-1}$) were run for quantification purposes. All samples were diluted (using a zinc acetate solution, 10.44 $\text{g}\cdot\text{L}^{-1}$ in deionized water to precipitate out sulphide as insoluble zinc sulphide) to fall within the range and filtered through 0.45 μm pore-size nylon filters (Osmonics). Removal of organics (for the benefit of anion exchange analysis) was achieved by passing samples through two Isolute® C18 (EC) columns (25 mg, 1ml) before injection into the instrument. A sulphate standard of 100 $\text{mg}\cdot\text{L}^{-1}$ was injected every 5 – 7 runs to assess column performance.

2. Turbidometric sulphate determination

The Hach Laboratory Supplies Sulphate kit was also used to quantify SO_4^{2-} levels. Samples were prepared in similar fashion as outlined above for HPLC analysis, however, samples were not passed through C18 columns.

Appendix E

Sulphide Determination

Reagents

1. N,N-dimethyl-p-phenylene diamine dihydrochloride solution (Solution A)

$(\text{CH}_3)_2\text{N} \cdot \text{C}_6\text{H}_4 \cdot \text{NH}_2 \cdot 2\text{HCl}$, 2 g; $6 \text{ mol} \cdot \text{dm}^{-3}$ HCl, 500 ml

2. Ferric chloride solution (Solution B)

Iron (III) chloride, 8 g; $6 \text{ mol} \cdot \text{dm}^{-3}$ HCl, 500 ml

3. Zinc acetate solution (Solution C)

Zinc acetate, $10.44 \text{ g} \cdot \text{L}^{-1}$ in distilled water

4. Sulphide stock solution

$\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, 0.750 g; oxygen-free distilled water, 1 000 ml

5. Oxygen-free water

A suitable volume of water was boiled for 30 min. Thereafter, the water was allowed to cool down to room temperature while continuously sparging oxygen-free nitrogen gas through it. The water was stored in a sealed bottle.

Method

100 μl Solution C was pipetted into a test tube whereafter a suitable sample volume made up to 5 ml using distilled water was dispensed into the tube. 0.5 ml Solution A and 0.5 ml Solution B was added, mixed and left to stand for 1 h. The absorbance was read at 670 nm using a Beckman Spectrophotometer (DU 530 UV/VIS). A blank was prepared in the same way substituting water for the sample. A calibration standard curve was prepared with concentrations ranging from 0 to $1 \text{ mg} \cdot \text{L}^{-1}$.

Appendix F

Raw Data: Field Operation

Table 1. COD (mg.L⁻¹) data for the plant during the initial stages of operation.

DATE	FEED COD	RUNNING COD	FINAL OUT COD	TIME h	CLARIFIER COD	± BATCH VOLUME m ³
02/11/25	9640					
02/11/27	7850		3680	48		2
02/12/02	8300		1420	120		2
02/12/04	5060		1390	48	1070	2
02/12/06	8820		1690	48		2
02/12/09	8000		1700	72		2
02/12/11	8880		1310	48	2760	2
02/12/17	11040				2164	
02/12/19		1676		48		
02/12/20	12400		1410	72		2
02/12/23			1438	72		2
02/12/24	19600					
02/12/27	20120		10430	72		2
02/12/30	19050		7310	72		2
03/01/03		5270		96		
03/01/06	14700 (pH 5.2)		2990 (pH 7)	168		2
03/01/07		6720 (pH 6.4)		16		
03/01/08		4850 (pH 6.8)		48		
03/01/10		2830 (pH 7.1)		96		
03/01/13		2100 (pH 7.5)		168		
03/01/14	15840 (pH 4.2) (20.2% pO ₂)		1580 (pH 7.5)	192		2
03/01/15		5890 (pH 7.6) (19.5% pO ₂)		24		
03/01/16		4330 (pH 7.6) (20.1% pO ₂)		48		
03/01/17		2130 (pH 7.5) (20.4 pO ₂)		72		
			1730 (7.6 pH) (20.4 pO ₂)	144		

Table 2. COD (mg.L⁻¹), pH, LEL (%) and pO₂ (%) data for batch volumes (m³) treated during the 48 h trial.

	20/01 to 22/01				22/01 to 24/01			
h	COD	pH	LEL	pO ₂	COD	pH	LEL	pO ₂
0	18300	4.5	9	20.2	19420	4.6	17	19.3
4	8780	5.3	4	20.4	10160	5.3	4	20.2
12	8150	5.4			9520	5.4		
16	7950	5.5			9640	5.4		
20	6740	5.6			8620	5.5		
24	5890	5.9	2	20.3	8160	5.5	8	15.5
28	5810	6.2	3	20.2	7790	5.5	5	20.2
32	5620	6.3	2	20.2	8970	5.5	3	20.2
36					7960	5.6		
40	4420	6.6			6530	5.3		
44	4010	6.7			6170	5.9		
48	3890	6.3	3	20.2	7390	6	3	20.3
52	3710	6.7	2	20.2	6720	6.4	2	20.2
vol m ³								
raw	1.5				1.74			
clarifier	0.26				0.19			
total	1.76				1.93			
	27/01 to 29/01				29/01 to 31/01			
h	COD	pH	LEL	pO ₂	COD	pH	LEL	pO ₂
0	18920	4.6	13	20.2	21000	4.5	12	19.9
1	14880	4.9	7	20.1	16380	4.7	6	20.1
2	12970	4.9	6	20.2	14400	4.8	5	20.2
3	13310	5	5	20.2	14360	4.9	5	20.2
4	12660	5	5	20.2	14240	4.9	5	20.3
12	11380	5.1			12610	5		
16	11380	5.15			11940	5.1		
20	10690	5.2			11740	5.2		
24	10470	5.2	4	20.2	11710	5.3	4	20
28	9630	5.3	4	19.9	11370	5.5	4	19.9
32	9520	5.4	4	20.2	10790	5.5	4	20.1
36					10350	5.7		
40	9010	5.5			10090	5.9		
44	8560	5.5			9830	6		
48	8560	5.6	3	20.2	9980	6.4	4	20.2
51	8330	5.7	3	20.2	9010	6.4	4	20
vol m ³								
raw	2.17				2.29			
clarifier	0.09				0.06			
total	2.26				2.35			

Table 2. COD (mg.L⁻¹), pH, LEL (%) and pO₂ (%) data for batch volumes (m³) treated during the 48 h trial.

	3/02 TO 5/02				5/02 TO 7/02			
h	COD	pH	LEL	pO ₂	COD	pH	LEL	pO ₂
0	17500	4.5	12	19.9	16960	4.6	14	19.9
1	15440	4.8	5	20.1	13040	4.9	6	20.2
2	13680	5	5	20.1	13620	4.95	6	20.2
3	13560	5.1	4	20.2	12880	5.05	5	20.2
4	13400	5.15	4	20.1	13840	5.15	5	20.3
12	11750	5.4			11310	5.4		
16	11740	5.65			10630	5.8		
20	11380	6			11220	6.1		
24	11050	6.25	4	20	10010	6.5	4	20
28	9960	6.3	5	20	11060	6.4	4	19.9
32	9930	6.4	4	20	10380	6.7	4	20.1
36	9670	6.6						
40	9250	6.85			9960	6.85		
44	9040	7			10180	7		
48	9070	7.1	3	20.1	9610	7.05	3	20.1
51	8600	6.8	3	20.2	8850	6.7	3	20.1
vol m ³								
Raw					1.87			
Clarifier	0.076				0.076			
Total	2 (Estimated)				1.946			
	10/02 TO 12/02				AERATED DRUM			
h	COD	pH	LEL	pO ₂	COD	pH		
0	18000	4.6	10	19.9	18440	4.6		
1	15340	5	4	20.3	18420	4.6		
2	12700	5.25	4	20.2	17640	4.6		
3	14000	5.5	3	20.3	17940	4.6		
4	12460	5.7	3	20.2	17100	4.5		
12	11040	6.6			16380	4.7		
16	10410	6.8			16440	4.7		
20	9460	7.2			17180	4.7		
24	9560	7.4	3	20.1	17780	4.75		
28	8970	7.1	3	20.1	16260	4.6		
32	8580	7.1	3	20.1	16480	4.6		
36	8310	7.15			16240	4.5		
40	7870	7.15			15500	4.5		
44	7550	7.2			16800	4.5		
48	7360	7.25	2	20.1	17160	4.6		
51	7890	7.35	2	20.1	17000	4.7		
vol m3								
Raw	1.79				0.2			
Clarifier	0.076							
Total	1.866				0.2			

Table 2. COD (mg.L⁻¹), pH, LEL (%) and pO₂ (%) data for batch volumes (m³) treated during the 48 h trial.

	12/02 to 14/02				AERATED DRUM			
h	COD	pH	LEL	pO ₂	COD	pH		
0	16100	4.7	10	19.8	18800	4.7		
1	15340	5.5	6	20.1	19180	4.9		
2	13260	5.45	5	20.1	18360	4.7		
3	13000	5.65	5	20.1	18060	4.7		
4	14320	5.9	5	20.2	18360	4.75		
12	12360	6.7			17400	4.8		
16	10620	6.9			17720	4.8		
20	10090	7.3			17220	4.8		
24	9910	7.3	4	20.2	18540	4.8		
28	9810	7.2	4	20.1	19060	4.8		
32	9550	7.2	3	20.3	18220	4.8		
36	9500	7.3			17520	4.85		
40	8930	7.3			16920	4.85		
44	9030	7.35			15760	4.85		
48	8300	7.4	3	20.2	16720	4.9		
51	7630	7.3	3	20.1	17500	4.8		
vol m ³								
Raw	1.91				0.2			
Clarifier	0 (forgot to add)							
Total	1.91				0.2			

Table 3. COD (mg.L⁻¹), pH, LEL (%) and pO₂ (%) data for batch volumes (m³) treated during the 72 h trial.

	18/02 to 21/02				Air flow	25/02 to 28/02				Air flow
h	COD	pH	LEL	pO ₂	m ³ .hr ⁻¹	COD	pH	LEL	pO ₂	m ³ .hr ⁻¹
0	16920	4.8	9	19.8	65	14220	4.91	10	20	65
6	10630	6.52			65	9830	6.2			
12	9550	6.77				9400	6.7			
18	8880	7.1				9080	6.95	3	19.9	65
24	8740	7.1	3	19.9	65	8080	7.15	3	20	65
30						7830	7.21			
36						7070	7.28			
42						6550	7.31	2	20	65
48	6590	7.2	2	20.2	65					
54	6380	7.2				5380	7.35			
60	5980	7.2				5270	7.35			
66	5440	7.2				4790	7.4			
72	5340	7.2	2	20	65	4690	7.41			
vol m ³										
raw	2.11					2.16				
clarifier	0.076					0 (forgot to add)				
total	2.189					2.16				
	4/03 to 7/03				Air flow	10/3 to 13/03				Air flow
h	COD	pH	LEL	PO ₂	m ³ .hr ⁻¹	COD	pH	LEL	pO ₂	m ³ .hr ⁻¹
0	20060	4.7	6	19.8	55	19160	4.48	12	19.5	60
6	9860	6.29				10670	5.13	3	19.6	60
12	9340	7.15				9640	5.29			
18	8980	7.45	2	19.9	55	9480	5.61			
24	8620	7.6				9010	5.95	3	19.6	60
30	7190	7.5				8000	6.95	4	19.7	60
36	6910	7.6				7170	7.33			
42	5870	7.4	2	19.8	55	6650	7.54			
48	4940	7.5				6030	7.54	3	19.6	60
54						5450	7.4	3	19.6	60
60						4610	7.55			
66						3900	7.55			
72	3740	7.9	2	19.9	55	3320	7.52	2	19.8	60
vol m ³										
raw	2.17					2.25				
clarifier	0.084					0.13				
total	2.26					2.38				

Table 4. COD (mg.L⁻¹), pH, LEL (%) and pO₂ (%) data for batch volumes (m³) treated during 72 h trial with pH correction applied.

	17/3 to 20/3 with pH correction (4% phosphoric acid)				air flow	25/3 to 28/3 with pH correction (4% phosphoric acid)				air flow
hr	COD	pH	LEL	pO ₂	m ³ .hr ⁻¹	COD	pH	LEL	pO ₂	m ³ .hr ⁻¹
0	19140	4.6	12	19.4	60	25460	4.5	13	19.9	60
6	10590	5.25				16700	5.12	5	20.5	60
12	9760	5.1				15600	5.1			
18	9460	5.12				14840	5			
24	8940	5.2				13100	4.9	3	20	60
30						12460	5.4			
36						12160	5.4			
42						11350	5.8			
48	6860	5.85				11080	6.2			
54	6310	6.41								
60	5630	6.54								
66	4870	6.61								
72	4700	6.78	1	20	60	8530	7.4			60
vol m ³										
raw	2.1					2.2				
clarifier	-					-				
total	2.1					2.2				