BIOLOGICAL DEGRADATION OF AZO DYES IN AN ANAEROBIC SYSTEM

by

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DECLARATION OF CANDIDATE

I, Cynthia Mary Carliell, declare that unless indicated, this dissertation is my own work and that it

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SUMMARY

Wastewater discharges from textile dyehouses are complex, variable, and highly coloured, generally containing dyes at concentrations of 10 to 200 mg/M, depending on the dyeing proces in operation. Although dilution of the effluent can, and does occur, colour is discernible at concentrations as low as 1 mg/M. Dilution of dyehouse effluent therefore tends to compound the treatment problem, by increasing the volume of coloured effluent.

Conventional treatment processes presently in use at waste water treatment works do not usually achieve satisfactory colour removal, resulting in coloured effluent being discharged from the treatment works. Consequently, downstream use of the treated water is limited, and the highly visible nature of the pollution source often gives rise to public concern.

Solutions to treatment problems are being sought through exploration of chemical, physical and biological treatment options. Chemical treatment processes such as the use of Fentons Reagent, and physical treatment such as the removal of dyes by filtration (reverse osmosis, crossflow microfiltration) are successful in removing colour. However, by-products such as possible dye intermediates (Fentons reagent) or dye concentrates (filtration) are alternative pollution sources which must be treated or disposed of.

Research into specialised biological treatment of dye-containing wastewater has shown potential for a complete treatment system ie. mineralisation of organic dye compounds, to inorganic constituents such as carbon dioxide, methane, and water.

Degradation of simple azo dyes by aerobic microorganisms has been reported in the literature, however, factors such as the restricted substrate specificity and highly bred nature of the bacteria, has rendered these processes impractical for large-scale waste treatment. In contrast to the substrate specificity demonstrated by aerobic dye-degrading microorganisms, anaerobic populations show potential for non-specific colour removal, although the nature of the decolourisation process, and the ability of anaerobic populations to mineralise intermediary dye metabolites, is uncertain.

Anaerobic treatment of dyes was chosen as the target of this research, and was investigated in two phases : (i) the ability of anaerobic microorganisms (enriched from digester sludge) to decolourise a reactive red dye viz. Procion Red HE-7B, and (ii) the ability,if any, of this anaerobic population to degrade/mineralise any dye metabolites originating from decolourisation of Procion Red HE-7B.

Research to date has been conducted in the form of batch studies in anaerobic serum bottles. Factors such as the order of decolourisation, and subsidiary rate limiting factors, have been addressed in the initial studies. The order of decolourisation of Procion Red HE-7B has been found to be first-order with respect to dye concentration, however, the decolourisation of Procion Red HE-7B does not appear to be a result of a catabolic pathway, and for this reason it is possible that the rate of decolourisation is pseudo-first order ie. factors other than dye concentration are indirectly responsible for the shape of the decolourisation curve. The literature favours the theory that decolourisation of azo dyes by anaerobic microorganisms is the result of dye reduction by reduced flavin nucleotides in the electron transport chain. To test this theory, competitive electron acceptors, such as nitrate and sulphate, are added to the assay bottles. To date only nitrate addition has been completed, and has been found to effectively inhibit decolourisation. This collaborates the above hypothesis, as nitrate is more favourable thermodynamically, and is therefore reduced preferentially in the electron transport chain.

Possible rate-limiting factors for decolourisation of Procion Red HE-7B, are as diverse as cell permeability, redox potential of the dye, and nature and concentration of an additional carbon source. The latter has been investigated by addition of glucose (non-limiting concentration) to the bottles in which decolourisation takes place. This has been found to result in an approximate 15-fold increase in decolourisation of Procion Red HE-7B. Alternate cabon rich sources that may be applicable in a wastewater treament system are currently being investigated. Microorganisms are being acclimated to utilise the carbon sources present in cotton scouring effluents, and preliminary results indicate the

ability of these microorganisms to decolourise Procion Red HE-7B at a rate comparable to that in the standard assay bottles.

Phase II ie. the mineralisation of resultant dye metabolites, will be investigated via Biochemical Methane Potential (BMP) tests, and through identification of dye metabolites, with the aim of determining the fate of these metabolites.

In addition, toxicity trials are to be conducted to assess the concentration of dye (and metabolites) that

ABSTRACT

Decolourisation of a reactive azo dye, Procion Red HE-7B was studied using serum bottle assays. Inoculum for the assays was obtained from laboratory digesters in which anaerobic digester sludge was incubated with Procion Red HE-7B for 4 months.

A standard set of operating conditions were developed to study the anaerobic decolourisation of Procion Red HE-7B. The rate of decolourisation in the standard assay system was determined to be first-order with respect to dye concentration, but was inversely proportional to the initial dye concentration in the system. This was not in agreement with first-order kinetics and was attributed to microbial inhibition, either due to increasing concentrations of Procion Red HE-7B and/or metabolites. These results were compared with those in literature and probable rate-limiting factors for decolourisation were identified as the rate of permeation of Procion Red HE-7B into the microbial cells, and the presence of supplemental carbon and/or additional electron acceptors.

Dye permeation was investigated using permeabilised biomass. Increased permeation of the dye into the microbial cells was found to inhibit decolourisation, suggesting that decolourisation occurred extracellularly. The rate of Procion Red HE-7B decolourisation was measured in the presence and absence of a supplemental carbon source (glucose 1 g/M) and was found to be limited in the absence of glucose. The addition of nitrate (as a competitive electron acceptor) to the assay system inhibited decolourisation for a period of time proportional to the concentration of nitrate in the system. In contrast the addition of sulphate to the system was shown to have no marked effect. It was proposed that nitrate reduction was preferential to Procion red HE-7B reduction which, in turn, was preferential to sulphate reduction. The role of system redox potential in the anaerobic decolourisation of Procion Red HE-7B was therefore investigated. It was found that a strictly anaerobic system was conducive to decolourisation.

The chemical reaction responsible for decolourisation was investigated using ultraviolet scanning. Decolourisation was found to be caused by reduction of the azo bonds and subsequent destruction of the dye chromophore. The fate of the ensuing metabolites was investigated with respect to their mineralisation potential in the anaerobic system, however, neither acclimated nor unacclimated biomass showed any capacity for mineralisation of Procion Red HE-7B.

The toxicity of Procion Red HE-7B to the anaerobic biomass was investigated by means of an anaerobic toxicity assay. Total gas production was monitored and maximum rate ratios were calculated to determine the level of inhibition. Acclimated biomass did not show significant inhibition at any of the test concentrations, however, unacclimated biomass was significantly inhibited at the higher dye concentrations.

Abiotic decolourisation of Procion Red HE-7B in the standard assay system was found to be caused by adsorption of the dye to the biomass (approximately 17 %) and decolourisation by the mineral salts medium (approximately 35 %). Adsorption isotherms developed for Procion Red HE-7B with anaerobic digester sludge as the adsorbent conformed to Freundlich and Langmuir isotherms.

A treatment process was investigated using organic-rich textile scouring effluents as carbon sources during decolourisation of Procion Red HE-7B. This system showed potential for decolourisation of the dye and for the reduction of the organic carbon in the scouring effluent.

GLOSSARY

Acclimation	The adaptation of a microbial community to degrade a previously recalcitrant compound, through prior exposure to that compound.
Activated sludge	A mixed association of prokaryotic and eukaryotic microorganisms, which aerobically decompose waste in an activated sludge effluent treatment system.
Adaptation	A change in the microbial community that increases the rate of transformation of a test compound, as a result of prior exposure to that test compound.
Adsorption (dye)	Binding of dye compounds to surfaces such as microbial cells or activated carbon, usually through electrostatic interaction between the charged support and the charged cell.
Aerobic	The condition of living or acting only in the presence of molecular oxygen.
Aerobe	A microorganism capable of growing and metabolizing in the presence of free oxygen ie. in an aerobic environment.
Algae	Organisms that perform oxygenic photosynthesis and possess chloroplasts. May be single- or multi-cellular organisms.
Anaerobe	A microorganism capable of growing or metabolizing in the absence of free oxygen ie. an anaerobic or anoxic environment. These microorganisms may be facultative or obligative, the latter will perish in the presence of free oxygen.
Anaerobic filter	A fixed film anaerobic digester which retains the microorganisms in the voids created by the packing media.
Anaerobic respiration	Respiration in which the final electron acceptor is an inorganic molecule (nitrate or sulphate) other than molecular oxygen.

Anoxic	An environment where oxygen is present in the form of compounds such as nitrate or sulphate.
Anthraquinone dyes	Dyes based on the structure of 9,10-anthraquinone, with powerful electron donor groups in one or more of the four alpha positions.
Azo dyes	Dyes which contain at least one azo group (-N=N-), and can contain up to four azo groups.
Azo reductase	The enzyme that catalyses the reduction of azo bonds.
Bacteria	Single-cell, prokaryotic microorganisms.
Batch culture	A closed culture environment in which conditions are continuously changing according to the metabolic state of the microbial culture.
Benzidine dyes	Dyes prepared from derivatives of the aromatic diamine benzidine (a carcinogen).
Biodegradable	A property which allows the microbial decomposition of an organic compound to inorganic molecules such as carbon dioxide, methane and inorganic salts.
Bleaching	The procedure, other than by scouring only, of improving the whiteness of textile material by decolourising it from the grey state, with or without the removal of natural colouring and or extraneous substances.
Carcinogenic	Cancer-causing.
Catabolism	The dissimilation of complex organic molecules, generally for the purpose of obtaining energy or simple compounds needed for synthesis of other organic matter.
Cationic dyes	Dye which contain a positive charge, either centered on one atom (usually nitrogen), or delocalized over many atoms.
Cell wall	The outer layers of bacterial cells comprise the cell wall. The cell wall functions to protect the bacterial cell from osmotic lysis in hypotonic environments, determines cell shape, and plays a role in movement and division.

Chemostat	A continuous culture system in which static conditions are maintained and the bacterial culture is kept in the logarithmic stage of growth.
Co-factors	A small non-protein inorganic component of an enzyme, frequently a metallic ion such as magnesium, zinc, copper or iron.
Denitrification	Microbial reduction of nitrates to free nitrogen, commonly observed with certain types of organisms utilizing anaerobic respiration.
Deoxyribosenucleic acid (DNA)	A single or double stranded macromolecular chain of nucleotides, the sequence of which determines the genetic code.
Desizing	A textile finishing process in which size is removed from the cloth or yarn to be treated.
Dyeing auxillaries	Chemicals used in the dyeing process to aid the dyeing of the cloth/yarn.
Electron transport (respiratory) chain	A chain of carrier molecules with fixed orientation in the cell membrane, through which electrons are transported and ATP generated.
Enrichment	Selection of microorganisms with certain characteristics, from a mixed culture, through manipulation of culture conditions.
Freeze-drying	The dehydration of frozen material (biological, pharmaceutical or foodstuffs) through sublimation.
Fungi	A diverse group of nonphotosynthetic, coenocytic microorganisms which usually have a vegetative structure known as a mycelium.
Gram negative	Bacteria that appear pink/red when Gram stained.
Gram positive	Bacteria that appear dark purple when Gram stained.
Gram stain	A staining technique that enables the differentiation of all bacteria into two basic groups viz. Gram negative or gram positive.
Halophilic	A property that enables microorganisms to tolerate relatively high sodium chloride concentrations in their environment.

Inducible enzymes	Enzymes that are not normally present in the microbial cell, but are synthesised in the presence of an inducer substrate.
Intercalation	Insertion of a dye between two base pairs in the DNA chain, which may give rise to error in DNA replication, and consequently, mutations.
Intermediates (dye)	The compounds used to synthesise dyes.
Kier liquor	The waste liquid which comes from scouring cotton with alkali, in specially constructed vessels known as kiers.
Medium	Mixture of nutrient substances required by cells for growth and metabolism.
Metabolism	The physiochemical transformations through which foodstuffs are synthesized into complex elements, complex substances are rendered into simple ones, and energy is made available for use by the organism.
Metabolites	Intermediate compounds formed during dye catabolism.
Microbial association	A mixed population of microorganisms which achieve mineralisation of organic compounds through cooperative metabolism.
Mineralisation	Microbial decomposition of an organic compound to inorganic constituents such as carbon dioxide, methane and water.
Mixed culture	Culture consisting of two or more types of microorganisms.
Mutagens	Certain chemical or physical agents that cause mutations to occur.
Mutation	A change in the sequence of bases in the bacterial genome, as a consequence of normal chromosomal replication, or exposure to mutagens.

Plasma membrane	The plasma membrane binds the protoplast, and is the cells principal osmotic barrier. It consists of a bilayer of phospholipids into which the membrane proteins are intercalated.
Plasmid	A covalently closed circular molecule of DNA that is extrachromosomal, autonomous and self-replicating.
Reactive dyes	Reactive dyes are coloured components capable of forming a covalent bond between the dye molecule and the fibre.
Recalcitrant	Resistant to microbial degradation.
Ribonucleic acid (RNA)	A single or double stranded macromolecular chain of nucleotides, the sequence of which can specify the order of amino acids in polypeptide synthesis.
Respiration	The oxidative breakdown and release of energy from nutrient molecules by reactions with molecular oxygen (aerobic respiration) or inorganic molecules such as nitrate (anaerobic respiration).
Scouring	A textile finishing process in which cotton cloth/yarn is scoured with hot alkali to remove natural waxes and pectins from the cotton, together with the spinning oils.
Sizing Agents (size)	Gelatinous film-forming substances that are applied to the individual yarns during weaving in order to coat and protect the yarns from the abrasive effects of the filling yarns, as these are positioned by the shuttle action of the weaving loom.
Sulphate-reducing bacteria	Bacteria that use sulphate as an electron acceptor during anaerobic respiration.
Terminal electron acceptor	The final electron and hydrogen acceptor in the electron transport chain.
Textile finishing	A collection of processes in which raw cloth/yarn is cleaned and prepared for dyeing and printing.

Xanthene dyes	Contain a xanthene chromophore in which two aryl nuclei are linked by oxygen to form a pyrone ring. Similar terminal groupings (amino, hydroxy, or both) are usually present.
Xenobiotic	A compound not found in nature.

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ADMI	American Dye Manufacturers Institute
ATP	Adenosine triphosphate
ASP	Activated sludge process
CI	Colour Index
CMC	Carboxymethyl cellulose
COD	Chemical oxygen demand
DNA	Deoxyribonucleic acid
DOC	Dissolved Organic Carbon
EPA	Environmental Protection Agency
ETAD	Ecological and Toxicological Association of the Dyestuffs Manufacturing Industry
FAD	Flavinadenine nucleotide
FMN	Flavin mononucleotide
IC	Inorganic carbon
IC_{50}	Inhibition concentration
MRR	Maximum rate ratio
NAD^+	Nicotinamide-adenine-dinucleotide (oxidised form)
NADH	Nicotinamide-adenine-dinucleotide (reduced form)
$NADP^+$	Nicotinamide-adenine-dinucleotide-phosphate (oxidised form)
NADPH	Nicotinamide-adenine-dinucleotide-phosphate (reduced form)

OC	Organic carbon
OD	Optical density
PAAB	*p*-Aminoazobenzene
PSB	Purple non-sulphur bacteria
PVA	Polyvinyl alcohol
RBC	Rotating biological contactor
RNA	Ribonucleic acid
SMA	Specific methanogenic activity
sp	species
spp	species (plural)
TOC	Total organic carbon
UV	Ultraviolet

NOMENCLATURE

c	Equilibrium concentration of dye in solution (mg/M)
C_o	Concentration of dye at time zero (mg/M)
C_t	Concentration of dye at time *t* (mg/M)
k	Rate constant for first-order reaction kinetics (/h)
n	Number of moles
P	Pressure (Pa)
R	Gas constant (J/mol/K)
t	Time (h)
T	Temperature (Kelvin)
V	Volume (kM)
x	Amount of dye adsorbed per unit mass of sludge solids (mg/g)
K, k	Empirical constants

CHAPTER ONE

INTRODUCTION

Increasingly strict environmental legislation, both in Europe and the United States of America, has led to textile finishing industries being labelled high priority industries with respect to pollution (Licis et al., 1991). The textile industry was one of 17 industries in the United States of America to be selected for a pollution prevention programme implemented by the Environmental Protection Agency (EPA) in 1991 (Licis et al., 1991). Similarly in Europe, environmental legislation has necessitated increasingly *green* textile processing. This has, and is being achieved, through recycling of chemicals and water, production and use of biodegradable textile chemicals and dyes, as well as efficient effluent treatment (Mohr, 1992). This green approach has initiated the discussion of quality labels for European textiles. This quality assurance label would be awarded on the basis of internationally recognised criteria and test methods with special focus on the ecological and physiological aspects of clothing (Mohr, 1992).

A recent article in the International Dyer (April 1993) referred to a new set of trade principles adopted by both United States of America (USA) and European textile leaders in December 1992. These trade principles were designed to *ensure that trade was fair and that no country profited from slave or child labour, or polluting the environment.* That is, countries exporting raw or finished textile products to Europe or the USA will have to comply with the legislation stated in these trade principles, regardless of the legislation (or lack of) in the country of origin. Therefore, South African textile manufacturers with an export market in Europe or the USA must comply with European regulations in order to compete with other export markets (Mohr, 1991). In addition, public pressure as a result of growing environmental awareness, and upgrading of environmental legislation in South Africa, necessitates an increasingly environmentally-conscious approach to textile finishing.

1.1 THE TEXTILE INDUSTRY

In order to comprehend the effluent problems facing the textile industry it is neccessary to be familiar with the processes which result in effluent production. **Fig 1.1** is a schematic representation of the main stages involved in the processing of natural fibres (wool and cotton) and synthetic fibres (Barnes et al., 1992), with E marking those processes giving rise to effluent requiring treatment.

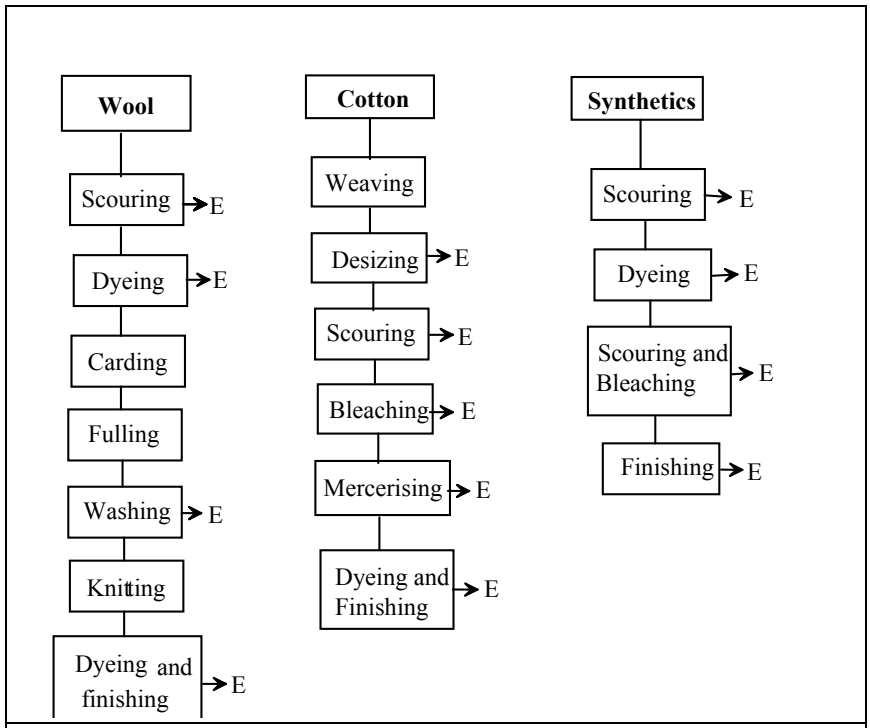

Fig 1.1 :
effluents requiring treatment.

As effluents emanating from the wet processing of *cotton* are the focus of this project, these processes are described in more detail below:

a) **Desizing.** This process is commonly the first wet stage in the processing of cotton. It involves the removal of size from the cotton fabric (using enzymes, acid or alkali) to ensure that subsequent chemical finishing processes function correctly. The cotton threads were initially coated with size to prevent breakages and impart a smooth finish during weaving. Sizes are organic compounds such as starch or starch-derivatives, cellulose-derivatives, polyacrylates and polyvinyl alcohol (PVA). Thus, desizing effluent generally has a high organic load and is characterised by high chemical oxygen demand (COD) values.

b) **Scouring.** Subsequent to desizing, any remaining natural impurities (i.e. organic components other than cellulose) are scoured from the cotton by a process of prolonged boiling in alkaline solutions, either in closed vessels known as kiers (Trotman, 1968) or in continuous reaction vessels. Scouring effluent is, therefore, characterised by high COD and pH values, and a strong yellow-brown colour.

c) **Bleaching.** This is used to improve the whiteness of the textile fabric and can be achieved with either oxidising or reducing agents. Bleaching effluents do not usually contain high concentrations of organics, however, where hypochlorite is used as a bleaching agent the presence of halogens in the effluent neccessitates a form of treatment.

d) **Mercerising.** Mercerising is the treatment of cellulosic fibres with a concentrated solution of sodium hydroxide, which swells the fibres and increases the strength and dye affinity of the fabrics. Mercerising effluent is characterised by high pH values.

e) **Dyeing.** Dyes used for the dyeing of cotton are direct, fibre reactive, sulphur and vat dyes (Refer to Appendix A for the classification and application of textile dyes). Fibre reactive dyes are rapidly replacing direct dyes (Burkinshaw, 1990) and are usually the principle dyes used for colouring cotton. Different dye classes require specific dyeing procedures, however, a common factor is that water is required for all forms of dyeing, either as a solvent or transport medium and, therefore, effluent is generated by all dyeing processes. The volume and characteristics of the effluent are determined by the type of dyeing process and the class of dye used.

Dyeing can either be performed in discontinuous batch dye machines or in a continuous range. The volume of dye-containing effluent resulting from batch dyeing is far greater than that resulting from continuous dyeing, as between four and ten rinses are required after batch dyeing to produce acceptable quality goods. The total volume of effluent emanating from a dyeing process is determined by the liquor ratio, i.e. *the volume of dye solution required to dye a kilogram of goods*. Liquor ratio values range from 8 : 1 to 20 : 1, with 10 : 1 being a convenient average (Buckley, 1992). The concentration of dye in the effluent is determined by the dye exhaustion properties, i.e. *the proportion of the dye that is fibre substantive,* and ranges from 95 to 98 % for acid, basic and disperse dyes, through 60 to 80 % for reactive dyes and 40 to 60 % for the balance of the dyes (Buckley, 1992). After discharging a batch dyebath 1 M of solution is usually retained per kg of goods i.e. 90 % of the unreacted dyes and auxiliary chemicals are present in the first drop (exhausted dyebath) giving rise to a low volume, concentrated, highly coloured form of effluent. Subsequent rinses become more dilute giving rise to large volumes of coloured effluent with a low organic load.

f) **Finishing.** This refers to any processes used to improve the quality of the fabric after dyeing.

1.2 LEGISLATION FOR DISCHARGE OF TEXTILE EFFLUENT

The Federal Republic of Germany (FRG), which has some of the most stringent effluent regulations in the world, ranks the following textile effluent characteristics/components in order of priority (personal communication, Envirocare division, Ciba Geigy):

a) colouration of the effluent;

b) toxicity of the effluent;

c) total organic carbon (TOC) content of the effluent;

d) adsorbable organic halogens in the effluent;

e) metals in the effluent; and

f) the salt content of the effluent.

This has resulted in the following textile effluents being forbidden to be discharged to a waste-water treatment works without pre-treatment :

a) no untreated washing water from printing;

b) no surplus of dye padding and finishing liquors;

c) no synthetic sizes with less than 80 % biodegradability; and

d) no chrome, arsenic or mercury.

Although legislation in the Republic of South Africa is not as stringent as that cited for the FRG, the following priority ranking is given to textile effluent characteristics in RSA (personal communication, L. Gravelet-Blondin, Department of Water Affairs and Forestry) :

a) colouration of the effluent;

b) salts, which increases in priority for inland textile mills; and

c) Toxicity.

Therefore, it can be seen that effluent colouration is of priority status in both Europe and South Africa. Legislation in South Africa states that discharged effluent must adhere to a general standard of zero colour, however, in practise the measurement of colour is complicated by inadequate analytical methods as well as natural colouration and suspended solids in receiving water bodies. Traditional analytical methods for measurement of water colour are calibrated against a yellow-brown standard (Hazen units) which is satisfactory for measuring the natural colour of water due to dissolved organic acids, but is unrelated to the spectrum of colours associated with dyeing. A method developed by the American Dye Manufacturers Institute (ADMI) has the advantage that it is independant of hue and can, therefore, be related to colour imparted by textile dyes. However, these colour measurements are still complicated by interfering solids (which must be removed by filtration) and insoluble colour bodies which contribute to the overall colour perception of the water but which are removed by filtration (Barnes et al., 1992). In addition, when colour is undesirable for aesthetic reasons it is extremely difficult to correlate analytical colour measurements with colour perception by the human eye. Therefore, in practise the zero colour standard may be modified so that the impact of the coloured effluent on the receiving water body is such that the total colour in the water is acceptable to all existing and potential downstream users.

No stringent limits are set for effluent TOC and COD in RSA, allowing textile mills to discharge high organic content effluents to a waste-water treatment works. However, the effluent charges are calculated on the organic load of the effluent (usually measured by COD) and, therefore, the discharge of desizing and scouring effluents usually results in extremely high effluent charges which could be dramatically reduced if pre-treatment of these effluents was implemented. Although the principle focus of this project was the removal of colour from textile effluents, COD reduction of high organic strength textile effluents has been considered as an aid to biological decolourisation, and is described in Chapter Five.

Chapter 1

1.3 DYE-CONTAINING EFFLUENTS

Dye-containing waste waters became a source of concern in the 1970's when some azo food dyes were implicated as being carcinogenic. This initiated concern about non-food dyes which led to two associations being formed to investigate environmental problems: The ADMI (American Dye Manufacturers Institute) and ETAD (Ecological and Toxicological Association of the Dyestuffs Manufacturing Industry). Textile dyes were evaluated for environmental impact by both these associations (Meyer, 1981) and, although the findings varied according to the class of dye tested, it was concluded that dyes and pigments would not be considered hazardous chemicals in general. However, colour is noticeable at dye concentrations of approximately 1 mg/M and, therefore, textile dyehouse effluent is highly visual even when diluted. Thus, the concentration and volume of dyehouse effluent discharged from textile industries every day often exceed the assimilative capacity of recieving water bodies, resulting in visible colour in this water. This results in aesthetic problems which initiates public concern, and moreover restricts the downstream applications of this water. The latter is extremely serious in water-restricted countries such as the Republic of South Africa (RSA) which rely on the extensive recycling of water to fulfill the ever increasing demand for water by the agricultural, industrial and domestic sectors.

1.3.1 Effluent Reduction / Treatment Options for Dye-Containing Effluents

Two phases are involved in reducing the pollution load of the dyeing process, waste minimisation and waste treatment. Waste minimization (clean technology) aims to decrease the concentration and volume of dye-containing effluent by recycling dyes, auxillaries and water, and reducing overall chemical consumption. Waste treatment involves the ultimate disposal of compounds that cannot be recycled.

Recycling of water can be achieved by removing dyes and auxillaries from solution, usually by physical methods such as filtration or adsorption. Suitable filtration techniques are reverse osmosis or cross-flow microfiltration. Adsorption of the dye compounds to activated carbon or other cellulosic low cost materials transfers the coloured component of the effluent to a solid form which can then be disposed of in a landfill site. In addition, chemical flocculants may be used to flocculate the dyes which are then removed from solution as a sludge. In some dyeing processes, re-use of the reclaimed dyes may be feasible, however, many textile manufacturers are reluctant to risk the quality of the dyeing process by using recycled dyes. In other cases, such as the recovery of cotton reactive dyes, re-use of the dye solutions is impossible due to hydrolysis of these dyes in the dye bath. This reaction also accounts for the relatively poor exhaustion encountered with this class of dye, resulting in highly coloured effluents emanating from reactive dyeing. In this case, waste treatment of the concentrated dye solutions must be implemented.

Waste treatment (with respect to the decolourisation of dye-containing effluent) can be divided into chemical or biological processes. Chemical treatment processes usually consist of oxidative

techniques such as ozonation which cleaves the conjugated bridges of the dyes, and ultraviolet irradiation combined with hydrogen peroxide, which cleaves the azo groups of azo dyes.

Options for the biological treatment of textile dyeing effluent may be single-phase aerobic or anaerobic processes, or multi-phase systems combining both aerobic and anaerobic processes. To date traditional aerobic biological systems, such as activated sludge processes, have not been effective in decolourising textile effluent. In fact, research has shown that very little decolourisation of anthraquinone or azo dyes occurs in these systems as a result of biodegradation (Meyer, 1981). However, other researchers demonstrated that aerobic microorganisms could be adapted to degrade simple azo dyes (Kulla, 1981) although the highly bred nature and stringent substrate specificity of those particular adapted microorganism led the reasearchers to conclude that they were not suitable for practical waste-water treatment. By contrast, anaerobic biological systems have demonstrated considerable potential for non-specific decolourisation of azo dyes (Brown and Laboureur, 1983a) but do not appear to have the ability to mineralise the resulting dye metabolites. Recent research (Haug et al., 1991) has shown that the combination of anaerobic and aerobic phases results in a biological treatment system that is capable of decolourising and mineralising a mordant textile dye.

The primary advantage of biological systems in comparison to chemical systems is that they are usually more economical in day to day running costs. Biological systems are essentially self-sufficent subsequent to initiation, whereas chemical systems require the constant input of chemicals such as flocculants or hydrogen peroxide.

When comparing the economic merits of various biological systems it can be seen that aerobic systems are disadvantaged by the high energy requirements of aeration, and the production of large volumes of waste sludge. Anaerobic systems are extremely economical with respect to running costs as aeration is not required, and the volume of waste sludge produced is minimal. Moreover, methane gas produced as an end-product of anaerobic digestion is sufficient to fulfill the energy requirements of pumping and heating, rendering the process self-sufficient. Therefore, anaerobic processes are becoming increasingly popular for waste water treatment and are replacing the traditional aerobic systems in a number of applications.

1.4 PROJECT OUTLINE

The objectives of this research project were to investigate the feasibility of biological anaerobic treatment for the decolourisation of dyes, with respect to the mechanism/s and rate-controlling factors of decolourisation, and the fate of the dye metabolites in an anaerobic system. The following approach was adopted to achieve these aims.

The project was divided into a number of interleading phases, the first phase involved the researching of retrospective and current literature which was compiled into the relevant literature reviews. Subsequent to reviewing the literature, decisions were made regarding the type of process and class of dye to be investigated.

An anaerobic biological system was chosen for investigation, primarily because anaerobic microorganisms show a greater ability for non-specific decolourisation of textile dyes than aerobic microorganisms, making this process more suited to the treatment of diverse effluents. Moreover, anaerobic treatment systems are better suited to the working schedule of a textile mill as they are able to survive periods of dormancy such as during the annual shutdown of the mill.

Due to uncertainty in the literature with respect to the mechanism of biological anaerobic decolourisation, it was decided to investigate anaerobic decolourisation in a controlled laboratory environment in order to elucidate the processes responsible for decolourisation. It was, therefore, neccessary to choose a target (representative) dye with which these fundamental studies could be performed.

The target dye was required to be representative of a dye class that was known to be problematic with respect to both loading and treatability. The literature reported that most decolourisation in an activated sludge plant is achieved through adsorption of the dye compounds to the sludge (Shaul et al. ,1986). Therefore, treatment problems arise when the dyes are extremely water soluble, and are not adsorbed to the sludge. In a report investigating the issue of colour removal at a publicly owned treatment works (McCurdy et al., 1991) it was stated that *the treament plant had no trouble treating the textile wastewater until the mills started using reactive dyes.* This stands to reason as reactive dyes are hydrophilic and, therefore, have a low affinity for the biological sludge, remaining in solution and causing the treated effluent to be coloured. Moreover, as stated previously, reactive dyes have relatively poor exhaustion properties in comparison to other classes of dyes, which results in highly coloured effluents.

It was, therefore, decided that the target dye for the initial research in this project would be chosen from the class of cotton reactive dyes. Furthermore, the reactive dye would have an azo chromophore, as azo dyes account for 60 to 70 % of all dyestuffs made, and are the most common class of chromophore for reactive dyes. Finally, a red dye was chosen, as this hue has been found to give rise to aesthetic problems at low concentrations, and is difficult to remove from treated water (data taken from the Umbilo Waste Water Treatment Works). For these reasons and because this dye was known to be commonly used in textile factories in the Pinetown and Hammarsdale textile regions, Procion Red HE-7B was chosen as the target dye for fundamental studies. Subsequent to the selection of this dye, a paper by Chadraborty (1990) confirmed that reactive HE dyes are slowly biodegraded in water in comparison to conventional reactive dyes due to the orientation of the two reactive groups at the different positions of the dye molecule and, therefore, that these dyes are problematic in waste-water treatment works.

The first experimental stage to be implemented consisted of enrichment programmes to yield microorganisms capable of fulfilling the requirements of the biological treatment process. As the requirement of the decolourisation process for additional organic substrate was uncertain, allowance was made for the fulfilment of this requirement at a later stage, through enrichment of microorganisms capable of utilising the organic content of textile finishing streams such as scouring

and desizing effluent. The rationale behind this approach was the possibility of an on-site treatment plant. Enrichment programmes were also implemented to select for dye tolerant anaerobic microorganisms with the ability to decolourise Procion Red HE-7B.

Once the enrichment programme was completed anaerobic decolourisation of Procion Red HE-7B was investigated with the aim of elucidating the mechanism responsible for decolourisation. This was undertaken by experimentally determining the kinetic order of decolourisation, and subsequently identifying probable rate-controlling factors for decolourisation. These rate-controlling factors were investigated with the dual objectives of: determining the mechanism of decolourisation; and identifying the factors that would be revelant to a waste-water treatment system. The fate and effect of Procion Red HE-7B in the anaerobic system was determined by measuring the toxicity of Procion Red HE-7B to the anaerobic microbial population, and investigating the fate of the dye metabolites in the anaerobic system by measuring their mineralisation potential.

Finally, microorganisms capable of degrading the organic content of a textile scouring effluent were tested for the ability to decolourise Procion Red HE-7B with the aim of developing a combined system for on-site treatment of textile effluent.

1.5 THESIS OUTLINE

The thesis begins with a review of current and retrospective literature on the subject of biological treatment of dye-containing effluents, which is presented in **Chapter Two. Chapter Three** is a discursive literature review presenting proposed mechanisms of azo reduction (anaerobic decolourisation of azo dyes) and aiming to identify a general accepted mechanism which could apply to the anaerobic decolourisation of Procion Red HE-7B. **Chapter Four** presents the results of the investigation into the decolourisation of Procion Red HE-7B, and is written in the form of a paper with the first section being an introduction, followed by the experimentation section, results, discussion and conclusions. **Chapter Five** follows the format of the preceding chapter and presents the results of the experimental work in which a combined process was developed involving the degradation of a scouring effluent with concomittant decolourisation of Procion Red HE-7B. The thesis is concluded with **Chapter Six**, a summary of the experimental work presented, and includes recommendations for future research.

CHAPTER TWO

BIOLOGICAL OPTIONS FOR THE TREATMENT OF DYE-CONTAINING EFFLUENTS

Traditional biological methods of waste-water treatment are not usually successful in decolourising dye-containing effluents from textile industries. This chapter presents a review of the literature with respect to traditional and alternative biological methods for decolourising and degrading dyes. All dyes mentioned in this chapter are listed in Appendix B, together with the chemical structures of the dyes where available.

2.1 PHYSICAL DECOLOURISATION BY ADSORPTION OF DYES TO MICROBIAL CELLS

It is possible to physically remove dyes from solution by means of low cost adsorbents. The saturated adsorbents can then be regenerated or disposed of in a landfill site. Low cost adsorbents are usually a form of cellulosic waste such as wood shavings, corn husks or bagasse, although, researchers have also investigated the use of microbial cells as adsorbents for water soluble dyes.

Hu (1992) investigated the adsorption of eleven reactive dyes by 22 strains of yeast and 25 strains of bacteria isolated from soil samples and the sludge of a textile waste-water treatment plant. The reactive dyes tested were 4 blue dyes , 3 red dyes (of which Procion Red G, CI 44 was one), 2 violet dyes and 2 yellow dyes. The names of these dyes were not given in the paper. Of the 47 strains of microorganisms tested, only 15 had the ability to reduce the colour of Procion Red G (by adsorption) within 8 d. The rest of the strains had no decolourising ability. A certain bacterial strain, identified as an *Aeromonas* sp, was found to decolourise Procion Red G within 6 d and was subsequently used for all tests. A reactive blue, yellow and another red dye were found to be partially decolourised by adsorption to the *Aeromonas* sp biomass, although, the remaining seven dyes were only slightly adsorbed.

The specific adsorption capacity of the microbial cells was found to be greatest during the early growing stages which, therefore, excluded the use of extracellular polysaccharides (usually produced in the stationary phase) as a means of adsorption. Hu (1992) therefore proposed that the principal site of adsorption of Procion Red G was the microbial cell walls of the *Aeromonas* biomass. The specific adsorption capacity of the biomass for Procion Red G was 27,41 mg dye/gram dried cell, and the percentage colour removal was 60,3 %.

It was concluded by Hu (1992) that adsorption of reactive dyes by bacterial cells appeared feasible but that further research, utilising raw textile waste water, was required to determine the applicability

of this process to waste-water treatment. The regeneration of spent adsorbents would also be investigated.

Fungal species, in particular *Myrothecium verrucaria,* have also shown promise for adsorption of dyes (Mou et al., 1991; Brahimi-Horn et al., 1992). The ability of this fungus to adsorb 3 dyes, Orange II, RS (H/C) and 10 B (H/C) was tested in static and shaking culture (Mou et al., 1991). It was found that adsorption was enhanced in static culture and no advantage was gained by shaking and/or aerating during the biodecolourisation process. Through visual inspection it was observed that initial dye adsorption was rapid, as indicated by the rapid coloration of the fungal mass. It was confirmed by spectrophotometric measurements that more than 50 % of the dye (for the RS red dye) was removed in the first few minutes after contact, and that equilibrium for optical density (OD) reduction was reached in about 10 h. The capacity of the fungal cells for RS dye molecules was found to be 4 g of dye adsorbed by 1 kg of wet mycelial cells. After adsorption of the dye to the mycelial mass was complete, the cell bound colour was then observed to either gradually disappear over a period of a week or longer, or to remain bound to the cell. This indicated that cell-bound colour loss was due to biodegradation of the dyes, the extent of which was probably related to dye structure. However, experiments with autoclaved cells showed that these cells were equally effective in decolourising the RS dye solution, which was viewed as a strong indication of adsorption over degradation as the primary mechanism of colour removal.

The mechanism by which the dyes became bound to *M. verrucaria* was investigated by Brahimi-Horn et al. (1992). They reported that the integrity of the microbial cells was important to retain optimal binding capacity, which indicated that some of the dye was internalised, although the bulk of the adsorbed dye was probably bound externally. The site of external binding was proposed to be the chitin component of the fungal cell walls. As chitin has a similar structure to cellulose, it is feasible that dyes (such as reactive dyes) may bind to chitin in a similar manner to cotton. However, it must be noted that reactive dyes in waste water are usually hydrolysed and have therefore lost the ability to bind with cellulose, and that these dyes may not be adsorbed successfully by the fungal mass. The addition of surfactants to the medium was found to reduce the binding efficiency of the cells which was suggested to result from a modification of the hydrophobic/hydrophilic environment which influenced cell binding rather than from a direct modification of the cell membrane structure. Re-generation of the mycelial adsorbent was achieved by extraction with methanol and the cells were re-cycled for further dye removal. The adsorption capacity of the cells was found to be slightly diminished subsequent to re-generation.

Laboratory scale tests were also performed by Mou et al. (1991) to determine the decolourising ability of *M. verrucaria* with respect to dye-containing waste water. Of particular interest is the comparison of decolourisation of a reactive red-dye containing waste liquor (from a dye manufacturing plant) by activated sludge biomass or by the bioagent (*M. verrrucaria*) over a 2 week incubation period. The bioagent showed 94 % reduction in sample OD whereas the activated sludge showed only 30 % reduction, proving that the fungal bioagent had an increased ability to adsorb the

reactive dye. It is not known whether the reactive dye in this waste water was hydrolysed or still in its reactive form.

The following waste-water

a) basic dye waste water from yarn dyeing, pH 4,5, dark blue;

b) direct dye waste water from yarn dyeing, pH 9,8, dark red;

c) T/R oxidative desizing and disperse dye waste water from yarn dyeing, pH 8,2, black;

d) reactive dye waste water, pH 11, dark red;

e) nylon acid dye waste water, pH 9,3, bright red;

f) sulphur dye : reactive/vat dye = 1:4, pH 10,2, black.

The results obtained when these waste waters were incubated statically for 2 weeks with 5 d old mycelia, are given in **Table 2.1**.

Table 2.1 : Decolourisation of textile waste waters by *M. verrucaria*

Wastewaters	**a**	**b**	**c**	**d**	**e**	**f**
% OD reduction	93	98	78	81	43	84

Acid dye waste water exhibited the poorest decolourisation. Basic and direct dye-containing waste waters showed 93 and 98 % OD reduction, respectively. The reactive dye waste water (which presumably contained hydrolysed reactive dye) also showed considerable decolourisation (81 % reduction in OD). As reactive dyes are usually not adsorbed to the activated sludge in waste water treatment works, these results are particularly interesting in demonstrating that the bioagent, *M. verruacaria*, had an enhanced ability for adsorption of reactive dyes.

Although the results presented in this section have been promising with respect to treatment of dye-containing waste waters by physical removal of the dyes, it must be remembered that the ultimate disposal of the dye-contaminated adsorbent still remains a problem, unless regeneration of the adsorbent can be achieved. In fact, adsorption of dyes to adsorbents, although removing the dyes from solution, results in the production of large quantities of dye-contaminated material and therefore, effectively increases the volume of waste to be disposed.

2.2 BIOLOGICAL DEGRADATION OF DYES IN AEROBIC SYSTEMS

An important property of textile dyes, which manufacturers and users have striven for, is resistance to oxidation. A garment saturated with water or perspiration and well inoculated with microorganisms is an excellent *culture medium*. Only compounds resistant to biochemical/oxidative degradation under these conditions will be fast and, thus, acceptable as stable dyes (Straley, 1984).

The above criterion clarifies why many commercial dyes are recalcitrant to oxidative microbial breakdown and, therefore, why existing forms of aerobic waste water treatment are not usually effective against dyehouse effluent.

2.2.1 The Fate of Textile Dyes in the Activated Sludge Process

The Ecological and Toxicological Association of the Dyestuffs Manufacturing Industry (ETAD) investigated the possibility of commercial dyestuffs being susceptible to aerobic degradation in an activated sludge plant, as part of their continuing programme of investigation into the environmental fate of dyestuffs (Pagga and Brown, 1986). The test simulated the conditions of an adapted activated sludge unit and was considered satisfactory for indicating the inherent biodegradability's of the test substances. It was acknowledged that the test could not always distinguish between true biodegradation and bioelimination (flocculation or adsorption onto the sludge), although, it was stated that if significant elimination was detected within the initial 3 h of the test, this was attributed to physical processes. No conclusion was forthcoming as to whether the substances removed by physical processes were subsequently biodegraded. In order to decrease the possibility of physical elimination of the dyestuffs, readily water-soluble dyestuffs were selected for this programme on the basis that they were less likely to be adsorbed to the sludge. In total, 87 commercial dyes were chosen to be tested (these were not listed in the paper), at a standard test concentration of 100 mg/M. The duration of the test was 42 d and the analytical methods used to determine dye degradation were extinction at the absorption maxima of the individual dyestuffs and dissolved organic carbon (DOC).

The results of the test yielded the following conclusions : that, even with adapted inoculum, no dyestuff tested showed any convincing evidence of genuine biodegradation; that some dyes did contain biodegradable components (biodegradation measured by DOC removal) but that these were not the chromophores; and finally, that partial colour removal was achieved by adsorption of the dyes to the sludge (even though they were water soluble) and subsequent removal by flocculation.

A project by the United States Environmental Protection Agency (EPA) titled *Fate of Water Soluble Azo Dyes in the Activated Sludge Process* (Shaul et al., 1986; 1988) investigated the partitioning of water soluble azo dyes in the activated sludge process (ASP). This was achieved by *spiking* specific azo dyes, at concentrations of 1 and 5 mg/M, into pilot-scale treatment systems and analysing liquid and sludge samples for residual dye. A total of 18 dyes were tested which are listed according to their fate in the activated sludge process, in **Table 2.2**.

Table 2.2 : The extent of bioelimination/degradation of 18 water soluble azo dyes in the activated sludge process (ASP).

Group One Dyes passing through ASP untreated	**Group Two** Elimination of dyes by adsorption	**Group Three** Dyes biodegraded in ASP
CI Acid Black 1	CI Acid Blue 113	CI Acid Orange 7
CI Acid Orange 10	CI Acid Red 151	CI Acid Orange 8
CI Acid Red 1	CI Direct Violet 9	CI Acid Red 88
CI Acid Red 14	CI Direct Yellow 28	
CI Acid Red 18		
CI Acid Red 337		
CI Acid Yellow 17		
CI Acid Yellow 23		
CI Acid Yellow 49		
CI Acid Yellow 151		
CI Direct Yellow 4		

The relatively high sulphonic acid substitution of the dyes in Group one was thought to hinder their removal by either adsorption or biodegradation in the ASP. The degree of sulphonation of an azo dye is known to increase the water solubility of that compound and consequently decrease the ability of that compound to adsorb to and penetrate microbial cell walls, thus limiting the chance of aerobic biodegradation.

The positioning of the sulphonic acid functional group(s) and the molecular weight of the compound was also speculated to have an effect on bioelimination. The compounds in Group Two were adsorbed to the sludge despite being highly sulphonated, which was attributed to the higher molecular weights of these compounds in comparison to those in Group One. The three remaining azo dyes tested were found to be biodegraded in the ASP, however, it was not known whether these compounds were mineralised in the system.

Shaul et al. (1986) developed adsorption isotherms for seven dyestuffs viz., CI Acid Blue 113, CI Acid Orange 7, CI Acid Red 1, CI Acid Red 88, CI Acid Red 151, CI Acid Red 337 and CI Acid Yellow 151. In all cases adsorption of the dyes to activated sludge conformed to the Freundlich isotherm equation.

It was concluded from this project that the structure of the dye compound determined the fate of the dyestuff in the activated sludge system, with high water solubility being the major factor in preventing an azo dye compound from being either adsorbed or biodegraded by the ASP. No trend could be found between adsorption and biodegradation of the dyes although sorption of the dye molecules was acknowledged to play a principal role in colour removal in the ASP. From these conclusion it can be deduced that water soluble azo dyes are not successfully treated in an activated sludge system. The recommendations for future reasearch included more tests to define relationships

between physical and chemical properties of the dyestuffs, and the fate of the compounds in the activated sludge process.

The fate of the reactive azo dyes, Reactive Black 5 and Navy 106, the latter being a mixture of three azo dyes, was investigated in a laboratory simulated activated sludge plant (Ganesh et al., 1992). The vinyl sulphone and hydrolysed forms of Reactive Black 5, and a Navy 106 washwater, were investigated for degradation by activated sludge. A rapid initial decrease in colour was measured upon addition of the vinylsulphone form of Reactive Black 5, which was attributed to sorption of the dye to the biomass. No significant evidence of biodegradation was observed for this dye and sorption was concluded to be the principal mechanism of colour removal. The hydrolysed form of Reactive Black 5 did not show evidence of sorption or biodegradation in a nine day study, i.e. very little colour removal is likely to be achieved in an activated sludge plant treating *hydrolysed* Reactive Black 5. As waste reactive dyes are hydrolysed, activated sludge plants are, therefore, probably not suitable for treatment of reactive dye waste water. Little colour removal was observed with the Navy 106 washwater.

The inhibitory effects of dyes on activated sludge microorganisms

As a number of dyes were found to be recalcitrant in the activated sludge process, concern arose as to the toxicity of these compounds to the waste-water bacteria. A screening method was developed by the ETAD (Brown et al., 1981) to determine the possible inhibitory effects of dyestuffs on aerobic waste water microorganisms. There were 202 dyestuffs screened for inhibitory effects and the results were reported as Inhibition Concentration (IC_{50}) values. The dyes chosen from the classes of acid, direct, disperse, reactive (including Reactive Red 141 (Procion Red HE-7B)), vat, mordant, pigment and solvent dyes all yielded IC_{50} values greater than 100 mg/M. That is, these dyes were not considered inhibitory to the waste-water microorganisms. However, a high percentage of the basic dyes tested were found to have IC_{50} values of less than 100 mg/M, which is in agreement with the findings of Ogawa et al. (1989) that basic dyes are inhibitory to microorganisms. It was concluded from these tests that the results agree with the general experience of the ETAD, that is, *although dyes may give concern at sewage works due to their colour, they should not give rise to concern regarding toxicity*.

2.2.2 Degradation of Azo and Triphenylmethane Dyes in Aerobic Aquatic Environments

The transformation rates of 2 triphenylmethane dyes (Basic Violet 1, Basic Violet 3) and an azo dye (Acid Orange 6) were measured in an aerobic system using field-collected and laboratory microbial cultures (Michaels and Lewis, 1986). The aim of this study was to determine the potential degradation of these compounds in an aquatic environment. Transformation of the Basic Violet dyes followed four phases, namely, initial sorption, initial sorption equilibrium, transformation (presumably degradation) and terminal equilibrium (i.e. the dyes were not completely transformed). The azo dye Acid Orange 6 did not show any measurable transformation which is consistent with the literature, i.e. that reductive cleavage of azo bonds occurs readily under anaerobic conditions, but the aerobic transformation is limited (Meyer, 1981).

It was concluded from these studies that some dyes may be slowly degraded by aerobic aquatic microbial populations, but that the toxicity of the persistent dyes may decrease the overall mineralisation of organic compounds in the aquatic environment by causing a reduction in concentration and species diversity of heterotrophic bacterial communities, unless these dyes are rapidly degraded by anaerobic populations.

2.2.3 Adaptation of Microorganisms to Degrade Simple Azo Dyes

Although tests with activated sludge showed that little degradation of azo dyes could be expected with aerobic microorganisms, a group of researchers at the Swiss Federal Institute of Technology aimed to *breed* aerobic azo-degrading microorganisms using simple azo compounds.

Kulla (1981) reported on the isolation of bacteria capable of utilising azo compounds as sole sources of carbon, nitrogen and energy. A simple azo compound, 4,4'- dicarboxyazobenzene, was initially used to isolate a *Flavobacterium* species capable of using this compound as a sole carbon, nitrogen and energy source. Adaptation of this bacterium to utilise various derivatives of Orange II (a commercially used textile dye) was then attempted in batch culture, however, no success was achieved and a chemostat system was developed to facilitate rapid selection of adapted microorganisms. The conditions in the chemostat were not sterile and gradually the *Flavobacterium* sp was outcompeted by a Pseudomonas-like organism, referred to as KF4. The breakthrough to growth with Orange II carboxy as sole carbon, nitrogen and energy source occurred after approximately 400 generations, during which time the characteristics of the pseudomonads did not change. The authors, therefore, proposed that the microorganisms capable of utilising Orange II carboxy as a sole carbon source (strain KF44) were direct descendants of strain KF4. Another Pseudomonas-like organism (K22) which was also capable of degrading Orange I carboxy was isolated after 100 generations.

When attempts were made to extend the substrate specificity of the two cultures (KF44 and K22), it was found that KF44 always retained the capability to metabolise the original substrates, although adapting to degrade more complex substrates, while K22 appeared to shift its specificity. As K22 contained no plasmids, it was postulated that one or more genes coding for the degradation of Orange II carboxy in KF44 was plasmid-borne, thereby allowing KF44 to retain its ability to degrade this compound.

Although a microorganism capable of mineralising the dye of choice was successfully developed, it was concluded that the strains KF44 and K22 were too highly bred to survive in waste-water treatment plants and, that the fairly stringent substrate specificity of the strain was not feasible for practical waste-water treatment.

Zimmermann et al. (1982) continued this research by isolating and characterising Orange II azoreductase, the enzyme initiating azo dye degradation by *Pseudomonas* KF44. The purpose of the study was to elucidate the substrate requirements of the enzyme and the following generalisations were found with respect to the structural features required of substrates:

a) A hydroxy group in the 2- position of the naphthol ring is a pre-requisite for the reaction to occur;

b) Charged groups in the proximity of the azo group hinder the reaction;

c) A second polar substituent on the dye molecule lowers its affinity to the enzyme and impedes the reaction; and

d)

Kulla et al. (1983) also found that substitution of sulphonate for carboxy groups on the Orange I and II dyes led to a disturbance of the degradative pathway. However, the enzymes initiating degradation (Orange I and II azoreductase) had no preference for carboxylated or sulphonated dyes and it was proposed that sulphanilic acid, the product of the initial fission reaction, was creating a blockage by being channelled into a dead-end pathway. This was postulated to interfere with mineralisation of the sulphonated dyes by preventing degradation of the aminonaphthol part of the dye molecule. These results were considered to be particularly significant as the sulphonated azo dyes served as models for commercially used textile dyes, which are known to be recalcitrant in aerobic waste-water treatment plants. Although it was not mentioned in this paper, it is possible that inhibition of the azo reduction reaction by the sulphonated dyes was also due to a decreased ability of these dyes to enter the microbial cells, as found by Wuhrmann et al. (1980).

Heiss et al. (1992) investigated the cloning of DNA from a *Rhodococcus* strain capable of decolourising azo dyes under aerobic conditions, to microorganisms previously unable to decolourise azo dyes. It was found that several of the strains possessing cloned DNA were subsequently able to decolourise the two test azo dyes, Orange II and Amido black. All the microorganisms capable of decolourising the dyes could decolourise both test dyes, and those microorganisms unable to decolourise the dyes could decolourise neither, suggesting that the same enzyme had a broad specificity and was responsible for decolourisation of both dyes. This is in contrast with the work by Kulla (1981) on Orange II degradation, in which the azo reducing enzymes were found to be substrate specific. In addition, none of the azo-reducing microorganisms could utilise the dyes as sole sources of carbon, nitrogen and energy (Heiss et al., 1992), which was also in contrast to the findings of Kulla (1981).

The papers reported in Section 2.2.3 show that decolourisation of azo dyes in an aerobic system is possible with specialised microbial populations. However, whether these systems would be suitable for waste-water treatment needs to be investigated with respect to the substrate requirements of the microbial population and the ability of these microorganism to survive in a treatment system. Mineralisation of the azo compounds must also be investigated.

2.2.4 Degradation of Azo Dyes by Fungi

The literature thus far in Section 2.2 has shown that azo dyes are generally resistant to biodegradation under aerobic conditions. However, the white-rot fungus *Phanerochaete chrysosporium* has shown considerable potential for the degradation of a wide range of dyes under

strictly aerobic conditions (Glenn and Gold, 1983; Cripps et al., 1990; Capalash and Sharma, 1992; Paszczynski et al., 1992; Pasti-Grigsby et al., 1992).

Glenn and Gold (1983) reported that *P. chrysosporium* degraded and decolourised 3 polymeric dyes (Poly B, Poly R and Poly Y) by means of secondary metabolic processes. A number of parallels were drawn between lignin degradation (which is also a secondary metabolic process) and decolourisation of the polymeric dyes. Firstly, decolourisation was repressed by nutrient nitrogen and only commenced after all the nitrogen in the cultures had been consumed and, secondly, oxygen appeared to be required to both induce and activate the catalytic system responsible for dye decolourisation. Both of these factors are valid for lignin degradation by *P. chrysosporium.* In addition, mutants lacking phenol oxidase and ligninolytic activity were not able to decolourise the dyes, whereas a phenotypic revertant strain was able to regain this capacity. From this evidence it was suggested that a single catalytic system was responsible for the degradation of lignin and the dyes but that specific reactions were likely to be dependent on the chemical and physical properties of the substrate.

Cripps et al. (1990) reported that *P. chrysosporium* could decolourise three azo dyes (Tropeolin O, Orange II and Congo Red) and a heterocyclic dye (Azure B). The structure of the dye was found to influence the extent of decolourisation, with the heterocyclic dye being readily degraded by the fungus while Congo Red, the largest and most complex dye, was not completely decolourised even after 12 d of incubation. These researchers agreed with Glenn and Gold (1983) that decolourisation of the dyes occurred via ligninolytic enzymes, and suggested that a number of enzymes or enzyme systems, which are expressed at the onset of the idiophase, must be involved in the degradation of the dyes.

Capalash and Sharma (1992) reported that degradation of the azo reactive dye, Reactofix Golden Yellow, as measured by decolourisation, occurred via the non-specific lignin degrading system of *P. chrysosporium*. The first step in dye degradation was suggested to be extracellular, due to the size of the compounds, and to be catalysed by enzymes such as lignin peroxidase. Paszczynski et al. (1992) and Pasti-Grigsby (1992) also suggested that peroxidase enzymes are involved in the initial azo dye biodegradation process when investigating the degradation of a number of sulphonated azo dyes by *P. chrysosporium*. They reported that all the dyes investigated were mineralised by the fungus and that the substitution pattern of the dyes did not significantly influence the susceptibility of the dyes to degradation.

Thus, the treatment of textile effluent with the fungus *P. chrysosporium* could be an effective means of colour and COD removal. However, disadvantages of this system compared with an anaerobic system could be high sludge production and high energy requirements for aeration, as the process is strictly aerobic.

2.2.5 Degradation of Azo Dyes by Algae

Jinqui and Houtian (1992) tested the ability of *Chlorella pyrenoidosa*, *C. vulgaris* and *Oscillateria tenuis*, to degrade 30 azo compounds. Fourteen of these azo dyes showed a percentage decolourisation of 50 % or higher, with results indicating that degradability was directly related to

the molecular structure of the azo compounds. Azo compounds with hydroxy or amino groups were generally more readily degraded than those with a methyl, methoxy, sulpho or nitro group. The presence of hydroxy or amino groups on the compound also appeared to partially counteract the inhibitive effect of sulphonate groups on azo reduction. The lower rate of degradation of sulphonated azo compounds was thought to be related to a decreased ability of the compounds to enter the microbial cells, as reported by Wuhrmann et al. (1980) with bacterial cells.

It was found that the sulphonated azo dye, Erichrome Blue SE could be utilised by the algae as a sole source of carbon and nitrogen, and that the degradation process was related to the physiological metabolism of the algae. It seemed likely, therefore, that the algal population had the ability to mineralise the azo degradation products. This was confirmed when aniline (a common azo degradation product) was found to be mineralised in all of the tests.

The azo reductase enzyme, responsible for the initial reduction reaction, was found to cleave the azo bonds of the compounds to yield aromatic amine intermediates which were subsequently mineralised by the algae. The activity of azo reductase increased with the addition of NADH or NADPH suggesting that these compounds were co-factors for azo reductase, acting as electron donors for the breaking of the azo bond. The algal azo reductase was more specific for NADPH than NADH. Moreover, it was found that unacclimated algae displayed a lower level of azo reductase activity than those previously exposed to azo compounds, suggesting that azo reductase is induced by the azo compounds.

The results of this research are particularly relevant to treatment of textile effluent in stabilization ponds. Traditionally, the algal component was thought to contribute only indirectly to organic degradation, by providing oxygen (through photosynthesis) to augment the degradative capabilities of the catabolising bacteria. However, these results show that the algal population is capable of mineralizing azo dyes in the textile effluent.

2.3 DECOLOURISATION OF AZO DYES IN AN ANAEROBIC SYSTEM

Decolourisation of azo dyes under anaerobic conditions is thought to be a relatively simple and non-specific process, involving fission of the azo bond to yield degradation products such as aromatic amines. This was initially studied with respect to mammalian metabolism, due to the increasing use of azo dyes as food colourants (Meyer, 1981). The ability of the azo bond to be reduced under anaerobic conditions (such as in the mammalian intestine) was cause for concern, as the by-products of azo fission (derivatives of aminoazobenzene) were suspected carcinogens. As early as 1911, Sidney and Porscher (cited by Meyer, 1981) found sulphanilic acid in the urine of dogs fed with the azo dye Orange I and postulated a reductive cleavage of the azo group as the initial step of degradation. This was later confirmed by other authors (Mueller and Miller, 1948, 1949; cited by Meyer, 1981) and was shown to be catalysed by azo reductase enzymes in the liver as well as anaerobic microorganisms of the intestinal tract. Subsequent work indicated that azo reduction by the gut microflora was an important and common metabolic process in mammals (Walker and Gingell, 1971) and early research involving anaerobic microbial azo reduction, therefore, concentrated on

catalysis by intestinal anaerobes. Since then, papers published on the microbial degradation of azo dyes in anaerobic systems have concentrated primarily on degradation of textile dyes as a potential means of decolourising textile effluent.

Various proposals have been given regarding the mechanism of anaerobic azo reduction. A common link between these theories is that azo reduction is thought to be concurrent with re-oxidation of reduced flavins of the electron transport chain, i.e. the azo compound is reduced as a terminal electron acceptor in the microbial electron transport chain. A detailed discussion with respect to the proposed theories of anaerobic azo reduction is presented in Chapter Three, therefore, this section aims only to provide an overview of the literature with respect to the results achieved by various groups of researchers examining azo dye decolourisation with anaerobic microorganimsm.

2.3.1 Degradation of Azo Dyes by Microflora of the Mammalian Intestine

Papers by Walker and Gingell (1971) and Gingell and Walker (1971) investigated the mechanism of azo reduction by *Streptococcus faecalis*, using the azo dye, Red 2G. Their results showed that soluble flavin, acting as a two electron donor, could rapidly reduce the azo dye non-enzymically, i.e. that the reduced flavins acted as electron shuttles, ferrying electrons from the flavoproteins of the microbial electron transport chain to the acceptor azo compound. The rate of formation of the reduced flavins was therefore a rate-limiting factor in azo reduction and, as the production of reduced flavins is dependant on catabolism of an electron donor, an additional carbon source was found to be required for azo reduction. Dubin and Wright (1975), working with *Proteus vulgaris* and the azo dyes, Amaranth, Orange II, Ponceau 3R, Ponceax SX, Sunset Yellow and Tartrazine, extended this theory to include a rate-controlling step which involved a redox equilibrium between the dye and an extracellular reducing agent. Therefore, the specific reduction potentials of the dye was proposed to be a rate-limiting factor in azo reduction. Dubin and Wright (1975) proposed that azo reduction was an extracellular reaction, although Larsen et al. (1976), investigating the azo reduction of Fast Red E, Amaranth, Ponceau 4R and Ponceau 6R by gut microflora, found that the rate-limiting factor for azo reduction was the ability of the dyes to penetrate the microbial cell walls. They, therefore, proposed that azo reduction occurred intracellularly. Finally, Chung et al. (1978) reported that *Bacillus thetaiotaomicron* decolourised the azo dyes Amaranth, Poneau SX, Allura Red, Sunset Yellow, tartrazine, Orange II and methyl orange, by means of a suitable electron shuttle. An interesting result from this work was that the presence of glucose was found to inhibit reduction of tartrazine by *B. thetaiotaomicron*, whereas most other researchers had found that glucose had an enhancing effect on azo reduction by stimulating production of soluble flavin.

2.3.2 Anaerobic Decolourisation of Azo Dyes by Single Microbial Species

Much of the experimental work involving the anaerobic decolourisation of dyes (predominantly azo dyes) was conducted using mono cultures. Species of *Bacillus* and *Pseudomonas* were found to be active in the anaerobic degradation of a number of dyes but other microorganisms, such as those belonging to the genus *Aeromonas* and purple non-sulphur photosynthetic bacteria, have been found to successfully decolourise a range of dyes.

Decolourisation of azo dyes by *Bacillus* sp.

One of the earliest reports of azo reduction by a *Bacillus* sp. was by Wilcox et al. (1971) in connection with the decolourisation of azo dyes used for dyeing incense cedar pencil slats. The dye solution consisted of CI Acid Red 14, CI Acid Orange 7 and CI Acid Black 1. The solution was stored in steel tanks between runs, at a temperature of 68 ^{o}C, however, during shutdown periods the temperature would often drop to 54 ^{o}C and decolourisation of the batch solution often occurred. A *Bacillus* sp was isolated from the batch and found to be capable of decolourising pure solutions of the red and black dyes in the laboratory, although no decolourisation of the orange component was observed. However, no residual orange colour could be detected *in vivo* from the mixture of dyes, indicating that the dye mixture was degraded more efficiently than the single dye solutions. The microorganisms were unable to utilise the dyes as sole carbon and energy sources in the laboratory and required an additional source of nutrition. It was subsequently found that sludge in the storage tank consisting of sawdust from the manufacturing operation, contributed the additional substrate required for decolourisation.

Horitsu et al. (1977) showed that *p*-aminoazobenzene (PAAB), a disperse dye with an azo chromophore, could be degraded by *Bacillus subtilis*. This occurred via reductive cleavage of the azo bond to produce aniline and *p*-phenylenediamine. Continuation of this research by Idaka et al. (1982) showed that *p*-aminoazobenzene was degraded by means of two pathways. The first pathway involved cleavage of the azo bond to yield aniline and *p*-phenylenediamine, with the aniline subsequently metabolised to acetanilide and the *p*-aminoacetanilide to *p*-phenylenediacetamide. The second pathway involved direct acetylation of PAAB to *p*-acetamidoazobenzene. No mineralisation of these degradation products was recorded.

Wuhrmann et al. (1980) investigated the anaerobic reduction of azo dyes utilised in the textile industry by a strain of *Bacillus cereus* isolated from soil. The bacteria were shown to have the ability to decolourise the azo dyes by reductive cleavage of the azo bonds. Decolourisation of these dyes did not occur when the supplemental carbon source (glucose) was absent and it was proposed that glucose was neccessary to sustain the metabolic state of the bacteria to facilitate the production of reduction equivalents (reduced flavins) which were required for decolourisation. Permeation of the dyes into the microbial cells was cited as the principal rate-limiting factor for decolourisation and this was confirmed by Meschner and Wuhrmann (1982). These researchers permeabilised the cells of *B. cereus* by treatment with toluene and reported that this significantly increased the rate of uptake of both sulphonated and carboxylated azo dyes from the external medium into the microbial cells. The mechanisms involved in the permeabilisation process were not resolved but two possible principles were proposed :

a) The toluene in the permeabilisation liquid removed part of the lipid fraction of the cell membrane and facilitated the passage of strongly polar, hydrophilic dye molecules; or

b) Some of the removed lipids may adsorb to the sulphonic acid groups on the dyes, decreasing the hydrophilic nature of these compounds and concomittantly increasing their permeability.

Yatome et al. (1991), investigating the degradation of azo dyes by *Bacillus subtilis,* also concluded that cell permeability was the principal rate-limiting factor in the microbial degradation of azo dyes. They reported that unsulphonated dyes were easily decolourised via reductive cleavage of the azo bonds, but that increasing sulphonation of the azo dyes decreased the rate of decolourisation. Some sulphonated azo dyes were not decolourised at all by cells of *B. subtilis.*

Decolourisation of dyes by *Pseudomonas* species

The pseudomonads are an extremely large, versatile and adaptable class of microorganisms and it is not surprising that *Pseudomonas* species have featured prominently in research involving the degradation of xenobiotic azo compounds.

The biodegradation of azo dyes by *Pseudomonas cepacia* 13NA was investigated by Ogawa et al. (1986; 1990) using the dyes, CI Acid Orange 12, CI Acid Orange 20 and CI Acid Red 88. A three-stage continuous culture system (with the flasks arranged in series) was found to be more successful then a one stage system, with respect to the rate of degradation of the dyes. This was attributed to :

a) An abundance of nutrients in the first stage of the cultivation system which resulted in a rapid increase in the bacterial population, increasing the number of potential azo degrading bacteria; and

b) A consequent paucity of nutrients in the second and third culture flasks which forced the microorganisms to assimilate the azo dyes for their growth, i.e. the azo dyes were degraded under nutrient limited conditions.

These results are unusual as a pre-requisite for degradation of azo dyes is usually that a supplemental carbon source is provided to sustain the metabolic activity of the azo reducing microbial population (Wuhrmann et al, 1980).

Ogawa and Yatome (1990) aimed to develop a waste-water clarification technology, for removal of dyes and other organic substances in a single operation, using *P. cepacia* 13NA. The mode of treatment chosen was a multi-stage rotating biological contactor, with discs on which *P. cepacia* 13NA was immobilized with K-carrageenan gel. A multi-stage reactor was chosen due to the findings of Ogawa et al. (1986). The researchers discovered that the natural organic substances in the effluent were preferentially assimilated and, consequently, significant biomass increase was achieved. However, little dye degradation occurred at this stage and it was realised that the dyes were degraded only by starved cells. For this reason, the first reactor was suited for growth of the microorganisms because of the rich nutrient quality of the effluent, while the third reactor was suited for the catabolism of the dyes because of poor nutrient quality. The researchers concluded that it may be possible to keep both the growth of the microbes and the degradation of the dyes high, by periodically changing the path of solution in the waste-water treatment plant. The dyes treated were C.I. Acid Red 88, C.I. Direct Blue 6 and PAAB and a retention time of 20 h was chosen as the dyes were known to be resistant to biodegradation. However, the overall biodegradation rate of the dyes

was poor and it was concluded that a longer retention time was neccessary to enhance the rate of elimination.

Yatome et al. (1990) investigated the degradation of several azo dyes by cell-free extracts from the azo reducing microorganism *P. stutzeri* to determine the roles of dye redox potential and hydrophobic character in the rate of azo reduction. They concluded that both parameters are useful in helping to gain an understanding of the relationship between the ease of degradation of dyes and their structure but that no definite rules for degradability of azo dyes could be formulated. Yatome et al. (1991) reported that the rate of degradation of azo compounds by whole cell culture of *P. stutzeri* was limited by the relative ease at which the compounds could permeate the cell.

Decolourisation of dyes by *Aeromonas* species

Reports of the degradation of azo dyes by *Aeromonas hydrophilia* var. 24B have been made by Idaka and Ogawa (1978) and Yatome et al. (1987).These findings were similar to other reports of anaerobic decolourisation of azo dyes.

Decolourisation of dyes by purple non-sulphur bacteria (PSB)

Guoquing et al. (1990) isolated a purple non-sulphur bacterium from activated sludge at a waste-water treatment works treating textile dye-containing effluent. This microorganism was tested for its ability to decolourise 14 commercial textile dyes at concentrations from 20 to 200 mg/M. The rates of decolourisation were found to differ for the various dye classes with dyes belonging to the direct, acid , basic, reactive and cationic dye classes generally being decolourised quickly and effectively. Of particular interest was the fact the a reactive dye, Reactive Red X-3B, could be decolourised with a 97,5 % efficiency. Only two dyes of the fourteen tested, a disperse blue dye and an alizanthrene blue dye, were found to be difficult to decolourise. The decolourising ability of the PSB was found to be considerably enhanced under *dark* anaerobic conditions, as opposed to *light* anaerobic conditions. Since photosynthesis can occur under light anaerobic conditions it may be speculated that this metabolic state is not conducive to decolourisation of the dyes.

Infrared analysis of the dyes showed that decolourisation was due to azo reduction, and that no mineralisation of the dyes occurred. These dyes were, therefore, not satisfactory as sole carbon sources for maintenance of the PSB. The addition of an auxiliary agent used in dyeing (sodium acetate) was found to be adequate as a nutritional source to sustain the decolourising efficiency of the bacteria.

2.3.3 Decolourisation of Dyes with Mixed Populations of Microorganisms in Anaerobic Digester Sludge

The ETAD performed a series of experiments to assess the primary biodegradability (decolourisation) of dyestuffs under conditions of anaerobic digestion (Brown and Laboureur, 1983a). The study was performed with 22 dyestuffs which were chosen as commercially important representatives of the major classes of dyestuffs. All dyestuffs evaluated were water-soluble and direct Red 7 was used as a positive control. The procedure followed was that specified in the ETAD

Ecological Method No. 105 (1982). The anaerobic sludge inoculum was obtained from a sewage digester at a local treatement works. This inoculum was incubated with a test medium containing glucose as a supplemental labile carbon source, in suitable anaerobic batch reactors. The dyes were added singly to individual reactors and monitored for decolourisation over a period of 42 d.

The results showed that the degree of primary biodegradability varied between, and within, the dye classes (dyes classified by application). The structure of the dye chromophore was more important than the dye application class in determining the ease with which decolourisation occurred. The dyes containing mono-, dis- and poly- azo chromophores showed a substantial degree of decolourisation, although, the dyes with anthraquinone chromophores exhibited a broad variation of results ranging from scarcely degraded to substantial degradation. Other dye chromophores which were represented were stilbene (53 % decolourisation), triphenylmethane (no decolourisation was measured as it was unstable in aqueous solution), phthalocyanine (36 % decolourisation), oxazine (62 % decolourisation), methine (35 % decolourisation) and nitro (62 % decolourisation initially reported but a coloured metabolite was subsequently formed).

The authors concluded that with the single exception of Acid Blue 80, an anthraquinone dye which showed only 7 % decolourisation, all the dyestuffs tested showed a substantial degree of colour removal, and that the breakdown of dyestuffs in the environment is likely to be initiated under anaerobic conditions.

Kremer (1989) reported that the anaerobic degradation of two monoazo dyes, Acid Red 88 and Acid Orange 7, occurred in anaerobic serum bottles inoculated with sludge from an anaerobic digester. The rate of decolourisation of the azo dyes was noted to be increased when supplemental carbon (other than the dyes) was present. The addition of supplemental carbon was also found to have an effect on the degradation products formed as a result of azo reduction, although no mineralisation of the dyes was seen to occur.

Degradation of hydrolysed Reactive Black 5 dye in an anaerobic digester (Ganesh et al., 1992) concurred with a decrease in colour (as measured by ADMI units). The hue of the wastwater was reported to change from blue to greenish-yellow and was thought to be indicative of the production of aromatic amines. As this greenish yellow colour persisted it was thought likely that mineralisation of the dyes did not occur.

It can, therefore, be concluded that reduction of azo dyes will occur readily when these dyes are incubated in an anaerobic treatment system in which supplemental labile carbon is present. However, the fates of the resultant dye metabolites in the anaerobic treatment system remains uncertain and is

2.4 THE FATE OF DYE METABOLITES IN BIOLOGICAL TREATMENT SYSTEMS

Decolourisation of dyehouse effluent by reductive cleavage represents a primary degradation step which involves no elimination of chemicals from the waste water and results in the production of

aromatic amine metabolites. Environmental protection requires the *mineralisation* of waste compounds and thus, conditions must be found which will permit microbial degradation of the amine moieties produced in the primary reductive reaction.

The ETAD extended the research begun with the assessment of the primary biodegradability of dyes, to include the biodegradability of some aromatic degradation products. Brown and Laboureur (1983b) investigated the aerobic biodegradability of the following unsulphonated aromatic amines: aniline, *o*-toluidine, *p*-anisidine, *p*-phenetidine, *o*-dianisidine and 3,3'-dichlorobenzidine. All these compounds are lipophillic aromatic amines and possible cleavage products from commercially available dyes. Aniline was used as the reference substance in the tests with the validity criterion specifying that *the level of aniline degradation must be at least 70 % by day 14*.

The results showed that aniline, *o*-toluidine, *p*-anisidine and *p*-phenetidine degraded in an aerobic system, and that degradation was complete, i.e. mineralisation of the compounds occurred. These compounds could, therefore, be utilised as sole sources of carbon and energy by the waste-water microorganisms. However, the extent of degradation of *o*-dianisidine and 3,3'-dichlorobenzidine showed a clear dependance on the presence or absence of yeast extract. It was found that yeast extract actually promoted biodegradation of the amines, possibly due to the provision of an essential growth factor. It is also possible that the yeast extract merely acted as a labile food source resulting in a large concentration of active bacteria which were then able to break down the amines.

Brown and Hamburger (1987) continued the ETAD investigation into the aerobic biodegradability of aromatic amines and also investigated the possibility of anaerobic degradation of these metabolites. The metabolites were prepared by anaerobically decolourising a number of sulphonated textile dyes and collecting the degradation products in the aqueous phase of the experimental systems. The aqueous phase (containing aromatic metabolites resulting from the degradation of sulphonated dyes) was then either incubated with activated sludge and subjected to aerobic degradation, or re-incubated in an anaerobic system. Degradation of the metabolites was assessed by monitoring the removal of DOC or by gas chromatographic analysis. The results showed that degradation of the dye metabolites occurred in the aerobic system, usually resulting in mineralisation, whereas no evidence of biodegradation of these compounds could be detected in the anaerobic system.. It was concluded that metabolites resulting from the reduction of azo compounds were unlikely to be degraded under anaerobic conditions, but could probably be degraded in acclimated aerobic systems.

Haug et al. (1991) reported that the metabolites resulting from the reduction of an azo dye Mordant Yellow 3, (6-aminonaphthalene-2-sulphonate and 5-aminosalicylate) were not degraded in the anaerobic system which gave rise to decolourisation. However, upon re-aeration of the culture these amines were mineralised by a bacterial association indicating that these compounds were more amenable to aerobic than anaerobic degradation.

Kremer (1989) reported that the anaerobic reduction of an azo dye, Acid Red 88, resulted in the production of naphthionic acid and 1-amino-2-naphthol. The metabolites were not mineralised when incubated in an anaerobic system with an additional carbon source, although transformation of these

compounds occurred during the incubation period. The fate of the aromatic amine metabolites, naphthionic acid and 1-amino-2-naphthol, is shown in **Fig 2.1.** The compounds marked with an asterisk were detected in the anaerobic system. It can be seen that 1-amino-2-naphthol (a metabolite resulting from reduction of AR88) was not detected in the anaerobic system and was, therefore, probably rapidly transformed to isoquinoline, 2-naphthol or 1,2 naphthoquinone, all of which were detected in the system. The other metabolite resulting from the reduction of AR88, naphthionic acid, was not transformed during the incubation period and was postulated to be channelled into a dead end pathway.

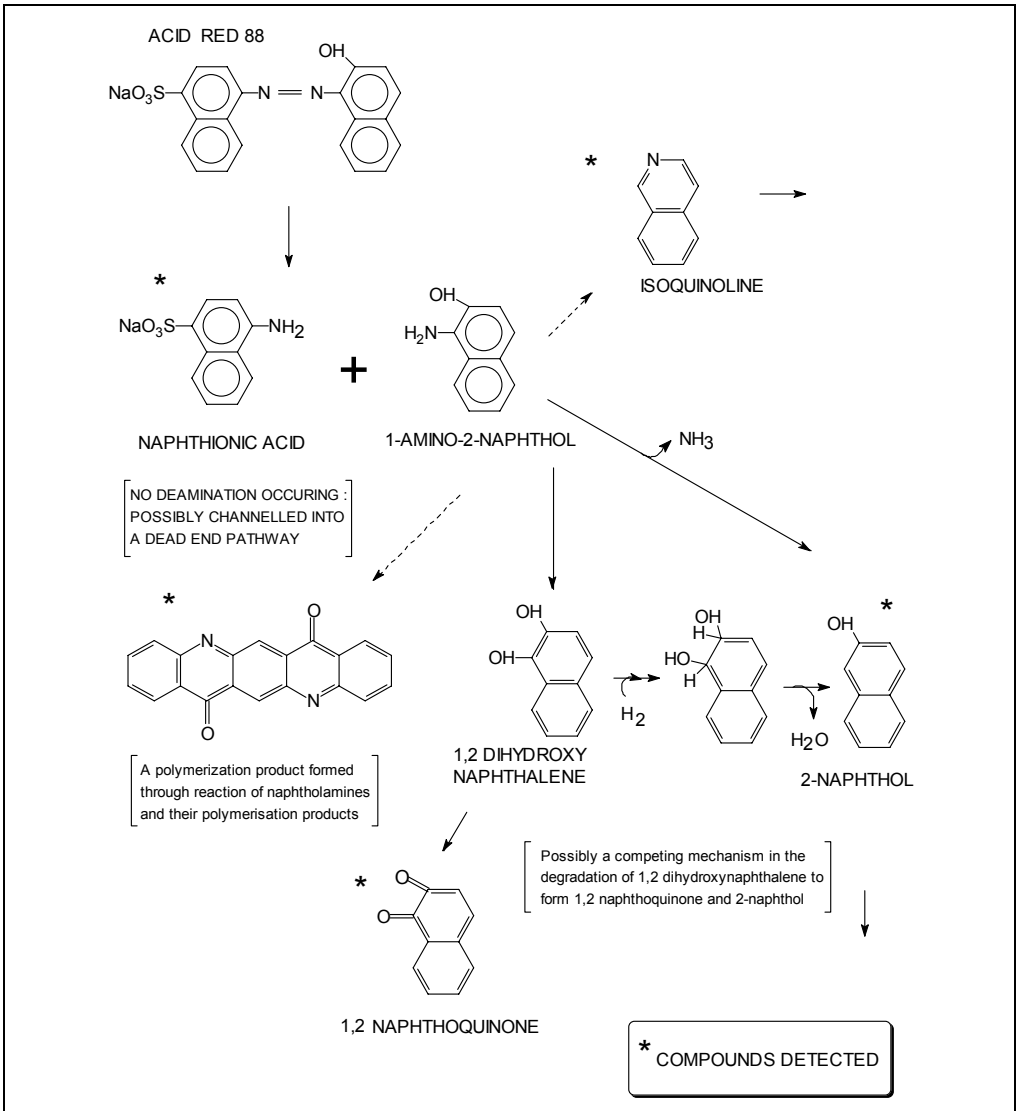

Fig 2.1 : Fate of Acid Red 88 metabolites in an anaerobic system.

2.4.1 The Potential of Specialised Anaerobic Microorganisms to Degrade Aromatic Compounds Structurally Similar to Azo Dye Metabolites

Research dealing specifically with the mineralisation of dye metabolites in biological systems has indicated that little biodegradation of these compounds would be expected in an anaerobic system. However, general research involving the degradation of aromatic compounds has shown that a

number of aromatic compounds previously thought to be dependent on oxygen for splitting the ring structure, can be degraded under anaerobic conditions. Since most dyes are aromatic in structure it was postulated that the aromatic dye metabolites could possibly be anaerobically degraded. A literature review was undertaken with the aim of determining whether dye metabolites are unlikely to be degraded in anaerobic systems, as concluded by Brown and Hamburger (1987), or whether these compounds have the potential to be degraded in specifically applied anaerobic systems.

The literature review concentrated on papers dealing with biodegradation of the following compounds:

a) Naphthalene and naphthol (including substituted compounds) which form the structural basis of numerous azo dyes;

b) Nitroaromatics such as aniline; and

c) Nitrogen heterocyclic compounds which form the reactive groups of reactive dyes.

Mihelcic and Luthy (1988) showed that degradation of naphthalene, acenaphthene and naphthol could occur under anaerobic conditions but was significantly improved in anoxic systems with denitrification conditions. The acclimation period for degradation of naphthalene and acenaphthene under denitrification conditions was reported to be two weeks, in comparison to the two day acclimation period required for aerobic degradation of these compounds. Mihelcic and Luthy (1991) investigating the degradation of naphthalene in soil water suspensions under denitrification conditions, confirmed that naphthalene was readily degraded under these conditions. Al-Bashir et al. (1990) showed that mineralisation of naphthalene to carbon dioxide occurred in parallel with consumption of nitrate in the system over a 50 d incubation period. They concluded that denitrifying redox conditions show significant potential for the biodegradation of low molecular weight polycyclic aromatic hydrocarbons. Garcia-Valdes (1988) isolated over 100 strains of microorganisms from anaerobic marine sediments in a heavily polluted area, that could utilise naphthalene as a sole source of carbon and energy. One of these isolates was identified as *Pseudomonas stutzeri,* a species previously shown to be capable of anaerobically decolourising azo dyes (Yatome et al., 1990). This correlation is interesting as the metabolites of the azo dye reduced by *P. stutzeri* were substituted naphthalenes, and the findings of Garcia-Valdes (1988) raises the question of whether these metabolites could have been degraded by this microorganism in an anaerobic system.

Nitroaromatic compounds such as aniline were reported to be degraded by the sulphate-reducing bacterium, *Desulfobacterium anilini,* under anaerobic conditions with carbon dioxide as the electron acceptor (Schnell and Schink, 1991). Hallas and Alexander (1983) also reported that aniline degradation could occur under anaerobic conditions with single species cultures of adapted microorganisms. However Horowitz et al. (1982) reported that several nitroaromatic compounds were highly resistant to microbial attack under anaerobic conditions.

Heterocyclic compounds, particularly those containing nitrogen (which commonly form the reactive groups of cotton reactive dyes), are susceptible to degradation under sulphate-reducing or methanogenic conditions (Kuhn and Suflita, 1989). Pyridine and indole have been reported to be metabolised under nitrate-reducing and methanogenic conditions respectively (Ronen and Bollag, 1991; Berry et al., 1987).

2.4.2 Degradation of Sulphonated Metabolites of Water-Soluble Dyes

Section 2.4.1 shows that the degradation of aromatic compounds such as naphthalene, aniline and nitrogen heterocyclic compounds, which form the structural basis of commercial textile dyes, does occur under anaerobic conditions. However, water-soluble textile dye intermediates are usually highly sulphonated, a property which is known to increase the recalcitrant nature of these compounds by decreasing their ability to permeate into microbial cells (Wuhrmann et al., 1980). In fact, naphthalene-sulphonic acids which are manufactured as pre-products for detergents and textile dyes have been classified as persistent xenobiotics (Quentin, 1978; cited by Luther and Soeder, 1991) due to the consistent recalcitrance of these compounds in both aerobic and anaerobic biological treatment systems.

Research involving the biodegradation of sulphonated aromatic compounds has shown that degradation of these compounds only occurs subsequent to the removal of the sulphonic acid group from the compound. The C-SO_3 bond is labilised by oxygenolytic cleavage (Brilon et al., 1981a) and is, therefore, a strictly aerobic reaction.

Aerobic degradation of naphthalene-sulphonic acids by a *Pseudomonas* sp was reported by Brilon et al. (1981a, 1981b). The sulphonic acid group was eliminated as hydrogen sulphite as a result of oxygenolytic cleavage of the C-SO_3 bond **(Fig 2.2)** and the naphthalene compound was mineralised as a sole source of carbon and energy.

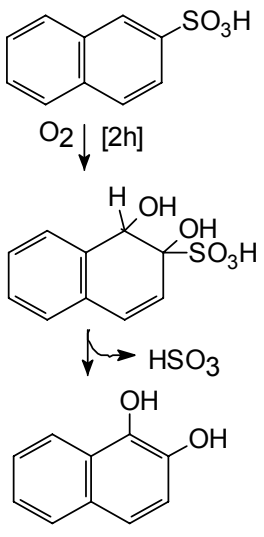

Fig 2.2 : Oxygenolytic cleavage of naphthalene-2-sulphonic acid and the subsequent liberation of hydrogen sulphite.

Zurrer et al. (1987) showed that nine substituted naphthalenesulphonic acids could be desulphonated while providing the sole source of sulphur for aerobic growth of a *Pseudomonas* and an *Arthrobacter* sp. None of these compounds served as a carbon source for these bacteria, i.e. they were not mineralised subsequent to desulphonation. Naphthalenesulphonic acids have also been shown to be adequate sources of sulphur for the green alga, *Scenedesmus obliquus*, which liberates the sulphate by oxygenolytic cleavage (Luther and Soeder, 1991).

However, no reports of the *anaerobic* degradation of sulphonated aromatic compounds have been made and, therefore, it seems unlikely at this time that mineralisation of the sulphonated degradation products of water-soluble dyes (such as acid, direct and reactive dyes) will take place in the anaerobic system that causes decolourisation.

2.5 A COMBINATION OF ANAEROBIC AND AEROBIC BIOLOGICAL SYSTEMS FOR THE DEGRADATION OF TEXTILE DYES

Anaerobic treatment of dyes has been found inadequate with respect to mineralisation of the degradation products and aerobic decolourisation of dyes does not seem feasible in practical waste-water treatment. Therefore, two-phase systems have been developed which incorporate an anaerobic stage for decolourisation and a subsequent aerobic stage for mineralisation of the degradation products.

Bhattacharya et al. (1990) investigated the fate and effect of water-soluble azo dyes in a two phase anaerobic-aerobic system. They reported 30 % to 50 % dye removal in the anaerobic stage and a further 1 % to 18 % removal in the aerobic stage. Unfortunately the DOC content of the effluent was not monitored and, therefore, the efficiency of the system for mineralisation of azo dyes cannot be assessed.

Haug et al. (1991) achieved mineralisation of the sulphonated azo dye, Mordant Yellow 3, by a 6-aminonaphthalene-2-sulphonate-degrading bacterial association. The system used to achieve this was a two phase anaerobic-aerobic system. Complete decolourisation was noted in the anaerobic phase, although the exact mechanism of decolourisation was not understood. The addition of glucose to the culture medium was shown to enhance the decolourisation process, which was attributed to two potential factors. Either the glucose could act as a donor of reduction equivalents, or its addition could result in more actively respiring cells thus rapidly depleting the medium of oxygen and facilitating the anaerobic transfer of reduction equivalents to the azo dye. The addition of flavin adeninenucleotide (FAD) to the culture medium clearly enhanced the reduction reaction, indicating that FAD was reduced in the microbial cells and the reduced FAD in turn spontaneously reduced the azo dye.

The reduction (cleavage of the azo bond) of Mordant Yellow 3 resulted in the production of two intermediates, 6-aminonaphthalene-2-sulphonic acid (6A2NS) and 5-aminosalicylate (5AS) which accumulated under anaerobic conditions. However, when aerobic conditions were restored the degradation products were mineralised by the microorganisms in this mixed culture. The proposed pathway for complete degradation of the azo dye, Mordant Yellow 3, is shown in **Fig 2.2**.

It should be noted that the unsulphonated metabolite (5-aminosalicylate) immediately underwent ring cleavage and degradation, while initial reactions involving the sulphonated metabolite (6-aminonaphthalene-2-sulphonic acid) were the oxygenolytic cleavage of the C-SO_3 bond and liberation of sulphite. This confirms reports by Brilon et al. (1981a, 1981b) that the sulphonic acid

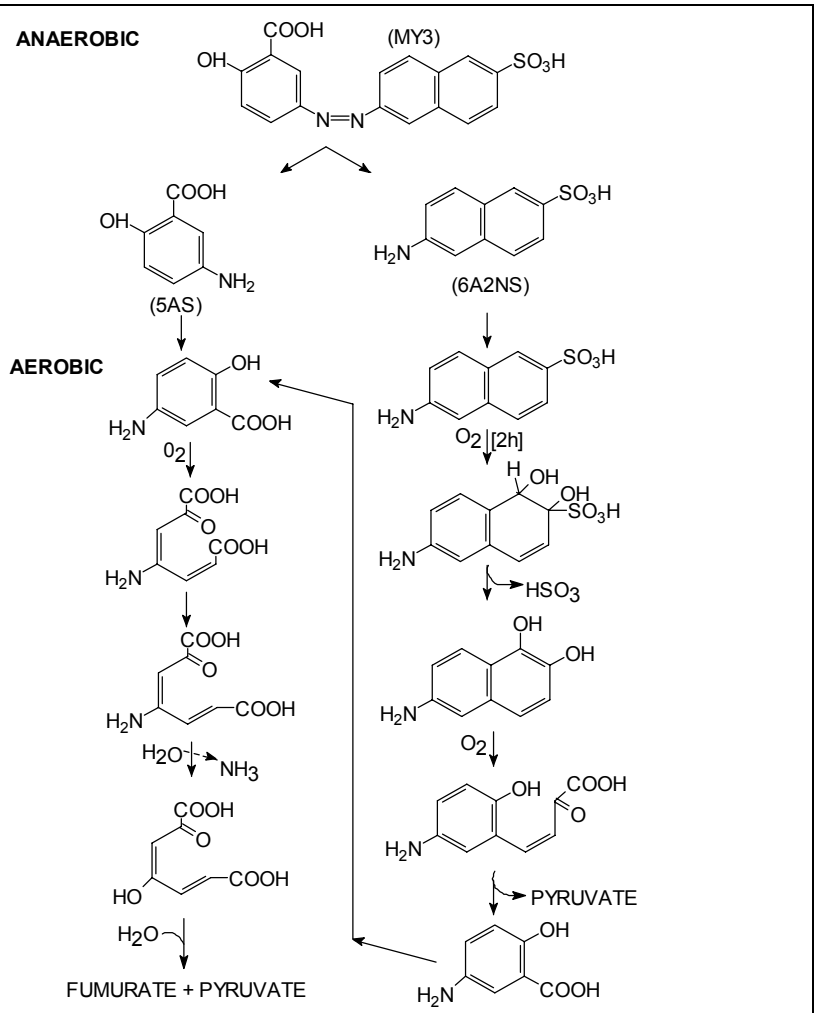

Fig 2.3 : Proposed pathway for degradation of the azo dye Mordant Yellow 3 (MY3) by a mixed microbial population (Haug et al., 1991).

Zaoyan et al. (1992) reported on the results of a dye waste-water treatment system consisting of an anaerobic rotating biological contactor (RBC) combined with an activated sludge process. This system was used to treat the waste water at a textile finishing mill producing polyester fibre cotton and cotton. The waste water consisted primarily of dyes (reactive, reductive, disperse, basic and naphthol), auxiliaries, size (usually PVA), detergents, acids, alkalis and salts.

The results showed that the anaerobic RBC was efficient in removing the colour from the effluent and also in degrading some complex organic matter (probably size and detergents). The aerobic activated sludge unit was responsible for removing the remaining organic matter from the effluent. The authors did not specifically measure the degradation of dye metabolites which originated in the anaerobic treatment stage and, therefore, it cannot be concluded that all the dyes treated were

completely degraded. An added advantage of this system, however, was that the waste activated sludge was recycled into the anaerobic phase, providing substrate for the anaerobic microorganisms and a means of sludge disposal.

Harmer and Bishop (1992) investigated the decolourisation and degradation of an azo dye, Acid Orange 7 (AO7), using laboratory-scale rotating drum biofilm reactors. The process was operated under aerobic bulk-liquid conditions, as it was proposed that the biofilm would provide both anaerobic and aerobic zones that would allow a two-phase mineralisation of AO7. The biomass was originally obtained from an activated sludge plant and, although an acclimation period was observed, it was found that unacclimated biomass possessed the ability to decolourise the azo dye. No degradation of AO7 occurred unless supplemental labile carbon was present in the synthetic waste water.

The results of these laboratory trials showed that decolourisation of AO7 occurred in anaerobic zones of the biofilm, producing 1-amino-2-naphthol and sulphanilic acid. No detectable levels of 1-amino-2-naphthol were found in any grab samples taken after decolourisation was complete, indicating that this compound was readily degraded in the system. Sulphanilic acid was present subsequent to decolourisation of AO7 but was later transformed in the bulk phase aerobic conditions of the treatment system. These results indicate that fixed film reactors operating with a bulk aerobic phase may be capable of both decolourising and mineralising azo dyes, in a single stage.

Loyd et al. (1992) investigated two phase anaerobic-aerobic treatment for a reactive dyeing waste water, Navy 106. Anaerobic pre-treatment was conducted in 2 M continuously stirred sequencing batch reactors, with a residence time of 12 h. The effluent from this process was subsequently subjected to aerobic treatment (activated sludge) in 4 M sequencing batch reactors over a period of 24 h. The results showed that the anaerobic treatment stage provided significant decolourisation of the waste water with little accompanying biodegradation, and succeeded in enhancing the extent and rate of subsequent aerobic biodegradation. It was reported that most of the total organic carbon removal occurred in the aerobic stage.

It can be concluded from the papers reviewed in Section 2.5 that a multi-phase biological process consisting of anaerobic reduction and subsequent aerobic mineralisation of the dye metabolites, is a promising form of treatment for dye-containing textile effluent.

2.6 COMBINED CHEMICAL-BIOLOGICAL PROCESSES FOR THE TREATMENT OF TEXTILE EFFLUENT

This section of the literature review investigates the combination of chemical pre-treatment techniques with conventional biological treatment systems, to optimise colour and total organic carbon (TOC) removal from textile effluents.

2.6.1 Chemical Reduction and Oxidation Combined with Aerobic Biodegradation

McCurdy et al. (1991) presented the results of a research project which aimed to develop a chemical pretreatment technique to remove the colour of a textile mill waste water effluent containing reactive

dyes. This effluent was then biologically treated (aerobically) to achieve the required TOC removal and, therefore, the effect of chemical pretreatment on the biological process was an important aspect of this project.

A reductive mechanism of chemical pretreatment was chosen with the principal aim of removing colour from the effluent. The reducing agents tested were sodium hydrosulphite, thiourea dioxide and sodium borohydride. The textile effluent chosen for this project contained three reactive azo dyes, Remazol Black, Remazol Red and Remazol Yellow (no classification numbers were given in the paper).

The preliminary results showed that all three reducing agents tested were successful in removing 11 % of the colour when treating 17 % of the textile waste water. Sodium hydrosulphite was chosen for future work and the results indicated that 62 % colour removal could be achieved when treating 100 % of the effluent. The fact that 100 % colour removal was not achieved was attributed to a residual recalcitrant component associated with the textile waste water. It should be noted that this recalcitrant component was probably the aromatic amine degradation products resulting from reduction of the azo bonds and, as these compounds are coloured (greenish-yellow), they would have contributed to the overall colour as measured by ADMI units.

This pretreated effluent was fed into aerobic sequencing batch reactors which comprised the biological treatment phase. The feed consisted of 75 % pretreated textile effluent and 25 % municipal waste water. It was found that reductive pretreatment of the textile effluent resulted in a biologically inhibitory environment which could have been due to unreacted reducing agent in the effluent, the creation of a reduced environment, and/or the presence of reduction by-products such as the aforementioned aromatic amines. For this reason it was decided to extend the pretreatment regime to include an oxidation step subsequent to reduction. The role of oxidation after reduction was to create an environment more conducive to biological treatment, oxidise unreacted hydrosulphite, and quinones.

Hydrogen peroxide was added after reduction with sodium hydrosulphite and was found to result in an oxidised environment more conducive to aerobic biological treatment. No additional benefits were gained by the oxidative pretreatment step as it did not contribute to the removal of colour from the effluent. A purely oxidative pretreatment step was also attempted using hydrogen peroxide alone but it was found that this removed little colour (less than 5 %) and did not enhance the biological degradation of the textile waste water.

2.6.2 Pretreatment of Textile Effluent with Ozone and Fentons Reagent Followed by Aerobic Biological Treatment

Powell et al. (1992) and Michelsen et al. (1992) reported on the use of oxidising agents to pretreat textile effluents containing reactive dyes and the subsequent treatment of this effluent in a conventional aerobic biological treatment system. The effluents chosen for the tests were exhausted dyebaths containing reactive (primarily azo) dyes. As the dyes to be treated were known to consist of large numbers of conjugated or aromatic bonds, the oxidants were chosen on the criterion that they

were to react with unsaturated systems. The oxidants chosen were ozone, which has been extensively used as a disinfectant for potable water systems and has recently grown in popularity for the treatment of industrial wastes, and Fentons reagent, which consists of hydrogen peroxide and ferrous salts. The biological treatment phase was simulated using aerated sequencing batch reactors with 2 d residence times. Municipal waste water was utilised to supplement the pretreated effluent to provide the necessary nutrients for the microorganisms.

The results of the oxidative pretreatment stage showed that good colour removal could be achieved with ozone or Fentons reagent when treating waste streams containing reactive dyes with azo chromophores. However, colour removal achieved with Fentons reagent and phthalocyanine reactive dyes was shown to be reversible when the pH of the treated solution was increased, indicating that the chromophores of these dyes were not destroyed in the oxidative process.

Ozone appeared to selectively oxidise the dyes and little DOC removal was achieved, whereas Fentons reagent did not seem to be selective for coloured organic matter. The level of colour removal achieved with Fentons reagent was therefore dependant on the amount of non-coloured organic matter in the waste stream and it was concluded that ozone would be preferred over fentons reagent for pretreatment of waste waters with a high organic load.

Pretreatment of the waste water with ozone or Fentons reagent did not seem to significantly enhance or inhibit subsequent biological degradation of the DOC in the waste water streams. Waste water pretreated with Fentons reagent appeared to show a lower rate of DOC removal than that pretreated with ozone, however, this was probably due to the fact that Fentons reagent removed a high percentage of the non-coloured labile organic matter, resulting in less DOC being readily available to the waste-water microorganisms.

2.7 CONCLUSIONS

The following conclusions have been drawn from the literature presented in Chapter Two :

a) Bacterial and fungal cells may be used as low cost adsorbents for the removal of dyes from solution. Specially selected fungal cells such as *Myrothecium verrucaria* have a far greater capacity for adsorption of water soluble dyes in comparison to activated sludge.

b) With respect to the aerobic degradation of dyes :

- Azo dyes are not significantly biodegraded in the activated sludge process, although some colour removal is achieved by adsorption of dyes to the biomass in the system.
- Although azo dyes are not biodegraded in activated sludge systems, these dyes are generally not toxic to the waste-water microorganisms.
- In aerobic aquatic environments simple triphenylmethane dyes (Basic Violet 1 and 3) are readily degraded but simple azo dyes (Acid Orange 7) are not transformed.
- Aerobic microorganisms have been adapted to degrade simple azo dyes such as Orange II. These microorganisms were identified as strains of *Pseudomonas* and it was

proposed that the ability to degrade Orange II was coded for by plasmid-borne genes. The enzymes responsible for azo reduction are inducible, with the azo dye being the inducer compound.

- The white-rot fungus *Phanerochaete chrysosporium* is able to decolourise and mineralise azo dyes through use of the lignin degrading enzyme system.
- Algae have been found to utilise azo dyes as sole sources of carbon and nitrogen.

c) With respect to the anaerobic degradation of dyes :

- Decolourisation of azo dyes under anaerobic conditions is a relatively simple and non-specific process that involves reduction of the azo bond and subsequent destruction of the chromophore.
- The microbial genera that have been found to degrade dyes in single species cultures under anaerobic conditions are: *Bacillus, Pseudomonas, Aeromonas* and unidentified purple non-sulphur bacteria.
- Azo dyes are readily degraded in anaerobic digestion systems, although the fate of the resulting metabolites is uncertain.

d)

- Aromatic amine metabolites are degraded in aerobic activated sludge systems but usually accumulate in the anaerobic treatment systems that caused decolourisation of the dyes.
- Unsulphonated structural components of dye metabolites, such as naphthalene, naphthol, aniline and nitrogen heterocyclic compounds, have been reported to be degraded in anaerobic or anoxic systems.
- Sulphonation of these compounds increases their recalcitrance in both aerobic and anaerobic systems, although, the ability of some aerobic microorganisms to oxygenolytically cleave the C-SO_3 bond and liberate sulphite allows the mineralisation of these sulphonated aromatic compounds under aerobic conditions.

e) With respect to the combination of anaerobic and aerobic systems for degradation of dyes :

- Anaerobic pretreatment (for decolourisation of dyes) followed by aerobic treatment to allow mineralisation of dye metabolites is a promising system for textile effluent treatment.
- An anaerobic RBC in combination with an aerobic activated sluge unit had the added advantage of low sludge disposal as the waste activated sludge was utilised as substrate in the anaerobic reactor.

- Fixed film reactors operated under aerobic bulk liquid conditions can decolourise azo dyes in anaerobic microzones with the metabolites being subsequently degraded in the aerobic areas of the system.

f) With respect to the combination of chemical and biological processes for the treatment of dye-containing effluent :

- Decolourisation of a reactive dye textile effluent through the use of reducing agents was found to render the decolourised effluent inhibitory to subsequent aerobic biological treatment. However, oxidation with hydrogen peroxide after reduction resulted in an environment more conducive to aerobic biological degradation.
- Pretreatment of dye-containing effluent with oxidising agents (ozone and Fentons reagent) achieved good colour removal and neither inhibited nor enhanced subsequent biological DOC removal in an activated sludge system.

CHAPTER THREE

PROPOSED MECHANISMS OF BIOLOGICAL AZO REDUCTION IN ANAEROBIC / ANOXIC SYSTEMS

Chapter Three takes the form of a discursive literature review dealing with the proposed mechanisms of biological azo reduction in anaerobic/anoxic systems. The purpose of this section was to collate the various mechanisms of anaerobic azo reduction that have been proposed in the literature, and to determine a general mechanism that could be applicable to decolourisation of Procion Red HE-7B in the standard assay system, which is reported in Chapter Four.

3.1 INTRODUCTION

Azo dyes, such as Procion Red HE-7B, can be decolourised in an anaerobic system by reduction of the azo bonds and subsequent destruction of the dye chromophore/s. This is a four electron process which proceeds through two stages. The first gives rise to an unstable, colourless compound **(Eqn 3.1)** which may revert to the original coloured form under oxidising conditions, or be further **(Eqn 3.2).**

$$R_1-N=N-R_2+2e^-+2H^+ \rightarrow R_1-NH-NH-R_2 \quad \textbf{(3.1)}$$

$$R_1-NH-NH-R_2+2e^-+2H^+ \rightarrow R_1-NH_2+R_2-NH_2 \quad \textbf{(3.2)}$$

(where R_1 and R_2 are various substituted phenyl and naphthol residues)

Proposed theories of microbially mediated azo reduction differ according to the site, the enzymatic nature and the rate-controlling factors. However, all the proposed theories indicate that the basic mechanism involves reduction of the azo bond, which is concurrent with re-oxidation of enzymatically-generated flavin nucleotides. The dye, acting as an oxidising agent for the reduced flavin decolourised.

This chapter begins with a section (3.2) on the role of enzymes in microbial azo reduction which aims to define (from the literature) the role of enzymes in the non-enzymatic azo reduction process. Section 3.3 explains the proposed role of soluble flavins in microbial azo reduction and Section 3.4 explains the proposed theories for intracellular and extracellular azo reduction, identifying the probable rate-limiting factors for each site of azo reduction. Finally, Section 3.5 examines the rate limiting effects of competitive oxidising agents in azo reduction and Section 3.6 the role of dye structure in microbial azo reduction.

3.2 THE ROLE OF ENZYMES IN MICROBIAL AZO REDUCTION

The role of enzymes in microbial azo reduction is uncertain. Some researchers, such as Kahn et al. (1983) (cited by Kremer, 1989), have stated that azo reduction is enzymatically catalysed by an enzyme referred to as azo reductase. It is not certain whether this is a single enzyme or a composite enzyme system but it is thought to have a broad substrate specificity and to be oxygen-sensitive, requiring anaerobic conditions for maximum activity. It is also not clear whether this azo-reductase enzyme directly catalyses the final transfer of electrons to the target azo compound or whether, as proposed by Gingell and Walker (1971); Larsen et al. (1976); Wuhrmann et al. (1980) and Haug et al. (1991), azo reduction occurs via enzymically-generated reduced flavins, but that the final step in the transfer of electrons occurs non-enzymically. The latter is generally referred to as *non-enzymatic* azo reduction and is mediated by a flavoprotein in the microbial electron transport chain. This flavoprotein catalyses the generation of reduced flavins (flavinmononucleotide (FMN) or flavinadeninenucleotide (FAD) by the re-oxidation of reduced nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH). These reduced flavins transfer electrons to the azo compound (the terminal electron acceptor), thereby reducing the azo bonds and being concurrently re-oxidised. A proposed mechanism for catalysis by azo reductase and NADPH is shown in **Fig 3.1** (Huang et al, 1968; cited by Kremer, 1989).

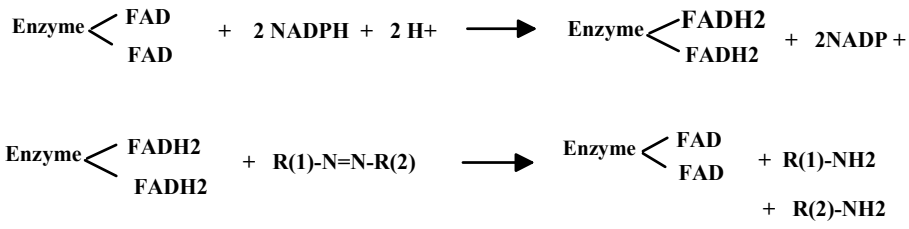

Fig 3.1 : Proposed mechanism of catalysis by azo reductase and NADPH.

These azo-reducing flavoproteins have been found to differ according to the class of microorganism in which they are found (Walker and Gingell, 1971). For example, *Streptococcus faecalis* and *Proteus vulgaris* both contain a flavoprotein which mediates azo reduction. However, whereas the enzyme in *P. vulgaris* has a FMN prosthetic group (Roxon et al. 1967; cited by Walker and Gingell, 1971), the enzyme catalysing azo reduction in *S. faecalis* is activated to a similar extent by riboflavin, FMN or FAD. In addition, the enzyme responsible for azo reduction in *S. faecalis* is more specific for NADH, although both NADH and NADPH are active as electron donors (Walker and Gingell, 1971), whereas *P. vulgaris* has a NADPH specific azo reducing enzyme (Roxon et al. 1967; cited by Walker and Gingell, 1971).

The reduced flavins responsible for the final electron transfer may either be enzyme bound (as in Fig 3.1) or may act as an unbound hydrogen transports system similar to a co-enzyme (Gingell and Walker, 1971).

3.3 THE ROLE OF SOLUBLE FLAVINS IN MICROBIAL AZO REDUCTION

Gingell and Walker (1971) showed that the addition of soluble flavin (FMN) to cell-free preparations of *S. faecalis*, enhanced the rate of reduction of the azo dye Red-2G. The anaerobic assay consisted of the cell-free NADH-generating system to which FMN and Red-2G were added, either simultaneously or sequentially. When FMN and Red-2G were added simultaneously FMN was partially reduced, reaching a plateau at which point all the dye was reduced. Only when all the dye was reduced did flavin reduction continue to its limiting value. The rate of Red 2-G decolourisation was dependent on the redox potential of the system, as given by the ratio of reduced to oxidised FMN in the plateau region. At the lower level (represented by the limiting value for enzymatic reduction of flavin) reduction of Red-2G was rapid, with the rate falling as the redox potential of the system rose. These results indicate that the redox potential of an anaerobic system could be a controlling factor in microbial azo reduction.

When Red-2G was added to an assay system containing enzymatically-reduced FMN, the dye was rapidly decolourised with simultaneous re-oxidation of the flavin, which suggested that the rate of azo reduction was directly related to the rate of generation of reduced FMN. This was proposed to be a rate-limiting factor in bacterial azo reduction, and was supported by other researchers such as Roxon et al. (1967) who reported that the presence of flavin in the medium was essential for reduction of tartrazine (an azo compound) by whole cell cultures of *Proteus vulgaris*. Chung et al. (1978) also noted a marked enhancement of azo reduction upon addition of flavin nucleotide and other electron carriers, and Wuhrmann et al. (1980) concluded that in the absence of oxygen, an azo compound would act as a sole electron acceptor and, therefore, the rate of azo reduction would be governed exclusively by the rate of fomation of the electron donor i.e. reduced flavin nucleotides. As the rate of formation of reduced flavins is directly related to the metabolic state of a microbial population, it is not surprising that azo reduction rates are sensitive to the amount of available respiration substrate in an anaerobic system (Haug et al., 1991; Harmer and Bishop, 1992), since catabolism of these substrates is ultimately responsible for the production of reduced flavins. Therefore, the rate of azo reduction will also be controlled by the availability of supplemental labile carbon in the system.

It was concluded by Gingell and Walker (1971) that soluble flavins can act as electron shuttles, under anaerobic conditions, to transfer electrons between NADH-linked flavoproteins that are normally involved in cellular electron transport and azo compounds which are acting as artificial electron acceptors.

3.4 THE SITE OF MICROBIAL AZO REDUCTION

Although Gingell and Walker (1971) had proposed that azo reduction was mediated by an electron shuttle, these results were obtained using cell-free extracts and did not establish whether this electron shuttle was capable of mediating extracellular azo reduction, or whether azo reduction was an intracellular process. Subsequently, theories have been proposed that support both these mechanisms.

3.4.1 Extracellular Azo Reduction

Dubin and Wright (1975) adopted the theory of Gingell and Walker (1971) and proposed an extracellular, non-enzymatic process in which soluble flavin acted as an electron shuttle between the extracellular dye and an intracellular reducing enzyme, the rate being controlled by generation of the reduced flavin. These researchers found a zero-order dependence of reduction rate on dye concentration, which was consistent with that reported by Gingell and Walker (1971), and proposed that the principal rate-controlling step involved a redox equilibrium between the azo dye and the reducing agent (FMN or FAD) i.e. that the redox potential of the dye to be reduced indirectly controls the rate of azo reduction.

The proposed mechanism of enzymic generation, and subsequent re-oxidation (by an azo compound), of a low molecular weight reducing agent (electron shuttle) is given is **Eqn 3.3** and **3.4.**

$$B + EH_2 \xrightarrow{k_1} BH_2 + E \qquad \textbf{(3.3)}$$

$$BH_2 + (R_1 - N = N - R_2) \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} B + (R_1 - NH_2) + (R_2 - NH_2) \qquad \textbf{(3.4)}$$

(Where BH_2 / B and EH_2 / E are the redox couples for the low molecular weight electron carrier and enzyme respectively)

Eqn 3.3 and 3.4 form a redox cycle with reducing power provided by bacterial metabolism. No net reduction of dye occurs until the concentration of BH_2 is such that the reduction potential of the BH_2/B couple approaches that of the dye, as shown in **Eqn 3.5**.

$$E = E^0 - 0,030 \log \frac{(BH_2)}{(B)} = E_m(dye) \qquad \textbf{(3.5)}$$

Subsequently, colour loss depends on the rate of formation of BH_2, which in turn depends on the concentration of B. If BH_2/B is constant, the concentration of B is controlled by the ratio BH_2/B which, according to Eqn 3.5, is determined by the dye redox potential. Thus the electrochemical properties of the dye may indirectly control the reduction rate by determining the concentration of B in the system, although the dye molecule is not itself involved in the rate limiting step. The rate of colour loss would then be zero-order with respect to dye concentration (until extremely low concentrations are reached) and dependent on dye redox potential.

3.4.2 Intracellular Azo Reduction

The dependence of azo dye reduction on the redox potential of the dyes (Dubin and Wright,1975) was tested by Yatome et al. (1991). The results showed that the rate of azo reduction was not controlled by the specific dye reduction potentials but by the degree of sulphonation of the dyes. This lead the researchers to conclude that azo reduction was limited by the rate of permeation of the dyes into the microbial cells and that azo reduction must be intracellular. These conclusions are supported by numerous authors reporting on the microbial reduction of azo dyes in anaerobic systems.

Research by Wuhrmann et al. (1980) revealed that some dyes, which were not decolourised by whole cells could be decolourised by cell extracts, which lead them to conclude that cell permeability (diffusion) was a pertinant governing factor in azo reduction rates. Additional evidence of cell permeability as a primary rate-limiting factor in microbial azo reduction was found in research by Meschner and Wuhrmann (1982), who managed to substantially increase the reduction rates of azo compounds by permeabilising bacterial cells prior to azo reduction.

Larsen et al. (1976) found that increasing sulphonation of azo compounds, which should increase the rate of degradation due to an increased withdrawal of electrons, instead has an inverse effect on their degradation rate. They attributed this to the reduced ability of the compounds to penetrate the microbial cell boundaries. Continuing in this line of research, Meyer (1981) reported that sulphonated azo compounds were slower to degrade than their carboxylated analogues, when incubated with a whole cell microbial culture. However, when the constraint of cell permeability was removed, the sulphonated compounds were found to be degraded on average 60% faster than the carboxylated analogues, proving that the ability of the dye compound to permeate the cell wall directly effected the rate of degradation.

In contrast to the zero-order kinetics of azo reduction reported by Dubin and Wright (1975) for extracellular azo reduction, and by Gingell and Walker (1971) for a cell-free system, Larsen et al. (1976), Wuhrmann et al. (1980), Meschner and Wuhrmann (1982) and Kremer (1989) observed first order kinetics of azo

3.5 THE EFFECT OF COMPETITIVE ELECTRON ACCEPTORS ON ANAEROBIC AZO REDUCTION

As azo reduction is proposed to occur through reduction of the dye as a terminal electron acceptor in the microbial electron transport chain, the presence of competitive electron accepting agents in the system would be expected to effect the rate of azo reduction. The sensitivity of the azo reduction process to the presence of oxygen can then be explained as a competition of the oxidants (azo dye and oxygen) for the reduced electron carriers in the respiration chain, with respiration of oxygen being the favoured reaction. The rate-limiting effect of nitrite (as a competitive electron acceptor) was also reported by Wuhrmann et al. (1980), who noted that decolourisation of an azo dye in a nitrate-containing system would not commence until denitrification was complete. That is, the nitrite appeared to be reduced preferentially to the azo dye, as a more favourable electron acceptor.

3.6 STRUCTURAL EFFECTS REGARDING PLACEMENT OF SUBSTITUENT GROUPS ON THE AZO DYE

Structural effects regarding placement of substituent groups on an azo dye have been noted by Meyer (1981). As a general rule, the closer the substituent group to the azo bond, the slower the rate of reduction. This was also noted by Larsen et al. (1976) who stated that the prominent factor determining the reduction rate of azo compounds was the electron density in the region of the azo group, while the additional factor of stabilisation by hydrogen bonding must be considered. It

would therefore be expected that an increase in the number of sulphonate groups (electron withdrawing groups) on a dye molecule, would consequently increase the reduction rate. However, this would however only hold true for permeabilised cells or cell free extracts, as a high degree of sulphonation reduces the ability of the dye compounds to penetrate the cell boundary, making cell permeability the rate-limiting factor for azo reduction.

3.7 CONCLUSIONS

a) The role of the flavoprotein enzyme in anaerobic microbial azo reduction is the generation of reduced flavins.

b) The reduced flavins may be enzyme bound, or unbound hydrogen carriers.

c) The reduced flavins transfer the electrons from the NADH-dependent flavoproteins to the azo compound, thus reducing the azo bond and becoming re-oxidised.

d) The rate of generation of reduced flavin is the principal rate-limiting step in extracellular azo reduction or azo reduction with cell free extracts, and is indirectly controlled by the redox potential of the compound to be reduced.

e) The rate of generation of reduced flavin is directly dependant on the metabolic state of the microorganisms and, therefore, since the presence of supplemental carbon is essential to maintain the metabolic activity of the microorganisms, this is a rate-limiting factor in azo reduction.

f) The site of microbial azo reduction has been proposed to be either intracellular or extracellular.

g) The rate-limiting factor for intracellular azo reduction is the rate of permeation of the azo compounds into the microbial cells.

h) Competitive electron accepting agents, such as oxygen or nitrite, affect the rate of azo reduction by competing for the source of reduction equivalents.

i) The structure of the azo compounds affects the rate of reduction, although this is often

CHAPTER FOUR

DECOLOURISATION OF PROCION RED HE-7B IN AN ANAEROBIC SYSTEM

4.1 INTRODUCTION

Procion Red HE-7B can be decolourised in an anaerobic system by reduction of the azo bonds and subsequent destruction of the dye chromophore/s. This azo reduction reaction can be caused by both chemical reducing agents and anaerobic microorganisms. Although chemical azo reduction is well documented, no single theory has been developed which adequately explains all the factors involved in microbial azo reduction. For this reason, the primary focus of the experimental work presented in this chapter was the elucidation and investigation of the mechanisms controlling microbial azo reduction in an anaerobic system.

Section 4.1 introduces the consecutive stages in the experimental investigation and is divided accordingly :

Section 4.1.1 introduces the concept of enriching microorganisms to develop or enhance the azo reduction reaction and Section 4.1.2 summarises the theories of anaerobic azo reduction which influenced the experimental approach for the investigation into the decolourisation of Procion Red HE-7B. As decolourisation in a biological anaerobic system can also be attributed to abiotic mechanisms, such as chemical reduction or physical adsorption of the dyes to the microbial cells, these mechanisms were investigated with respect to decolourisation of Procion Red HE-7B and are introduced in Section 4.1.3. Following on from this, the fate and effect of Procion Red HE-7B in a biological anaerobic system are discussed. Section 4.1.4 contains a summary of the possible fates of degradation products resulting from reduction of azo dyes in biological anaerobic treatment systems and Section 4.1.5 introduces the possibility of azo dyes (or their degradation products) being inhibitory to the anaerobic microbial population.

4.1.1 The Effect of Prior Exposure of Anaerobic Biomass to Procion Red HE-7B on the Efficiency of Azo Reduction

Adaptation of a microbial community to degrade a previously recalcitrant compound, through prior exposure of the microorganisms to this compound, is known as enrichment or acclimation. The literature has shown that anaerobic decolourisation of textile dyes can occur with unacclimated microorganisms, although prior exposure of microorganisms to particular azo dyes has been shown to play a major role in enhancing degradation (Meyer, 1981). These adapted microorganisms may also have increased tolerance to previously inhibitory concentrations of dyes (Ogawa et al., 1988, 1989). It is possible that exposure of biomass to dyes may even facilitate the development of microorganisms able to mineralise dye metabolites and, in fact, recommendations for future research, by Kremer (1989) included specific enrichment programmes to improve the dye mineralisation ability of

anaerobic microorganisms.

Although preliminary experimental results indicated that decolourisation of Procion Red HE-7B could occur with unacclimated biomass, enrichments were performed with the aim of :

a) improving the rate of decolourisation;

b) improving degradation and, possibly, mineralising the degradation products resulting from the decolourisation of Procion Red HE-7B; and

c) improving microbial tolerance to inhibitory concentrations of dye and/or degradation products.

The effectiveness of this acclimation period was tested with respect to the above objectives. The experimental method for comparison of decolourisation rates for acclimated and unacclimated biomass is presented with details of the enrichment programme, in Section 4.2.1. However, the experiments performed to test whether acclimation had improved the ability of the biomass to (a) mineralise Procion Red HE-7B degradation products; and (b) tolerate inhibitory concentrations of Procion Red HE-7B, are dealt with in Sections 4.2.4 and 4.2.5, respectively. The results are also presented in the respective sections. This format was chosen to avoid repetition of experimental methods and results. Discussion of all results is included in the general discussion (Section 4.4).

4.1.2 Biological Decolourisation by Anaerobic Microorganisms

As indicated in Section 4.1, the process responsible for biological decolourisation of azo dyes under anaerobic conditions is subject to debate. Theories proposed for the mechanism/s of azo reduction can be broadly categorised into two groups :

a) intracellular azo reduction; and

b) extracellular azo reduction.

In both cases the mechanism proposed for azo reduction is similar, in that reduction of the azo bond is concurrent with re-oxidation of enzymatically-generated reduced flavin nucleotides. The dye, acting as an oxidising agent for the reduced flavin nucleotides of the electron transport chain, is reduced and, consequently, decolourised, as is shown in **Fig 4.1**. The disparity arises as to whether azo reduction takes place within the microbial cell, or in the extracellular medium.

Rate-determining factors for azo reduction differ according to whether azo reduction is proposed to be intra- or extra-cellular. Generally, intracellular azo reduction has been found to be first-order with respect to dye concentration (Larcen et al., 1976; Meyer, 1981; Wuhrmann et al., 1981; Yatome et al.,1991), with the principal rate-limiting factor of decolourisation being permeation of the azo dyes through the cell boundaries (Meschner and Wuhrmann, 1982). Extracellular decolourisation has been found to be zero-order with respect to dye concentration (Dubin and Wright, 1975). Electron transfer is thought to be catalysed by an extracellular reducing agent which acts as an electron shuttle between the dye and cellular reducing enzymes. The rate-limiting factor for non-enzymatic azo reduction is proposed to involve a redox equilibrium between dye and reducing agent (FAD or FMN), which results in the rate of reduction of an azo dye being governed by its redox potential.

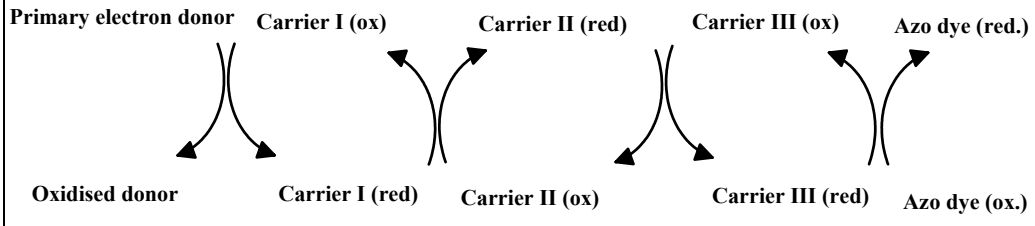

Fig 4.1 : Schematic respresentation of an electron transport chain with an azo dye as the terminal electron acceptor.

Both intracellular and extracellular decolourisation rely on the production of enzymatically-generated reduced flavin nucleotides. These reduced flavins are formed in catabolic processes and therefore the availability of respiration substrates should also be considered as a rate-controlling step in azo reduction (Haug et al.,1991).

The experimental approach adopted in this chapter aimed to elucidate the mechanism of biological decolourisation of Procion Red HE-7B, so that the suitability of this process for large scale decolourisation of textile dyes could be assessed. Thus, decolourisation of the azo dye was investigated in a standardised laboratory system with the aim of identifying the order of decolourisation (with respect to dye concentration) and, from these results, identifying possible rate-limiting factors for further investigation. The aims of the latter experiments were two-fold, that is, to understand which rate-limiting factors should be taken into account when designing a waste-water treatment plant and to aid in identifying the controlling mechanism/s for decolourisation of Procion Red HE-7B. Experimental materials and methods are presented in Section 4.2.2 and experimental results are presented in Section 4.3.2. Discussion of these results is included in the general discussion (Section 4.4).

4.1.3 Abiotic Decolourisation

Abiotic decolourisation can be attributed to physical removal of dye from solution, either by adsorption to the biomas or flocculation/precipitation by a chemical agent, or to chemical reduction of the dye.

Decolourisation by Adsorption

Research into the decolourising abilities of biological sludge has led to the conclusion that considerable decolourisation of textile effluent can occur through adsorption of dyes to the biomass (Pagga and Brown, 1986; Ganesh et al., 1992; Shaul et al., 1988) the extent of which is primarily governed by the class of dye in the waste water. For example, reactive dye waste water discharged to a treatment works contains primarily hydrolysed dye which is no longer able to react with the fabric. These hydrolysed dyes are water soluble and, therefore, only a small percentage of the dye adsorbs to the sludge. However, dyehouse waste water containing dyes which are predominantly hydrophobic (for example, disperse dyes) will attain a high level of colour removal by adsorption of these dyes to the biological sludge in the treatment system. As discussed in Chapter Two, decolourisation by adsorption of dyes to the biomass is the primary and often, sole, mechanism of decolourisation

operating in an activated sludge plant.

Adsorption of dyes to biomass in a treatment system can be measured experimentally and adsorption isotherms developed from the results. The experiments consist of the equilibration of sub-samples of sludge at a constant temperature (isothermal) with a number of aliquots of solutions containing different concentrations of the adsorbate (dye) of interest. The graphical plot of the amount adsorbed by the sludge against the equilibrium solution concentration is termed the adsorption isotherm. If the adsorbent is characterised by monolayer adsorption, these plots usually have a steep portion at relatively low solution concentrations (i.e. when more adsorption sites are available than can be filled by adsorbate) followed by a less steeply rising curve as the concentrations become higher (i.e. the adsorbent becomes saturated) (Holford, 1974). The Langmuir and Freundlich equations are the most widely used isotherm equations for description of such curves (Holford, 1974). The principal function of an adsorption isotherm is that it allows the prediction of adsorption for values of solute concentrations not used experimentally.

Langmuir equation : This equation expresses the relationship between the amount adsorbed, *x,* at an equilibrium concentration, *c*, as is given in **Eqn 4.1**:

$$x = \frac{kKc}{1+Kc} \qquad \textbf{(4.1)}$$

x = amount of dye adsorbed per unit mass of sludge solids (mg/g)

c = equilibrium concentration of dye in solution, mg/M

k, K = empirical constants

A linear plot is given either by *c/x* against *c* , or *x/c* against *x.* From either of these graphs values of *K* and *k* can be determined from the linear slope and intercept, respectively (Holford, 1974).

Freundlich equation : The Freundlich equation is given by the following relationship **(Eqn 4.2)** :

$$x = ac^b \qquad \textbf{(4.2)}$$

x = amount of dye adsorbed per unit mass of sludge solids (mg/g)

c = equilibrium concentration of dye in solution, mg/M

a, b = empirical constants

The logarithmic form of the Freundlich equation is employed to obtain a straight line relationship and is given in **Eqn 4.3** :

$$\log(x) = \log(a) + (b)\log(c) \qquad \textbf{(4.3)}$$

This may be illustrated graphically by a plot of log (*x*) versus log (*c*). The slope of this line equals (*b*) and the intercept equals *log (a*).

Two experiments were performed to determine the adsorption characteristics of Procion Red HE-7B. The first measured adsorption of Procion Red HE-7B to biomass in the standard assay system (Appendix C) and the second measured the extent of adsorption of Procion Red HE-7B (with anaerobic biomass as the adsorbent) in a system for the prediction of adsorption isotherms (Shaul et al., 1986). The experimental materials and methods for this work are presented in Section 4.2.3 and the results in 4.3.3. These results are discussed in Section 4.4.

Decolourisation in the mineral salts medium

It is possible that ions in the mineral salts growth medium (Appendix C) could reduce the azo dye in the low redox potential environment of an anaerobic digester, although this type of decolourisation would often be reversible upon atmospheric exposure. In addition, physical removal of the dye from solution by precipitation or flocculation could be responsible for abiotic colour loss. The extent of colour removal that could be solely attributed to decolourisation in the mineral salts medium was investigated under standard assay conditions. Experimentation is presented in Section 4.2.3 and the results in Section 4.3.3.

4.1.4 Identification and Fate of Procion Red HE-7B Breakdown Products

Anaerobic decolourisation of reactive azo dyes involves the reduction and consequent splitting of the dye chromophore. This process eliminates the colour from the effluent but produces intermediary compounds which may be broadly classified as sulphonated aromatic amines. Degradation products (metabolites) resulting from the decolourisation of Procion Red HE-7B **(Fig 4.2)** are primarily sulphonated aminonaphthalenes and aminonaphthols, nitroaromatics such as aniline and nitrogen heterocyclic

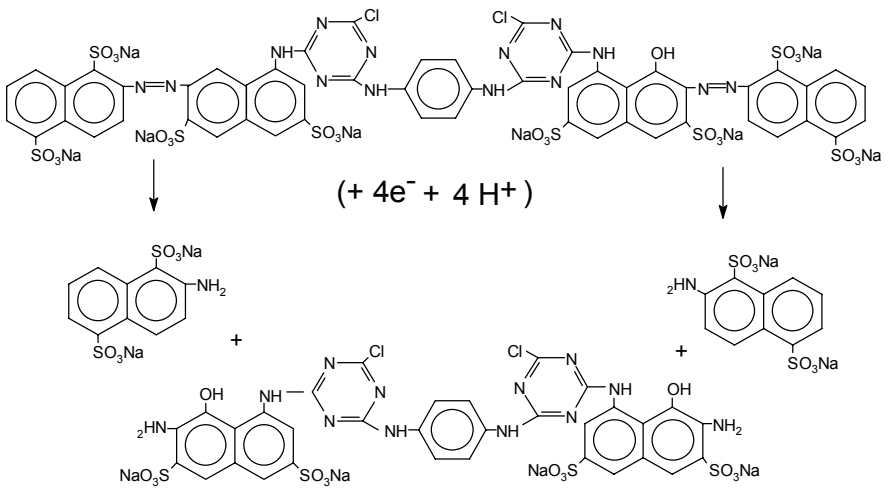

Fig 4.2 : Decolourisation of Procion Red HE-7B to yield dye intermediates.

Sulphonated aromatics such as napthalenesulphonic acids (NSS) have been classified as persistent xenobiotics (Quentin, 1978 cited by Luther and Soeder, 1991) and pass through waste-water treatment plants without significant biodegradation (Zahn and Wellens, 1979 cited by Luther and Soeder, 1991). Some classes of aromatic amines are also suspected carcinogens (Weisburger and Weisburger, 1966; Chung, 1983). Therefore, although decolourisation is a primary step in the treatment of textile dyehouse effluent, environmental protection requires the *mineralisation* of waste compounds and conditions must be engineered that will permit microbial catabolism of the resulting dye metabolites.

Research into the biodegradability of aromatic amines (as degradation products of textile dyes) has shown that aerobic conditions are more amenable to mineralisation, with little or no degradation of these compounds occurring under the anaerobic conditions that give rise to decolourisation. The Ecological and Toxicological Association of the Dyestuffs Manufacturing Industry (ETAD) reported that little degradation of dye degradation products occured in the anaerobic systems tested, as evidenced by a large build-up of these compounds in the systems. They concluded that aerobic treatment was the more feasible option for mineralisation of dye degradation products (Brown and Hamburger, 1987). Haug et al. (1991) also concluded that a two phase anaerobic-aerobic system was neccessary for mineralisation of the sulphonated azo dye, Mordant Yellow 3.

However, anaerobic degradation of aromatic compounds (mono- and poly- cyclic) similar in structure to the aromatic degradation products of textile dyes, has been reported by Evans (1977), Sleat and Robinson (1984) and Berry et al. (1987). Guoqing et al. (1990), reported that *latest results show that aromatic compounds, as the products of the reductive splitting of bisazo dyes, are likely to undergo further anaerobic degradation*. Such reports have motivated a renewed examination of the feasibility of mineralisation of azo dyes in anaerobic systems.

Thus, a specifically enriched anaerobic association may be able to mineralise dye compounds in an anaerobic treatment system. The advantages of such a system compared to a two phase

anaerobic-aerobic system would include energy production in the form of biogas and low sludge production. However, other considerations such as the residence time required for dye mineralisation to occur under anaerobic conditions may be impractical for a full size plant, neccessitating the implementation of a two-phase anaerobic-aerobic system.

The first stage of experimentation with Procion Red HE-7B aimed to prove that azo reduction was responsible for the decolourisation observed in the standard assay system. This was achieved by performing scans of absorbance versus wavelength (190 to 600 nm) using centrate from serum bottles in which decolourisation was occurring. The second phase of experimentation was to determine whether mineralisation of Procion Red HE-7B would occur over an extended incubation period of 52 d. This experiment also involved the use of acclimated and unacclimated biomass to determine whether acclimation of the biomass resulted in the ability to mineralise Procion Red HE-7B degradation products. The mineralisation experiment was monitored by measurement of total gas production. The volumes of gas produced in bottles containing Procion Red HE-7B were compared with those measured for the controls (containing no dye). The details of experimental materials and methods are presented in Section 4.2.4 and the results are presented in Section 4.3.4.

4.1.5 Inhibitory Effects of Procion Red HE-7B on an Anaerobic Microbial Population

Textile dyes are known to exert inhibitory effects on the activity and growth of microorganisms. The extent of inhibition is governed by parameters such as dye type and concentration and the class of microorganism. In general, high concentrations of dyes are usually required for inhibition to occur, although with some cationic species (e.g. triphenylmethane dyes) inhibition is noticeable at low concentrations (Meyer, 1981). Dye structure has been shown to affect the level of inhibition. Azo dyes such as Tropeolin O exhibit a mean microbial survival rate of 92 % at 5 mg/M, while triphenylmethane dyes such as Basic Violet 3 exhibit only a 20 % survival rate at identical concentrations (Michaels and Lewis, 1985). With respect to the class of microorganisms, gram negative species generally exhibit a greater resistance to dyes than gram positive species (Fung and Miller, 1973).

The mechanism of growth inhibition caused by dye toxicity was investigated by Ogawa et al. (1988, 1989). Inhibition of *Bacillus subtilis* by basic dyes was related to decreasing ratios of RNA : DNA, which concurred with increasing dye concentration. This indicated that the dyes acted preferentially to limit protein synthesis, rather than inhibit cell division, which resulted in a variation in cell shape when bacteria were grown in the presence of basic dyes. Cell division inhibition was found to be caused by stabilization of the DNA double helix which, in turn, inhibited enzymatic activities and prevented DNA replication. Stabilization was caused by intercalation of the dyes between the base pairs of DNA. It was reported by these authors that acclimation of bacteria to growth in the presence of inhibitory dyes reduced the toxic effects of the dyes and partially restored the physiological activity of the cells.

Textile effluent entering a waste-water treatment works is usually characterised by high volumes of dilute waste water and should, therefore, not pose a toxicity problem in the biological treatment

processes. However, concentration of dye-containing effluent by flocculation, membrane filtration or segregation of concentrated exhausted dyebaths, prior to treatment in a specialised biological treatment system would, accordingly, increase the inhibitory nature of the effluent. It was, therefore, neccessary to determine the toxicity of Procion Red HE-7B to both acclimated and unacclimated anaerobic biomass by means of an anaerobic toxicity assay.

Anaerobic toxicity can be defined as the adverse effect of a substance on the predominant methanogens in an anaerobic population of microorganisms (Jerris and Mcarty, 1965; cited by Owen et al.,1979). This can be determined through batch techniques which make use of serum bottles and syringe gas measurements, as described in papers by Owen et al. (1979) and Shelton and Tiedjie (1984). A second batch technique, known as the *specific methanogenic activity test* (SMA), makes use of a modified Warburg respirometer to measure gas volume (Chernicharo and Campos, 1991; Campos and Chernicharo, 1991). Both these techniques have a similar approach. The compound to be tested has assay concentrations selected to provide a range from non-inhibitory to severely toxic. In general, five to ten assay concentrations and three controls are selected. The assay bottles contain an anaerobic inoculum, mineral salts medium, inhibitory substance (at the concentrations chosen) and a suitable source of substrate. The control bottles lack the inhibitory compound to be tested. The bottles are incubated at a required temperature and gas production is measured once or twice daily for the first seven days and periodically thereafter.

Total gas production data, or methane production data, are employed for determining the relative rates of metabolism of the feed source among samples. The maximum rate of gas production is computed for each sample over the same time period and the data are normalized by computing ratios between respective rates for samples and the average of the controls. This ratio is designated the maximum rate ratio (MRR). Since measurement of gas production is relatively accurate, a MRR of less than 0,95 suggests possible inhibition and one less than 0,9 suggests significant inhibition (Owen et al., 1979). Data interpretation can be complicated by gas production from sample decomposition and by varying rates of carbon dioxide and methane production.

The batch method of Owen et al. (1974) was used to test the anaerobic toxicity of Procion Red HE-7B. This test also aimed to determine whether prior exposure of the biomass to Procion Red HE-7B increased the resistance of the biomass to any inhibitory effects of the dye. The experimentation is presented in Section 4.2.5 and the results in Section 4.3.5.

4.2 EXPERIMENTATION

A standard set of operating conditions was developed for laboratory studies of Procion Red HE-7B decolourisation and are described in Appendix C. In the experimentation sections which utilised these conditions the details are referenced to Appendix C, with any amendments described in the relevant experimental section. All experiments were performed in triplicate unless otherwise specified.

4.2.1 The Effect of Prior Exposure of Anaerobic Biomass to Procion Red HE-7B on the Efficiency of Azo Reduction

This section is divided into :

a)

b) the experimental procedure for testing whether acclimated biomass could decolourise Procion Red HE-7B at a higher rate than unacclimated biomass.

Enrichment programme for Procion Red HE-7B

The enrichments were performed in two batch digesters; a round-bottomed reactor (5 M) with flange (Quickfit); and an aspirator bottle (10 M). The inoculum was obtained from anaerobic digesters at the Umzinto and Umbilo Waste-water Treatment Works. These works were chosen as sources of inoculum as they treat a significant volume of textile effluent and the microoganisms in the sludge could have become adapted to tolerate and/or degrade textile dyes. A 40 % (v/v) inoculum was used in the enrichments (including undigested organics in the sludge). A defined medium of trace elements, minerals and vitamins was prepared according to Owens et al. (1979) (Appendix C) and added to the digesters with the inoculum. The digesters were overgassed with oxygen-free nitrogen (Fedgas) for 30 min at a flow rate of 0,5 M/min and incubated, without shaking, in the dark at 32 ^{o}C. Gas was released continuously from the digesters into gas traps. The traps consisted of upturned measuring cylinders clamped in beakers of solution (20 % (w/v) NaCl and 0,5 % (w/v) citric acid). The volume of solution displaced was equivalent to the volume of gas produced.

Once the digesters were stable (approximately 15 d of incubation) Procion Red HE-7B dye solution was added to the reactors to give an initial concentration of 10 mg/M. Dye solution was added at monthly intervals over the 4 month enrichment period and the final concentration of Procion Red HE-7B (after 4 months of incubation) was calculated to be 200 mg/M, assuming that no degradation of the dye had occurred. A cumulative concentration was calculated, although decolourisation was seen to occur, as it was not known whether the resultant dye metabolites were degraded or merely accumulated in the digester, and there was uncertainty concerning the concentration at which these metabolites would become toxic to the microorganisms.

Dye solution

The commercial (impure) form of Procion Red HE-7B (ICI) was used in the enrichment programme.

The effect of prior exposure of anaerobic biomass to Procion Red HE-7B on the rate of azo reduction.

The conditions and methods of the standard assay, described in Appendix C, were followed for these experiments in which rates of decolourisation of Procion Red HE-7B were compared for acclimated and unacclimated biomass, i.e. biomass previously exposed to Procion Red HE-7B in the enrichment programme and biomass that had not been previously enriched for Procion Red HE-7B. An amendment to the standard assay procedure was the method of serum bottle inoculation. As the two sources of inoculum (acclimated and unacclimated biomass) were to be obtained from digesters with different liquid : volume ratios, a standard volume of 30 mM inoculum could not be used. A volume of 30 mM inoculum (equivalent to that in the standard assay) was centrifuged and the biomass pellet weighed. It was found to give a wet weight of approximately 5 g and this mass was chosen as the standard inoculum size per serum bottle for this experiment.

4.2.2 Biological Decolourisation of Procion Red HE-7B

A series of experiments was undertaken to define the nature of anaerobic decolourisation. The first experiment aimed to determine the order of decolourisation for Procion Red HE-7B with respect to dye concentration. From this study probable rate controlling factors were identified to be: cell permeability; presence of additional carbon source; and addition of competitive electron acceptors. These factors were investigated to determine which theory of microbial azo reduction (Chapter Three) supported the results observed in the standard assay system. The role of redox potential in azo reduction was investigated both when Procion Red HE-7B was present as a sole electron acceptor and when competitive electron acceptors such as nitrate or sulphate were added.

Standard assay conditions were followed and are described in Appendix C. A number of ammendments were made to these conditions to suit the individual experiments, which are explained below :

The order of Procion Red HE-7B decolourisation, with respect to dye concentration

Procion Red HE-7B concentrations of 150 and 200 mg/M were used, in addition to the 100 mg/M concentration used in the standard assay. Three dye concentrations were utilised to assess the effect of increased dye concentration on the rate of decolourisation of Procion Red.

Rate of decolourisation of Procion Red HE-7B as a sole carbon source

The rate of decolourisation of Procion Red HE-7B was measured in the presence of an additional carbon source (glucose) as per standard assay conditions and compared with decolourisation rates in serum bottles with no additional carbon source. That is, no glucose was added to the experimental bottles, which were otherwise prepared according to standard assay conditions.

The effect of cell permeability on rate of Procion Red HE-7B decolourisation

Standard assay conditions were not followed in these experiments and the full experimental materials and procedure are described in this section. Cells were permeabilised using the technique of Meschner

and Wuhrmann (1982) (reported below with some amendments) so that decolourisation rates of Procion Red HE-7B

Cell permeabilisation

Cells from the laboratory enrichment digester were separated from the preculture medium by centrifugation (5 000 rpm for 20 min), washed in phosphate buffer (0,025 M, pH 6,86) and centrifuged again (5 000 rpm for 20 min). A 30 g (wet weight) pellet of washed cells was resuspended by vigorous agitation in 250 mM of phosphate buffer (0,025 M, pH 6,86) containing 1 mmol/M MgCl2 and 20% (v/v) toluene. The suspension was immediately cooled to 2 ^{o} C by plunging into crushed ice and centrifuged at 10 000 rpm for 30 min. The supernatant was separated into a toluene and a water phase in a separation funnel (ca. 5 min). The cooled cell pellet was then divided into two; one half was resuspended in the water phase of the permeabilisation mixture and the other in phosphate buffer (0,025 M, pH 6,86).

The cell pellet was resuspended in the water phase of the permeabilisation mixture since it was found by Meschner and Wuhrmann (1982) that although the aqueous phase of the permeabilisation mixture was inactive as a dye reductant, permeabilised cells resuspended in this mixture reduced the dyes at a much higher rate than cells suspended in phosphate buffer. As boiling of this aqueous phase did not change its reduction enhancing effect on the cells, they assumed that treatment of the bacteria in the water-toluene mixture leached out some heat resistant lipophilic compound that was essential for dye transport through the cell envelope.

The permeabilised cell mixture (either suspended in the permeabilisation solution or in phosphate buffer, 0,025 M) was decanted into serum bottles (100 mM into each bottle). Glucose was added to the bottles to give an initial concentration of 1 g/M. Control bottles consisted of non-permeabilised cells suspended in 100 mM phosphate buffer with 1 g/M glucose. The bottles were overgassed with nitrogen (Fedgas) at a flow rate of 0,5 M/min, sealed, crimped and incubated statically in the dark, in a water bath at 32 ^{o}C. After 18 h of incubation a solution of Procion Red HE-7B was added to each bottle by syringe to give an initial dye concentration of 100 mg/M. Samples were withdrawn by inserting a hypodermic needle through the septa of the serum bottles and withdrawing 2,5 mM of sample into a syringe. These samples were prepared and analysed according to the analytical procedure described in Appendix C.

Measurement of metabolic activity of permeabilised cells after exposure to Procion Red HE-7B.

The contents of the serum bottles were centrifuged at 5 000 rpm for 20 min to separate the biomass and the centrate. The biomass pellets were washed by re-suspending the pellets in phosphate buffer (0,025 M) and centrifuging this mixture at 5 000 rpm for 20 min. This was repeated 5 times for the permeabilised cells, until no visible colour was present in the centrate. The biomass pellets were then suspended in 100 mM of mineral salts medium (Appendix C) containing 1 g/M of glucose, which was decanted into serum bottles. The serum bottles were overgassed with oxygen-free nitrogen, sealed and incubated in the dark, in a waterbath at 32 ^{o}C. Gas production was monitored by inserting a hypodermic needle connected to a ground glass syringe through the butyl rubber septa of the serum

bottles and allowing the pressure to equilibrate to atmospheric through the release of gas into the syringe.

Rate of decolourisation of Procion Red HE-7B in the presence of additional electron acceptors

Two oxidising agents (nitrate and sulphate) were added to the assay bottles to assess the effect of the presence of additional electron accepting compounds on the reduction of Procion Red HE-7B. Experimental bottles were prepared and pre-incubated according to conditions described in Appendix C and the following amendments to the standard assay conditions were made after completion of pre-incubation.

Addition of nitrate

A concentrated stock solution of sodium nitrate was prepared by dissolving 850 mg of sodium nitrate in distilled water in a 10 mM volumetric flask, to give a 1 000 mM (1 M) stock solution (A). This stock solution (A) was diluted 1:1 to give a 500 mM stock solution (B) and 1: 9 to give a 100 mM stock solution (C). One mM volumes of stock solutions A, B or C were added by syringe to the pre-incubated serum bottles to give initial nitrate concentrations of 10, 5 or 1 mM. Control bottles contained no nitrate. A volume of 2 mM Procion Red HE-7B stock solution (as per standard assay conditions) was added to each serum bottle and decolourisation monitored according to the analytical procedure described in Appendix C.

Addition of sulphate

A concentrated stock solution of sodium sulphate was prepared by dissolving 1420 mg of sodium sulphate in distilled water and making up to volume in a 10 mM volumetric flask, to give a 1 000 mM stock solution (A). This stock solution (A) was diluted 1:1 to give a 500 mM stock solution (B). One mM of appropriate stock solution was added to the assay bottles by syringe to give initial concentrations of 5 and 10 mM sulphate. Control bottles contained no sulphate. A volume of 2 mM Procion Red HE-7B stock solution (as per standard assay conditions) was added to each serum bottle and decolourisation

The role of redox potential in azo reduction

These experiments required continuous monitoring of the redox potential in the anaerobic system so that any changes in redox potential could be recorded. Preliminary experiments using serum bottles neccessitated the samples to be withdrawn from the anaerobic environment for the redox potential to be determined. This was not satisfactory for the following reasons: firstly, the redox electrode required approximately 30 min to stabilise; and secondly, although a blanket of nitrogen was used to prevent oxygen contamination of the samples this was inadequate over the time period required for the electrode to stabilise and, consequently, readings were erratic. For these reasons the following system **(Fig 4.3)** was developed.

Equipment for on-line measurement of redox potential

The anaerobic system consisted of a cylindrical reactor (1 M) and flange (Quickfit) with ground glass ports for insertion of electrodes and collection of gas and liquid samples. A magnetic stirrer was used

to mix the contents of the digester. A PHM82 standard Radiometer pH meter, fitted with a combined platinum-calomel redox electrode (PK 1401) was used for on-line redox potential measurements. The electrode was inserted into the digester through a ground glass port which was subsequently sealed. Redox potential readings (mV) were relayed to a data collection programme and stored in a *.PRN file. Silicone tubing and a 3-way valve connected to a syringe (Aldrich Chemical Company) were used to withdraw samples from the digester without introducing air into the system. Gas produced in the digester was transferred through silicone tubing into a gas trap with a solution of 20 % (w/v) sodium chloride and 0,5 % (w/v) citric acid.

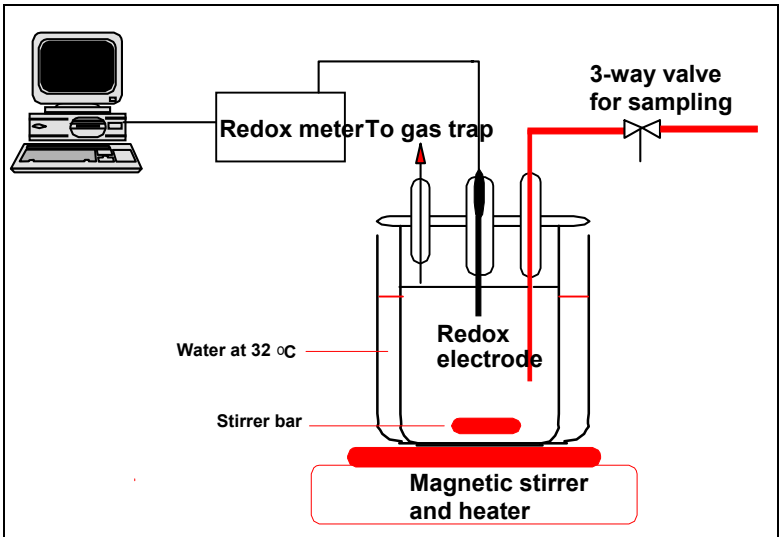

Fig 4.3 : System for on-line measurement of redox potential in an anaerobic digester containing Procion Red HE-7B.

Experimental procedure for on-line measurement of redox potential

Conditions in the digester were identical to standard assay conditions i.e. a 30 % (v/v) inoculum of biomass was introduced into the digester together with mineral salts medium and 1 g/M of glucose. The digester was sealed, overgassed with oxygen-free nitrogen and pre-incubated for 18 h in a beaker of water at 32 °C. The digester was gently stirred during pre-incubation by means of a magnetic stirrer.

Once pre-incubation was completed the redox electrode was introduced into the digester and allowed to stabilise for 30 min, after which one of three experimental routes was followed :

a) Procion Red HE-7B solution was added to the digester (by means of the 3-way valve) to give an initial concentration of 100 mg/M;

b) sodium nitrate solution was added to the digester to give an initial concentration of 20 mM, together with Procion Red HE-7B as in (a); or

c) sodium sulphate solution was added to the digester to give an initial concentration of 5 mM, together with Procion Red HE-7B as in (a).

The digester was incubated at 32 °C with on-line measurement of redox potential (mV). Samples were withdrawn from the digester at regular intervals and analysed for Procion Red HE-7B concentration

(mg/M) by means of the analytical procedure described in Appendix C.

4.2.3 Abiotic Decolourisation

Abiotic decolourisation of Procion Red HE-7B was investigated with respect to physical removal of colour i.e. adsorption of the dye to the microbial cells and colour removal in the mineral salts medium. Adsorption of Procion Red HE-7B was measured in the standard assay system to provide a control for biological decolourisation and in a system for the development of an adsorption isotherm.

Adsorption of Procion Red HE-7B in mineral salts medium

Standard assay conditions (Appendix C) were followed for the measurement of adsorption of Procion Red HE-7B to the microbial biomass. The following amendments were made to these conditions :

a) the sludge inoculum was inactivated by autoclaving at 121^{o}C for 15 min to ensure that any decolourisation observed was a result of adsorption of dye to the biomass and not biological activity;

b) the experiment was performed under sterile conditions to prevent the growth of other microorganisms which could cause decolourisation of the dye; and

c) adsorption of Procion Red HE-7B to the microbial biomass was also measured in a saline solution which was known to have no decolourising action. This was used as a control to determine the influence of the minerals salts medium on adsorption of Procion Red HE-7B to the biomass.

To determine whether the adsorbed portion of Procion Red HE-7B was degraded in the standard assay system, methanol extractions were carried out using : (a) autoclaved biomass from the adsorption experiment, to determine the concentration of adsorbed dye that could be extracted from the biomass; and (b) active biomass used to degrade Procion Red (soluble) to below 5 mg/M, to determine whether any dye would be released from the cells of active microorganisms.

The biomass mixtures were centrifuged at 5 000 rpm for 20 min to remove any liquid and the cell pellets re-suspended in 50 mM of methanol. The biomass-methanol mixture was left to stand for 30 min at ambient temperature, after which time the methanol was remove by centrifugation at 5 000 rpm for 20 min.

Procion Red HE-7B adsorption isotherm

The method used to determine an adsorption isotherm for Procion Red HE-7B was based on that of Shaul

Experimental procedure

Sludge adsorbent was prepared by freeze drying biomass from the Procion Red HE-7B enrichment digesters. Freeze drying was chosen as the drying method to prevent microbial disruption. A mass of 0,2 g of adsorbent was added to each of sixteen 250 mM erlenmeyer flasks. Five solutions of Procion Red HE-7B were prepared by adding 5, 10, 15, 20 and 25 mg of the commercial dye preparation to 1 M volumetric flasks and making up to volume with distilled water. Dye solution (100 mM) was

added to the erlenmeyer flasks containing the sludge adsorbent so that each concentration of Procion Red HE-7B was represented in triplicate. A single erlenmeyer flask (containing only sludge adsorbent and 100 mM distilled water) was prepared in order to assess any absorption interference due to colour imparted from the sludge. Control flasks contained 100 mM of Procion Red HE-7B solution but no adsorbent. All flasks were incubated on a rotary shaker at 32 ^{o}C for 24 h.

Analytical procedure

Samples were withdrawn after 24 h, centrifuged at 6 500 rpm for 20 min and the supernatant analysed at 520 nm for absorption intensity. These results were converted into dye concentration (mg/M) from a standard curve (Appendix C). The results were analysed according to the Freundlich and Langmuir adsorption isotherms.

Decolourisation in the mineral salts medium

Standard Assay conditions were followed, as given in Appendix C, with one amendment i.e. no inoculum was added to the assay bottles. These serum bottles contained only mineral salts medium (sterile), glucose and Procion Red HE-7B (100, 150 and 200 mg/M) so that the decolourising effect of the mineral salts medium could be assessed.

4.2.4 Identification and Fate of Procion Red HE-7B Degradation Products

This section of experimentation investigated the following :

a) that decolourisation of Procion Red HE-7B occurred through azo bond cleavage and resulted in degradation products such as those shown in Fig 4.2; and

b) whether these compounds were mineralised during a 52 d incubation period.

Azo bond cleavage was investigated using ultraviolet scanning techniques. Mineralisation of the degradation products was investigated as a continuation of the anaerobic toxicity assay (Section 4.2.5), by extending the incubation period to 52 d and monitoring the serum bottles for gas production. The aim of this study was firstly, to determine whether mineralisation of Procion Red HE-7B did occur during the incubation period and, secondly, to compare the ability of acclimated and unacclimated biomass to mineralise the degradation products.

Cleavage of Procion Red HE-7B azo bonds

Standard assay conditions (Appendix C) were followed to prepare an experiment to measure decolourisation of 100 mg/M of Procion Red HE-7B.

Analytical procedure

Samples were prepared according to Appendix C. The ultraviolet spectra of the samples were determined by scanning the samples for absorbance from 190 nm to 600 nm using a Varian MS-200 spectrophotometer. The results obtained were qualitative and depicted cleavage of the azo bonds with an increasing level of decolourisation.

Mineralisation of Procion Red HE-7B degradation products

The experimental serum bottles used in the anaerobic toxicity assay (as described in Section 4.2.5) were incubated for a period of 52 d. During incubation total digester gas was measured by the method of Owen et al. (1979) in which a hypodermic needle connected to a ground glass syringe was inserted through the rubber septum of each of the serum bottles, allowing gas to be expelled into the syringe until the bottles had equilibrated to atmospheric pressure.

Total gas produced (mM) was measured to determine whether the serum bottles containing Procion Red HE-7B had significantly greater gas production than the control bottles containing no dye, which would indicate mineralisation of Procion Red HE-7B degradation products. The theoretical gas yield for mineralisation of Procion Red HE-7B degradation products was calculated to determine the volume of digester gas which would be expected for mineralisation of Procion Red HE-7B.

4.2.5 Inhibitory Effects of Procion Red HE-7B on an Anaerobic Microbial Population

The inhibitory effects (if any) of Procion Red HE-7B and/or Procion Red HE-7B degradation products on an anaerobic microbial population was investigated by the following anaerobic toxicity assay method :

Experimental procedure

Five concentrations of Procion Red HE-7B were chosen for the toxicity assay, namely, 20, 50, 100, 200 and 500 mg/M. A defined medium consisting of mineral salts, trace elements and vitamins (Appendix C) was prepared and a known mass of dye (dry weight) added to five separate aliquots of assay medium to give concentrations of 20, 50, 100, 200, and 500 mg/M. Inoculum for the toxicity assay was obtained either from the Procion Red HE-7B enrichment digesters (acclimated biomass) or a laboratory digester containing sludge from Umbilo and Umzinto Waste-water Treatment Works (unacclimated biomass).

The anaerobic toxicity assay was performed in serum bottles (120 mM) (Aldrich Chemical Company). Acclimated or unacclimated inoculum (30 mM) was added to each serum bottle and the volume in the serum bottles was made up to 100 mM by adding 70 mM of assay medium (containing Procion Red HE-7B) to give final concentrations of 20, 50, 100, 200 and 500 mg/M. Control bottles were prepared by adding 30 mM of acclimated or unacclimated biomass to serum bottles (120 mM, Aldrich Chemical Company) and making up to a volume of 100 mM with assay medium, no dye was added to the control bottles. The bottles were overgassed at a flow rate of 0,5 M/min for 15 min with high purity nitrogen (Fedgas). The serum bottles were sealed with butyl rubber caps and aluminium crimp seals and 4 mM of acetate-propionate solution was added by hypodermic needle and syringe to give 75 mg acetate and 26,5 mg propionate per bottle. After 1 h of incubation the pressures inside the serum bottles were equilibrated to atmospheric by insertion of a hypodermic needle through the butyl rubber septa.

Gas production from degradation of acetate and propionate was found to be limited and unsuitable for obtaining accurate gas volume measurements. For this reason glucose (100 mg) was added to the

serum bottles in addition to the acetate and propionate.

Analytical procedure

Two analytical methods were followed. The first measured the incremental volume of gas produced and the second the percentage methane in the incremental gas volume.

Gas volume

Digester gas produced was measured daily by insertion of a hypodermic needle (20-gauge) and syringe through the butyl rubber septum of each serum bottle. The syringe was initially flushed with nitrogen gas and lubricated with distilled water. Readings were taken at the incubation temperature with the syringe held vertically. Volume determinations were made by allowing the syringe plunger to move freely and equilibrate between the bottle and atmospheric pressure. Gas was exhausted after each measurement.

Percentage methane in the digester gas

The percentage methane content of the digester gas was measured using a Varian (3 300) gas chromatograph equipped with a thermal conductivity detector (TCD) which was used to detect methane, carbon dioxide and nitrogen, and a Varian (3 700) gas chromatograph equipped with a flame ionisation detector (FID) to detect methane. A stainless steel packed column (poropak N, 6' by 1/8", 80 to 100 mesh) was used for separation of methane, carbon dioxide and nitrogen with the following conditions :

<u>Conditions for the Varian 3 300 and TCD</u>

Column oven	:	50 °C
Detector	:	200 °C
Filaments	:	250 °C
Injector	:	100 °C
Range	:	10^{-9}
Attenuation	:	64

<u>Conditions for the Varian 3 700 and FID</u>

Column oven	:	50 °C
Detector	:	100 °C
Injector	:	100 °C
Flow rate (nitrogen)	:	15 mM/min
Range	:	10^{-9}
Attenuation	:	64

The residence times of methane and carbon dioxide in the column were approximately 0,53 and 1,81 min respectively, although, water vapour present in the gas samples required a run time of 20 min to be released from the column under these operating conditions. A temperature ramp was, therefore, used to remove the water vapour and the following analytical method was developed:

Initial column oven temperature : 50 °C

Hold time	:	2 min
Temperature ramp to 100 °C		
Rate	:	10 °C/min
Hold time	:	5 min

Samples of digester gas were withdrawn from the serum bottles (directly after the bottles had been equilibrated to atmospheric pressure) by inserting the needle of a gas-tight syringe (100 μM) through the butyl rubber septa of the serum bottles and withdrawing 70 μM of headspace gas. A volume of approximately 20 μM of gas in the syringe was wasted to achieve an accurate volume of 50 μM, which was injected into the gas chromatograph. Peak area was recorded on a Varian integrator with the attenuation set at 64 and the chart speed at 0,5 cm/min.

Calibration curves were prepared by injecting increasing volumes of high purity methane (Fedgas) and plotting the peak areas versus volume (μM) of methane injected. The peak area obtained for methane in the digester gas was related to a volume of 100 % methane by use of the calibration curve. This volume was converted to a percentage (v/v).

4.3 RESULTS

This section presents results obtained from laboratory investigation of Procion Red HE-7B decolourisation in a biological anaerobic system. The sections begin with the results of the enrichment programme with respect to the rate of decolourisation (Section 4.3.1). Section 4.3.2 presents results from the investigation of the nature of biological decolourisation and Section 4.3.3 results from the identification and fate of the degradation products of azo reduction. The final section contains results from an anaerobic toxicity assay conducted with Procion Red HE-7B. All results are discussed in the general discussion section (Section 4.4).

4.3.1 The Effect of Prior Exposure of Anaerobic Biomass to Procion Red HE-7B on the Rate of Decolourisation.

Fig 4.4 shows that prior exposure of the anaerobic biomass to Procion Red HE-7B in the enrichment digesters did not increase the rate of decolourisation of Procion Red HE-7B. The rate constants for decolourisation by acclimated and unacclimated biomass were - 0,289/h and - 0, 310/h, respectively which shows a slightly lower rate of decolourisation for the acclimated biomass. Experimental data are presented in Appendix D.

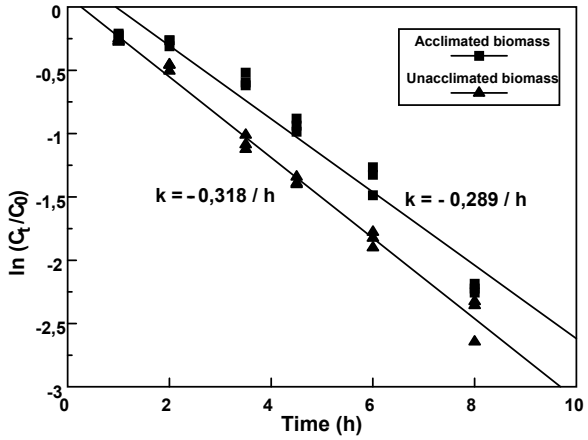

Fig 4.4 : Rate constants for decolourisation of Procion Red HE-7B by acclimated and unacclimated biomass with standard assay conditions.

4.3.2 Biological Decolourisation of Procion Red HE-7B

The results of the investigation of the nature of biological decolourisation of Procion Red HE-7B in the standard assay system are presented in the following order. The first experiment determined the order of decolourisation with respect to dye concentration. From these results the following possible rate-determining factors were chosen for investigation : cell permeability, presence of electron donor i.e. glucose; and presence of additional oxidising agents (nitrate or sulphate). The final experiment measured changes in redox potential in the standard assay system during decolourisation.

Experimental data are presented in Appendix D.

The order of Procion Red HE-7B decolourisation with respect to dye concentration

The exponential regressions of Procion Red HE-7B (mg/M) versus time (h), for initial dye concentrations of 100, 150 and 200 mg/M, are plotted in **Fig 4.5.** To account for the decolourising action of the mineral salts medium (Section 4.3.3) in which the experiment was performed, decolourisation during the first 15 min was attributed solely to chemical decolourisation and Co was taken as the concentration of Procion Red HE-7B after 15 min of incubation. The exponential curves suggest a first-order relationship of Procion Red HE-7B decolourisation versus time, with respect to (soluble) dye concentration. That is, the rate of dye degradation at any time was directly dependent on the concentration of dye in the system.

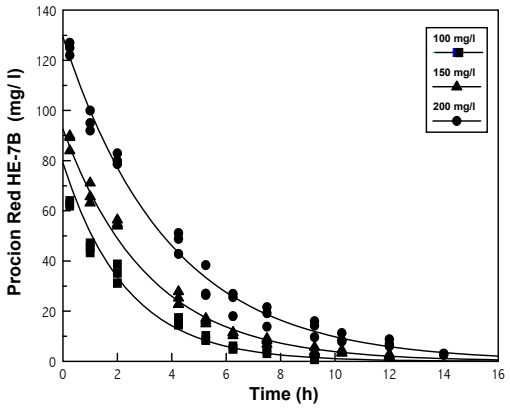

Fig 4.5 : Exponential regression of Procion Red HE-7B (mg/M) with time (h) showing possible first order reaction with respect to dye concentration. Decolourisation is shown with initial dye concentration of 100, 150 and 200 mg/M.

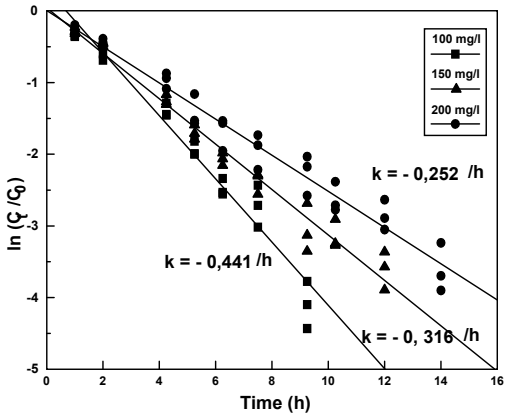

Fig 4.6 : ln (C_t/C_o) versus time (h) is plotted for initial dye concentrations of 100, 150 and 200 mg/M, confirming that Procion Red HE-7B decolourisation could be first-order with respect to dye concentration.

To confirm whether decolourisation of Procion Red HE-7B was first order with respect to dye concentration, ln (C_t/C_o) [where C_t is the dye concentration at time t and C_o the dye concentration at time zero] was plotted against time (h). This plot was expected to be linear if the reaction was first order with respect to dye concentration. As can be seen in **Fig 4.6**, linear relationships of ln (C_t/C_o) versus time were obtained for all the intial dye concentrations and the rate constant (k) was calculated from the slope of the line. The rate constant for a first-order reaction should be independent of initial concentration, as the half-life is independent of the initial concentration (Brown, 1988). However, **Fig 4.6** shows decreasing values of k and, therefore, decreasing rates of decolourisation) with increasing initial dye concentrations.

Procion Red HE-7B decolourisation with permeabilised cells

Cell permeability was investigated as a rate-limiting factor in the decolourisation of Procion Red HE-7B. Cells were permeabilised by the method of Meschner and Wuhrmann (1982) and permeabilised cells were re-suspended in :

a) the aqueous phase of the permeabilisation mixture. The reason for this was that Meschner and Wuhrmann found that permeabilised cells, re-suspended in this mixture, decolourised dyes at a much higher rate than cells re-suspended in phosphate buffer; and

b) phosphate buffer (0,025 M).

Non-permeabilised cells were used as a control for this experiment and were suspended in phosphate buffer (0,025 M).

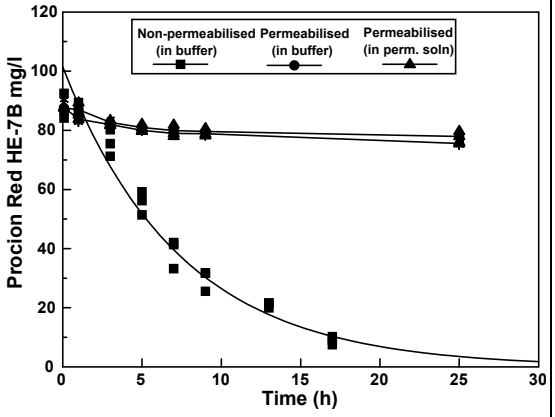

Fig 4.7 : Decolourisation of Procion Red HE-7B (100 mg/M) with inoculum consisting of : cells permeabilised and suspended in phosphate buffer; cells permeabilised and suspended in perm. solution and non-permeabilised cells in phosphate buffer.

Table 4.1 : Gas production (cumulative) from non-permeabilised and permeabilised cells.

Prior to addition of Procion Red HE-7B			
Time (h)	**N-P**	**P-B**	**P**
18	4	4	4
Subsequent to addition of Procion Red HE-7B, biomass washed and suspended in mineral salts medium + glucose			
24	12.5	0	0
48	20.5	8	0
120	25.5	18.5	0

N-P = non-permeabilised biomass

P-B = permeabilised biomass suspended in buffer

P = permeabilised biomass suspended in permeabilisation liquid

Fig 4.7 shows that decolourisation of Procion Red HE-7B by the non-permeabilised cells was exponential over time. The permeabilised cells did not show any marked decolourisation of the dye which suggested that the permeabilisation process could have had a detrimental effect on the microorganisms. However, gas produced by the permeabilised microorganisms during pre-incubation (**Table 4.1**) indicated that the permeabilised microorganisms were metabolically active prior to addition of Procion Red HE-7B. To determine whether addition of the dye had inhibited the permeabilised microorganisms, the permeabilised biomass was centrifuged (to remove the soluble dye remaining in the serum bottles after 25 h of incubation) and washed with phosphate buffer (0,025 M) (to remove any dye adhering to the microbial cells). It must be noted that the permeabilised biomass had to be washed five times to remove all visible dye from the cells whereas two washes had previously been found adequate to remove adsorbed dye from autoclaved (non-permeabilised) biomass. The washed biomass was re-suspended in mineral salts medium and glucose and monitored for gas production. These results are presented in **Table 4.1** and show that the permeabilised cells did not produce any gas for the first 48 h whereas the non-permeabilised cells produced 20 mM of gas during the initial 48 h of incubation. The permeabilised cells originally suspended in buffer were able to resume metabolic activity after 48 h of incubation, producing 8 mM of gas, although those cells, originally suspended in the aqueous phase of the permeabilisation mixture, did not show any signs of metabolic activity during the 120 h incubation period.

Rate of decolourisation of Procion Red HE-7B as a sole carbon source

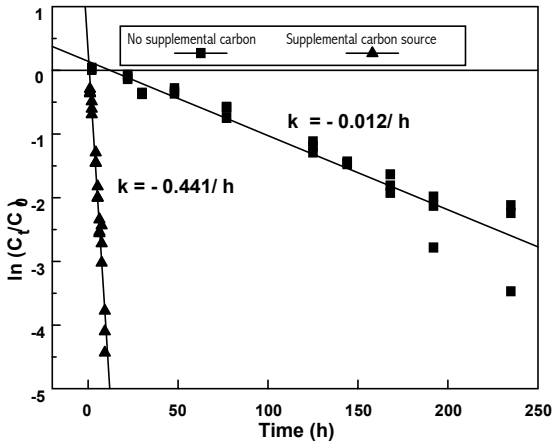

Fig 4.8 : Decolourisation of Procion Red HE-7B (100 mg/M) in the presence and absence of a supplemental carbon source (glucose, 1 g/M).

Fig 4.8 shows that decolourisation of Procion Red HE-7B could occur in the standard assay system when the dye was present as the sole carbon source and that this reaction could be related to first-order kinetics. However, this reaction proceeded at a very low rate when compared with the rate of decolourisation in assay bottles containing glucose at 1 g/M. The rate constant (k) measured without glucose was only 2,72 % of that measured in the presence of glucose. No gas production was measured during the time period required for decolourisation (or for 5 days after decolourisation was complete) although gas bubbles were noted in the biomass.

Rate-limiting effects of additional electron acceptors

Two electron acceptors, nitrate and sulphate, were used to determine the effect of the presence of an additional electron acceptor (i.e. other than Procion Red HE-7B) on the rate of decolourisation of Procion Red HE-7B.

Nitrate as an additional electron acceptor

Fig 4.9 shows how the presence of nitrate in the standard assay system inhibited decolourisation of Procion Red HE-7B for a period of time which will be termed the *lag phase*. The duration of these *lag phases* were directly related to the concentration of nitrate in the system, which is well illustrated by comparing the duration of the *lag phase* in the presence of 5 mM nitrate (approximately 12 h) to that in the presence of 10 mM nitrate (approximately 25 h). This two-fold increase in nitrate concentration resulted in a doubling of the *lag phase*. The close correlation between nitrate concentration and the duration of the lag phase (in which no Procion Red HE-7B reduction occured) suggests that nitrate reduction was occurring preferentially to reduction of Procion Red HE-7B in the standard assay system.

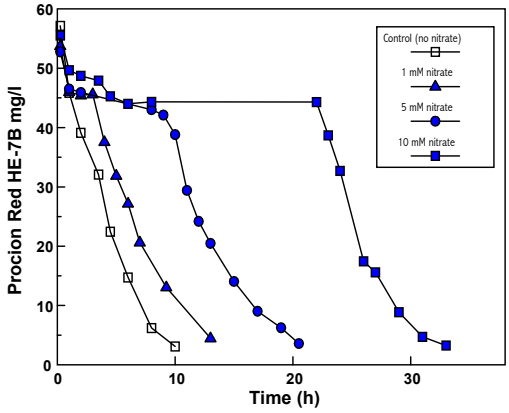

Fig 4.9 : Procion Red HE-7B decolourisation in the presence of 0, 1, 5 and 10 mM nitrate showing *lag phases* before the onset of exponential decolourisation, the duration of which corresponded to the concentration of nitrate present.

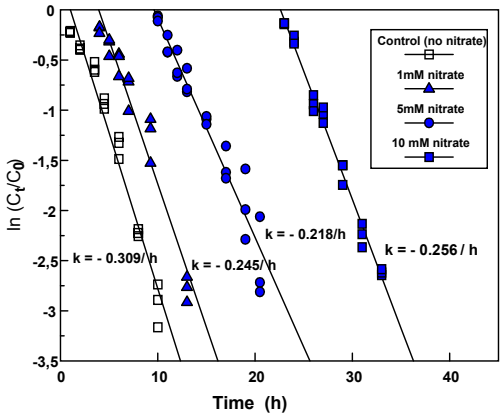

Fig 4.10 : ln (C_t/C_o) versus time plotted for decolourisation of Procion Red HE-7B in the presence of 0, 1, 5 and 10 mM nitrate. Time zero was taken as the sampling time recorded directly before the onset of decolourisation.

Once decolourisation of Procion Red HE-7B commenced, an exponential relationship of dye concentration versus time was observed **(Fig 4.10)**. The control (no nitrate) had the highest rate of decolourisation while the rate constants for the nitrate-containing samples were similar, irrespective of the initial nitrate concentration.

Sulphate as an additional electron acceptor

The addition of sulphate to the serum bottles containing Procion Red HE-7B had no effect on the rate of decolourisation. This was demonstrated by the fact that there was no significant difference in reaction rates for the control bottles (no sulphate) and those containing 5 or 10 mM sulphate **(Fig 4.11)**.

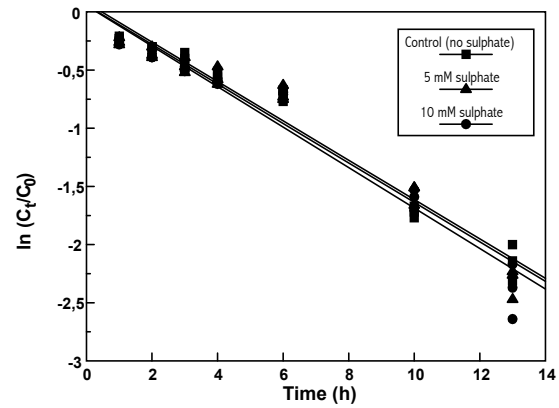

Fig 4.11 : Decolourisation of Procion Red HE-7B in the presence of 0, 5 and 10 mM sulphate.

Trends in redox potential for microbial decolourisation of Procion Red HE-7B

The redox potential of an anaerobic digester was monitored during decolourisation of Procion Red HE-7B (100 mg/M) when Procion Red HE-7B was present as the sole electron acceptor, or in the presence of nitrate (20 mM) and sulphate (5 mM). **Fig 4.12** shows the decolourisation of Procion Red

HE-7B when the dye was present as a sole electron acceptor. The redox potential of the anaerobic system was monitored continuously during decolourisation and for approximately 20 h subsequent to decolourisation. The results show that the redox potential of the system decreased from approximately - 375 mV (addition of Procion Red HE-7B) to approximately - 475 mV by the end of the 5 h decolourisation period. A redox potential reading of approximately - 475 mV was maintained until 12,5 h of incubation, at which point the redox potential of the system can be seen to increase fairly rapidly to approximately - 450 mV. This reading then remained steady until the end of the monitoring period (22,5 h).

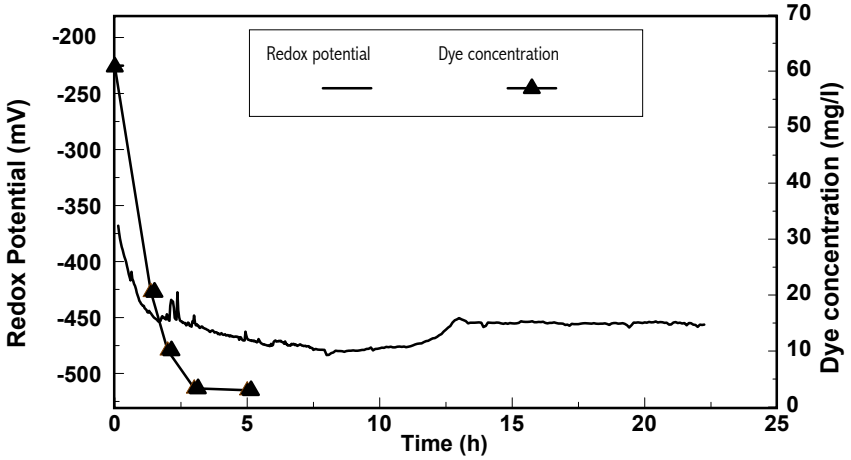

Fig 4.12 : Redox potential (mV) measured in an anaerobic digester during decolourisation of Procion Red HE-7B (approximately 5 h) and for 20 h subsequent to the completion of decolourisation.

Fig 4.13 Shows the redox potential (mV) and Procion Red HE-7B concentration (mg/M) for an anaerobic digester containing Procion Red HE-7B and nitrate. The redox potential of the system (prior to addition of dye and nitrate) was approximately - 475 mV. An initial colour loss was recorded in the first five hours subsequent to addition of dye and nitrate to the digester, during which time the redox potential remained below - 400 mV. Once the redox potential increased to approximately - 375 mV, decolourisation appeared to be inhibited. The redox potential continued to rise to - 225 mV, at which point the readings remained steady (within a range of - 225 to - 200 mV) for approximately 40 h of incubation. During this time no decolourisation was recorded and it may be speculated that nitrate reduction coincided with this redox potential plateau. It must also be noted that the duration of the *lag phase* was approximately 40 h (i.e. 2 h per mM nitrate) which was consistent with results shown in section 4.3.2. After 40 h of incubation the redox potential of the system decreased sharply to reach - 450 mV within 3 h. Once the redox potential decreased below - 450 mV decolourisation of Procion Red HE-7B was initiated. Decolourisation was achieved in approximately 5 h during which time the redox potential decreased to - 500 mV.

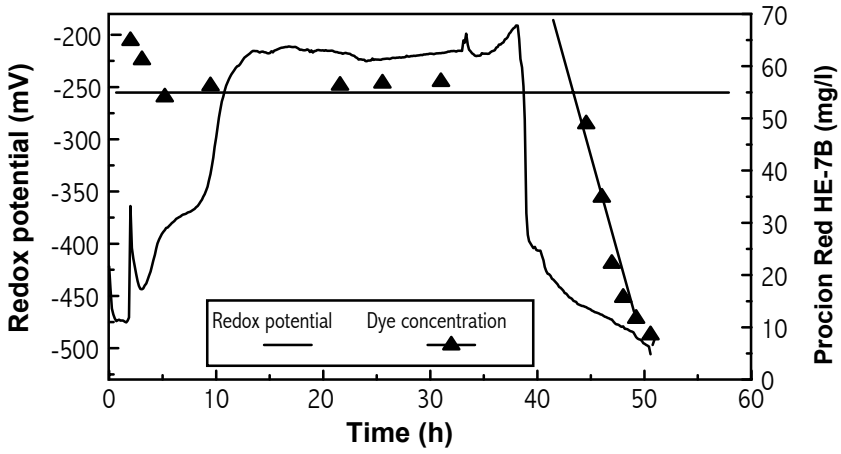

Fig 4.13 : Redox potential (mV) measured in an anaerobic digester containing nitrate (20 mM) and Procion Red HE-7B (100 mg/M).

Fig 4.14 shows the change in residual dye concentration and Eh, for an anaerobic system containing Procion Red HE-7B (100 mg/M) and sulphate 5 mM . The redox potential of the system was fairly high (- 300 mV) before addition of the dye. Subsequent to addition of Procion Red HE-7B and sulphate the redox potential decreased rapidly to - 450 mV. Decolourisation of the dye was completed in approximately 6 h and the redox potential remained steady at - 450 mV until 15 h of incubation at which point a sharp decrease in potential was recorded (below - 500 mV). An increase in redox potential was recorded after approximately 20 h of incubation and the Eh of the system remained steady at approximately - 475 mV for the next 50 h.

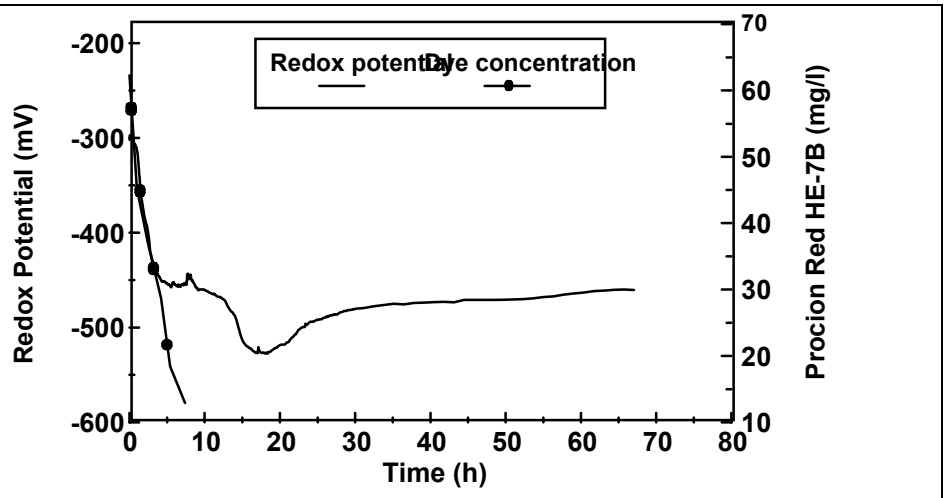

Fig 4.14 : Redox potential (mV) measured in an anaerobic digester containing sulphate (5 mM) and Procion Red HE-7B (100 mg/M).

4.3.3 Abiotic Decolourisation of Procion Red HE-7B

This section presents the results from an investigation of possible abiotic mechanisms of decolourisation that could occur in the standard assay system. The mechanisms investigated can be divided into :

a) adsorbance; and

b) decolourisation of the dye in the mineral salts medium.

Experimental data for this section are presented in Appendix E.

Asorption of Procion Red HE-7B with anaerobic biomass as the adsorbent

Adsorption of Procion Red HE-7B was investigated with respect to the extent of adsorption occurring in the standard assay system and the fate of the adsorbed dye. Adsorption was also investigated with the aim of developing an adsorption isotherm for Procion Red HE-7B, with digester sludge as the adsorbent.

Adsorption of Procion Red HE-7B in mineral salts medium and saline solution

Fig 4.15 shows that adsorption of Procion Red HE-7B to the inactivated (autoclaved) biomass did occur although this was not instantaneous and required approximately 6 h to reach equilibrium. An increased level of decolourisation was observed in serum bottles where the adsorbent was incubated with mineral salts medium, in comparison with the serum bottles in which the adsorbent was incubated with saline solution. Control bottles (results not shown) in which Procion Red HE-7B (100 mg/M) was incubated with either sterile mineral salts medium or sterile saline solution gave similar results, indicating that the increased colour removal observed in the mineral salts medium was due to a component of the medium and not to increased adsorption of the dye to the biomass adsorbent.

Once it had been established that adsorption of Procion Red HE-7B to the biomass in the standard assay system was responsible for a percentage of the recorded decolourisation it was neccessary to determine the fate of the adsorbed dye in an actively decolourising system i.e. whether the adsorbed dye was, subsequently, decolourised or could be extracted from the biomass after decolourisation of the soluble dye was completed. A second experiment was, therefore, performed to determine whether dye could be extracted from :

a) inactivated biomass adsorbent; and

b) biomass that had actively decolourised a solution of Procion Red HE-7B (100 mg/M).

Methanol extraction of the autoclaved sludge resulted in some dye removal whereas extraction of the active sludge showed no release of dye from the biomass. The results were obscured by a strong yellow colour which was extracted from both types of inoculum and, therefore, only represented a qualitative experiment. In fact, more successful results were obtained by washing the biomass with saline solution as a considerable amount of dye was released from the autoclaved sludge, whereas no dye was released from the active sludge. Although this was only a qualitative experiment the results

indicated that the adsorbed portion of Procion Red HE-7B was probably decolourised. Decolourisation by adsorbance was, therefore, not dissociated from biological decolourisation of Procion Red HE-7B in rate-determining experiments.

Development of an adsorption isotherm for Procion Red HE-7B

Fig 4.16 is the plot of Procion Red HE-7B adsorbed/mass of adsorbent versus the equilibrium concentration of Procion Red HE-7B in solution. This plot shows a typical pattern of monolayer adsorption i.e. a steep portion with relatively low solution concentrations (when more adsorption sites were available than could be filled by adsorbate) followed by a less steeply rising curve as the solution concentrations increased. The amount of dye adsorbed reached a maximum value at moderate concentrations (17 to 20 mg/M) and remained constant with a further increase in dye concentration, as the adsorbent was saturated.

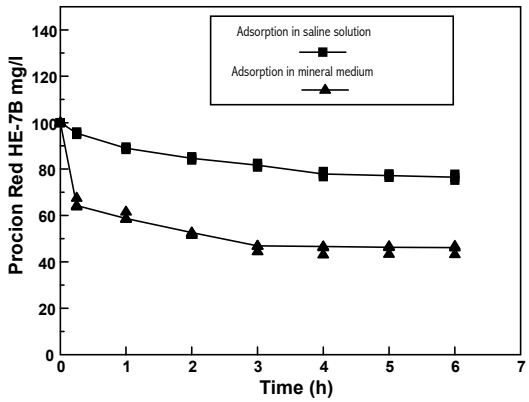

Fig 4.15 : Adsorption of Procion Red HE-7B to inactivated (autoclaved) biomass in saline solution and mineral salts medium.

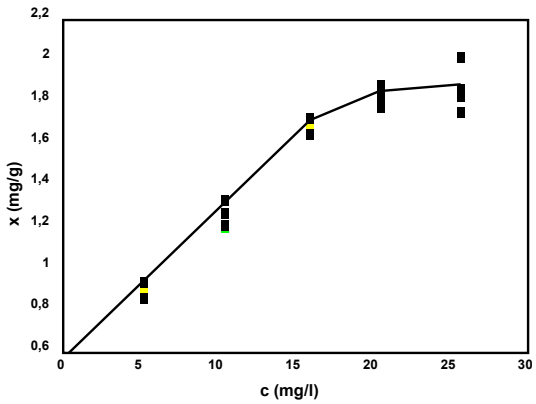

Fig 4.16 : Procion Red HE-7B adsorbed per mass of sludge adsorbent plotted versus the equilibrium concentration of Procion Red HE-7B in solution.

Fig 4.17 shows the linear relationship between x (mg/g) and c (mg/M) when Procion Red HE-7B adsorption data is represented by the logarithmic form of the Freundlich adsorption isotherm equation. The linear regression has an R-Square value of 0,95541. The slope of the line (b) is 0,50469 and indicates adsorption intensity. The y-intercept (a) is - 0,96092 and indicates adsorption capacity. **Fig 4.18** shows the linear relationship of c/x versus c when Procion Red HE-7B is represented by the Langmuir adsorption isotherm. The linear regression has an R-Square value of 0,95729. The slope of the line (K) is 0,1849 and the y-intercept (k) is 2,06165.

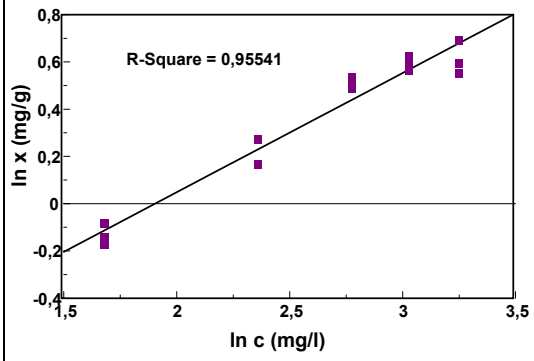

Fig 4.17 : Log-log plot of the Freundlich adsorption isotherm.

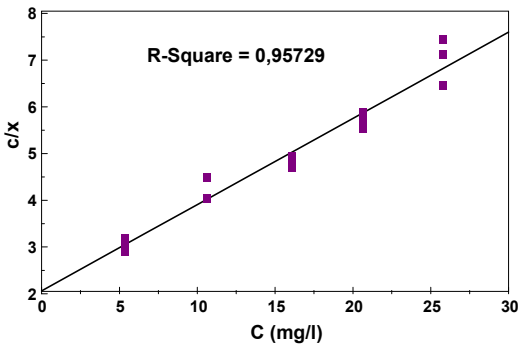

Fig 4.18 : The Langmuir adsorption isotherm.

Decolourisation in the mineral salts medium

As shown in Fig 4.19, approximately 35 % of the dye was removed by contact with the mineral salts medium. Since this colour removal remained constant and occurred instantaneously this form of decolourisation, although accounted for in a colour balance, was excluded from the results when calculating the order and rate of dye degradation by the microbial inoculum.

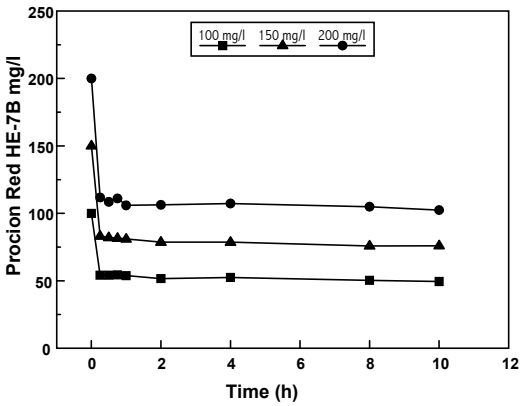

Fig 4.19 : Decolourisation of Procion Red HE-7B in sterile mineral salts medium. Initial dye concentrations of 100, 150 and 200 mg/M.

4.3.4 Identification and Fate of Procion Red HE-7B Degradation Products

The objectives of this study were two-fold. Firstly, to determine whether azo reduction was responsible for decolourisation of Procion Red HE-7B in an anaerobic biological system and secondly, to determine the fate of these degradation products in the anaerobic system during an extended incubation period of 52 d.

All calculations and raw data for this section of experimental results are presented in Appendix F.

Experiments to determine whether azo reduction was responsible for biological decolourisation of Procion Red HE-7B

Fig 4.20 shows the results of ultraviolet scanning of samples taken from anaerobic serum bottles prepared according to the standard assay procedure. The samples taken prior to decolourisation exhibited a broad peak (475 to 600 nm) which corresponded to the azo bonds of the dye chromophore. Samples taken subsequent to decolourisation no longer exhibited a peak in this region of the spectrum, indicating that decolourisation of Procion Red HE-7B in the standard assay system occurred through reduction and consequent splitting of the azo bonds in the dye chromophore.

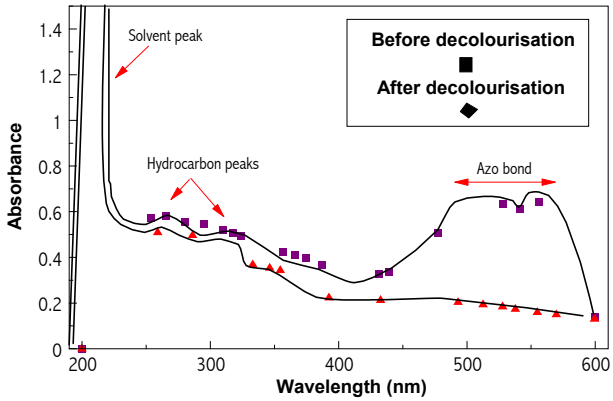

Fig 4.20 : Scans of samples from serum bottles containing Procion Red HE-7B, before and after decolourisation.

Fate of Procion Red HE-7B degradation products in an anaerobic system

Solutions of Procion Red HE-7B (20, 50, 100, 200 and 500 mg/M) were incubated with either acclimated or unacclimated biomass for a period of 52 d. At the initiation of the incubation period glucose, acetate and propionate were added as substrate for the biomass. **Fig 4.21** shows the total gas production during 52 d of incubation for the serum bottles containing 0 (control), 20, 50, 100, 200 and 500 mg/M of Procion Red HE-7B inoculated with either acclimated or unacclimated biomass. The total gas produced by the acclimated biomass was far greater than that by the unacclimated biomass but this could not be attributed to dye inhibition of the unacclimated biomass as the unacclimated control bottles produced on average similar volumes of gas compared with the bottles containing Procion Red HE-7B. No significant difference in total gas production could be seen between the control bottles and those incubated with Procion Red HE-7B (acclimated biomass), indicating that mineralisation of the Procion Red HE-7B degradation products did not occur when incubated anaerobically for 52 d with acclimated biomass.

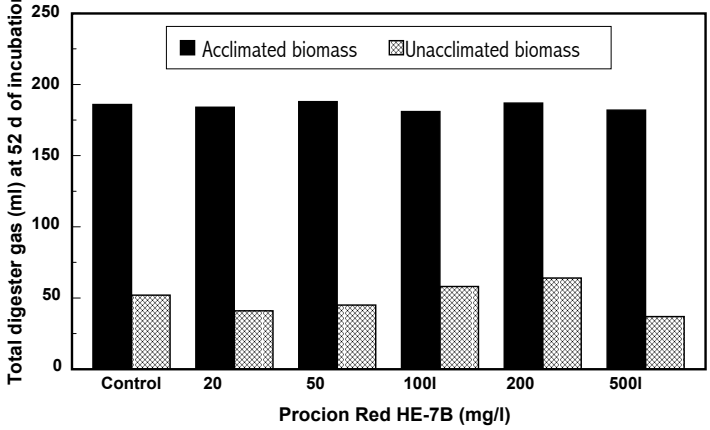

Fig 4.21 : Total gas produced by acclimated and unacclimated biomass during 52 d of incubation.

Calculations of theoretical gas production for mineralisation of Procion Red HE-7B **(Table 4.2)** show that digester gas volumes of 3,7 to 92,5 mM would be expected for mineralisation of Procion Red HE-7B. At the lower volumes it would be impossible to distinguish mineralisation of Procion Red HE-7B from experimental variation, although, at the higher assay concentration (500 mg/M assay bottles) a significant volume of gas would be expected as a result of Procion Red HE-7B mineralisation and could be easily detected by the analytical techniques employed.

It may be argued that mineralisation of the dye would not be expected in the presence of labile carbon sources unless this was a co-metabolic process. As the additional substrates were exhausted by the acclimated biomass at approximately 37 d this would mean that the effective incubation period in which mineralisation could have occured was only 15 d. Therefore, the possibility of mineralising Procion Red HE-7B in an anaerobic system cannot be overruled without additional investigation. As the unacclimated biomass did not exhaust the additional sources of substrate in the 52 d incubation period no conclusions can be made as to the mineralising ability of this biomass for Procion Red HE-7B.

Table 4.2 : Theoretical gas volumes for mineralisation of Procion Red HE-7B in assay bottles.

	20 mg/M	**50 mg/M**	**100 mg/M**	**200 mg/M**	**500 mg/M**
Methane (mM)	2.43	6.05	12.17	24.33	60.83
Carbon dioxide (mM)	1.27	3.18	6.35	12.7	31.75
Total volume (mM)	**3.7**	**9.26**	**18.52**	**37.03**	**92.58**

4.3.5 Inhibitory Effects of Procion Red HE-7B on an Anaerobic Microbial Population

The objectives of this study were two-fold: to determine whether Procion Red HE-7B was inhibitory to the anaerobic biomass of the standard assay; and to determine whether prior exposure of the biomass to Procion Red HE-7B increased the resistance of the acclimated biomass to the inhibitory effects of the dye. An anaerobic toxicity assay was performed to achieve these objectives. The toxicity assay consisted of a range of five concentrations of Procion Red HE-7B viz. 20, 50, 100, 200 and 500 mg/M which were incubated with either acclimated or unacclimated biomass (fed with

acetate, propionate and glucose) in anaerobic serum bottles. During incubation gas production in the serum bottles was monitored to determine whether the presence of Procion Red HE-7B was inhibitory to the methanogenic population.

Figs 4.22 and **4.23** show gas production (cumulative) for acclimated and unacclimated biomass respectively, during the 52 d incubation period. To calculate the levels of inhibition for samples containing Procion Red HE-7B, the maximum rate of gas production for each sample had to be calculated over the same time period. The time periods chosen for computation of these rates are shown between the vertical lines in Fig's 4.22 and 4.23, and the plots used to calculate these rates are shown in **Figs 4.24** and **4.25.**

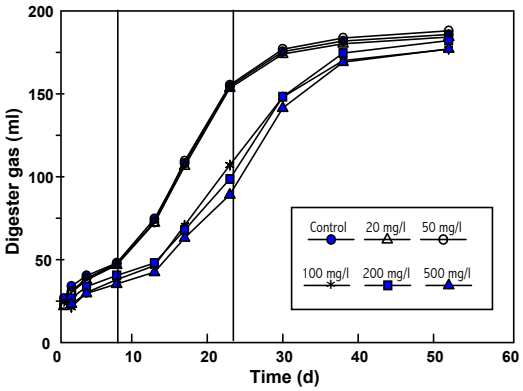

Fig 4.22 : Cumulative gas production for serum bottles inoculated with acclimated biomass and fed with acetate, propionate and glucose. Control bottles contain no Procion Red HE-7B and assay bottles contain 20, 50, 100, 200 and 500 mg/l dye.

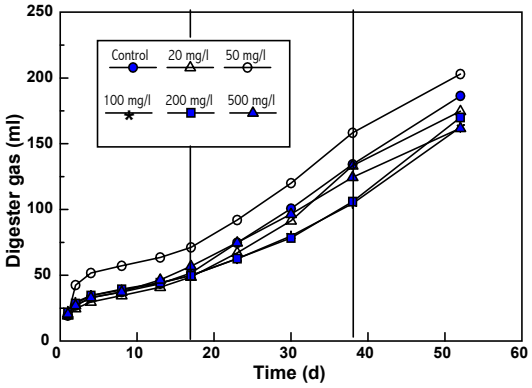

Fig 4.23 : Cumulative gas production for serum bottles inoculated with unacclimated biomass and fed with acetate, propionate and glucose. Control bottles contain no Procion Red HE-7B and assay bottles contain 20, 50, 100, 200 and 500 mg/l dye.

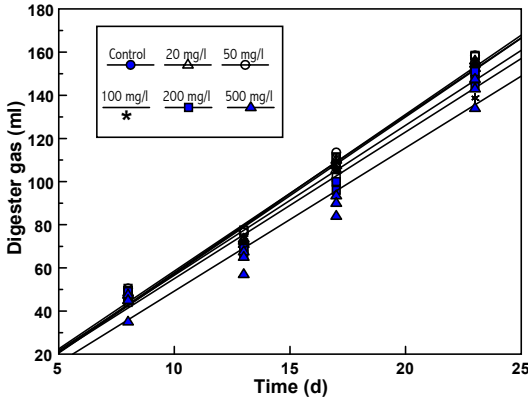

Fig 4.24 : Rates of gas production (acclimated biomass) calculated for the time period when gas production rates were linear i.e. before substrate concentration became limiting.

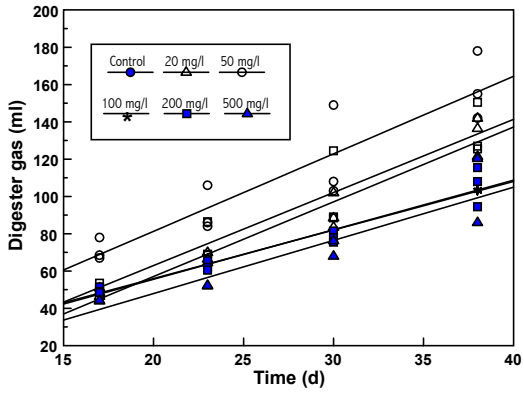

Fig 4.25: Rates of gas production (unacclimated biomass) calculated for the time period when gas production rates were linear i.e. before substrate concentration became limiting.

The maximum rates of gas production for all serum bottles were calculated and the data normalised by computing ratios between respective rates for samples and the controls. This ratio was designated as MRR. A MRR of less than 0,95 suggested possible inhibition, and one less than 0,90 suggested

significant inhibition (Owen et al., 1979). The MRRs calculated for Procion Red HE-7B are shown in **Table 4.3** and **Fig 4.26**.

Table 4.3 : Maximum Rate Ratio's (MRR) for the anaerobic toxicity assay with Procion Red HE-7B

	Maximum Rate Ratio (MRR)	
Procion Red HE-7B (mg/M)	**Acclimated biomass**	**Unacclimated biomass**
Control (0)	1.000	1.000
20.000	1.014	1.023
50.000	1.022	1.060
100.000	0.965	0.664
200.000	0.944	0.678
500.000	0.920	0.727

The MRRs for the anaerobic toxicity assay with Procion Red HE-7B showed that, for biomass previously exposed to the dye, concentrations of 20, 50 and 100 mg/M were not inhibitory. Higher concentrations of Procion Red HE-7B (200 and 500 mg/M) resulted in MRRs of 0,944 and 0,92 respectively which indicated possible inhibition of the anaerobic biomass by the increasingly concentrated dye, although, no significant inhibition of the acclimated biomass was recorded in this toxicity assay. However, for serum bottles containing unacclimated biomass, MRRs indicating significant inhibition were recorded for concentrations of Procion Red HE-7B above and including 100 mg/M. No inhibititory effects were recorded with the lower dye concentrations. These results, therefore, suggested that prior exposure of the biomass to Procion Red HE-7B increased the resistance of this microbial population to inhibitory concentrations of dye.

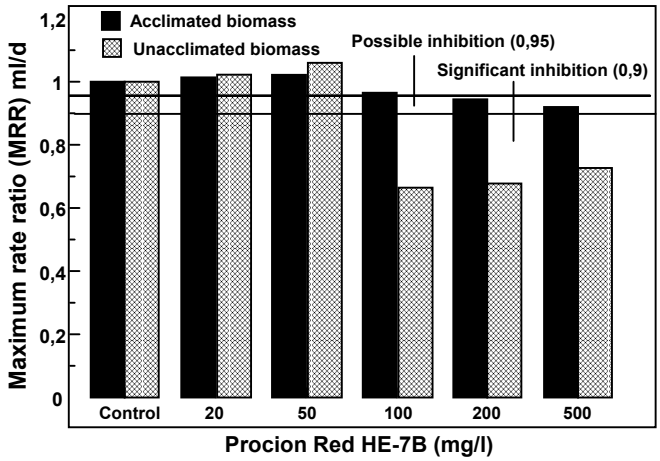

Fig 4.26 : Maximum rate ratio's for anaerobic toxicity assay with Procion Red HE-7B.

Although anaerobic toxicity assays are usually conducted using the analytical criterions of total gas production, the results may be complicated by varying ratios of carbon dioxide to methane, which may cause subtle inhibition effects to be overlooked. For this reason the Procion Red HE-7B toxicity assay aimed to employ methane production data for determination of MRR's, by analysing the

percentage methane in the digester gas. However, due to difficulties with analytical equipment, methane percentages were only measured on 3 days of the assay, viz. days 3, 8 and 30 and methane production data could not be employed for analysis of the assay. The available data has been presented in **Figs 4.27** and **4.28,** and show percentage methane in the digester gas for acclimated and unacclimated biomass respectively.

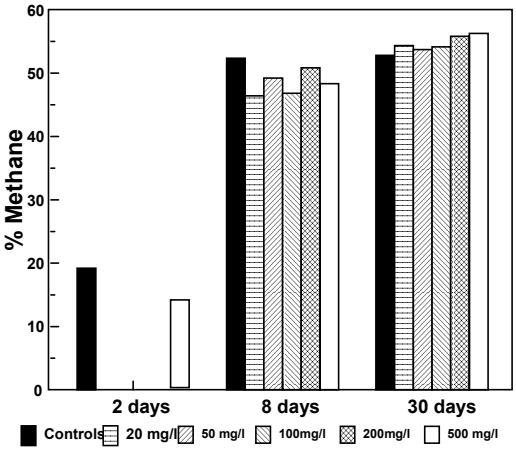

Fig 4.27 : Methane content (%) of digester gas produced in anaerobic toxicity assay with acclimated biomass.

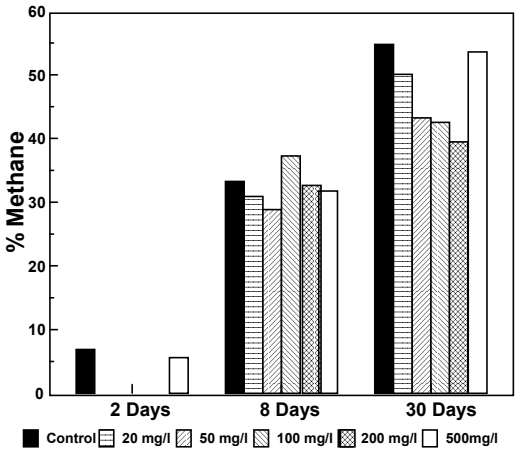

Fig 4.28 : Methane content (%) of digester gas produced in anaerobic toxicity assay with unacclimated biomass.

Fig 4.27 shows that no marked difference could be detected between the methane content of gas produced in the assay bottles (containing Procion Red HE-7B) and the control bottles containing no dye. **Fig 4.28** does show marked differences in the methane contents for the various samples, although no trend could be discerned from these results.

4.4 DISCUSSION

This section discusses the experimental results in Chapter Four, beginning with the outcome of the enrichment programme, followed by a discussion aimed at elucidating the mechanism of decolourisation of Procion Red HE-7B under anaerobic conditions and ending with a discussion on the role of abiotic decolourisation in the biological system.

4.4.1 Prior Exposure of Anaerobic Microorganisms to Procion Red HE-7B

As outlined in the Section 4.1.1, an enrichment programme for Procion Red HE-7B was performed with the objectives of improving the rate of decolourisation of Procion Red HE-7B, selecting for microorganisms with the ability to mineralise the degradation products from decolourisation of Procion Red HE-7B, and improving the tolerance of the microorganisms to inhibitory concentrations of dye and/or dye degradation products.

Prior exposure of the anaerobic microorganisms to Procion Red HE-7B was expected to improve the rate of decolourisation of the dye, however, the measured rates of decolourisation (Section 4.3.1) indicated that the acclimated microorganisms were less proficient in decolourising the dye than the unacclimated microorganisms. It was, therefore, suggested that prior exposure to Procion Red HE-7B (or the dye metabolites) caused the microorganisms to be stressed, resulting in an overall lowering of the metabolic state of the acclimated microorganisms and thereby slowing the rate of decolourisation.

Alternatively, inoculating the serum bottles by mass (wet weight) of inoculum could have been subject to error if the biomass activities of the acclimated and unacclimated digesters differed significantly. For example, if cell death occurred in the enrichment digester a percentage of the wet weight inoculum was probably inactive. If 10 % of the acclimated inoculum was inactive, the rate of decolourisation by acclimated biomass would only be 90 % of that recorded for the unacclimated biomass (assuming 100 % activity for the unacclimated biomass). As the difference between -0,289 and -0,318 is approximately 10 %, the reaction rates measured would not be considered to differ significantly. It is, therefore, recommended that, for experiments in which different sources of biomass are to be utilised, biomass activity should be determined prior to inoculation by methods such as measurement of dehydrogenase activity.

Although this enrichment programme did not improve the ability of the microorganisms to degrade Procion Red HE-7B, other researchers have reported success in this field. Heiss et al. (1992) reported that previous exposure of an azo reducing *Rhodococcus* strain to a particular azo dye improved the ability of the microorganisms to degrade that dye by stimulating production of an inducible azo reducing enzyme. Haug et al. (1991) showed that enrichment of a bacterial population with a particular sulphonated azo compound increased the ability of this population to decolourise other sulphonated azo dyes. They proposed that this occurred through the development of an inducible membrane transport system which was able to take up sulphonated compounds into the cell. As cell permeability is cited as the principal rate-limiting factor in microbial decolourisation of sulphonated azo dyes (Meschner and Wuhrmann, 1982) this is in keeping with the theory. However, as discussed

in Section 4.4.2, it is debatable whether cell permeability is a major rate limiting factor in the anaerobic decolourisation of Procion Red HE-7B, which could explain why prior exposure of the anaerobic population to Procion Red HE-7B did not increase the rate of decolourisation of the dye.

With respect to the selection of microorganisms capable of mineralising Procion Red HE-7B, experiments in Section 4.3.4 showed that mineralisation of Procion Red HE-7B did not occur in the 52 d incubation period with either acclimated or unacclimated biomass. Although the literature had reported that aromatic compounds such as naphthalene, naphthol and aniline could be degraded under anaerobic/anoxic conditions, a principle difference between these compounds and the degradation products of Procion Red HE-7B is the highly sulphonated nature of the latter. Sulphonation tends to increase the recalcitrant nature of these compounds by decreasing the ability of these compounds to permeate through the microbial cell walls (Meschner and Wuhrmann, 1982; Haug et al., 1991). Microbial degradation of sulphonated naphthalene compounds has been reported by Brilon et al. (1988a,b) but desulphonation was found to be essential before these compounds could be utilised as sources of carbon and energy. As molecular oxygen is essential for oxygenolytic cleavage of the sulphonate bonds, in order to liberate the sulphonate groups as sulphite, it does not seem likely that mineralisation of sulphonated dyes will occur under the strict anaerobic conditions required for decolourisation. Therefore, it is probable that the results achieved in these experiments correctly indicate that mineralisation of Procion Red HE-7B degradation products is unlikely to occur in the anaerobic system responsible for azo reduction. However, any problems with the mineralisation experiment or improvements that could be made to the original enrichment programme must be taken into consideration before drawing any conclusions.

Firstly, with respect to the mineralisation experiment, supplementary labile carbon was present in the growth medium for 75 % of the incubation period, which would probably have been degraded preferentially to the recalcitrant aromatic products unless co-metabolic degradation occurred. Therefore, the absolute time period in which mineralisation of the degradation products was most likely to occur was only 15 d of the total 52 d incubation period. In this time a population shift would have had to occur to favour those microorganisms capable of degrading the degradation products and, in addition, synthesis of suitable catabolic enzymes would have been required if the enzymes were inducible. Only upon completion of these requirements would degradation and possibly, mineralisation, have occurred.

With respect to the enrichment programme, it must be taken into consideration that supplemental labile carbon (originating from the digester sludge inoculum) was present throughout the enrichment period i.e. the microbial population was never exposed to Procion Red HE-7B as a sole carbon source. Therefore, enrichment schemes utilising Procion Red HE-7B as a sole carbon source could have significantly improved results compared with the original enrichment programme. To further increase the efficiency of the enrichment programme, with respect to mineralisation of Procion Red HE-7B, isolation of the various degradation products resulting from azo reduction should be undertaken, and separate enrichment schemes implemented for each degradation product. This would facilitate the development of microbial associations capable of catabolising each degradation product and would

give valuable information such as the relative degradabilities of these compounds and rates of degradation. Some enrichment schemes should target the compound as a sole carbon source and others should include supplementary labile carbon to allow for co-metabolic catabolism of the target compound.

The redox state of the enrichment system should also be taken into consideration, as a methanogenic system may not be optimal for catabolism of the Procion Red HE-7B degradation products. For example, the sulphonated aminonaphthalene compound may be more efficiently catabolised under denitrification conditions than other anaerobic conditions, as was discovered for naphthalene (Mihelcic and Luthy, 1988) whereas the nitroaromatic compound may be degraded under anaerobic conditions with carbon dioxide as the electron acceptor, as was found for aniline (Schnell and Schink, 1991). Nitrogen heterocyclic compounds, such as those forming the reactive groups of Procion Red HE-7B, have been reported to be metabolised under both nitrate reducing and methanogenic conditions (Ronen and Bollag, 1991). Degradation of these compounds in an aerobic system should also be considered as reports by the ETAD (Brown and Laboureur, 1983b) found the biodegradability of primary aromatic amines to be enhanced under aerobic conditions.

Rather than monitoring mineralisation by digester gas production, suitable analytical techniques such as high performance liquid chromatography (HPLC) should be employed to trace the catabolic pathways of the Procion Red HE-7B degradation products. This would elucidate, for example, whether certain products were channelled into *dead-end* pathways, as was found by Kremer (1989) with naphthionic acid. Kulla (1981) also suggested that sulphonated aromatic compounds resulting from cleavage of azo bonds were channelled into *dead-end* pathways. Build-up of these compounds in treatment systems could, therefore, be a serious problem resulting in digester failure if increasing concentrations of these compound were not detected. A simpler approach for monitoring mineralisation would be to assay total organic carbon in the digester, although some researchers have reported difficulties with this method as the readings are often complicated by an overall increase in TOC in the digester, possibly due to cell lysis, which conceals any TOC reduction that could be attributed to catabolism of the dye (Ganesh et al., 1992).

Therefore, successful mineralisation of Procion Red HE-7B could necessitate a multi-component system to allow for the different operating conditions required to catabolise the individual degradation products. These could be the provision of supplemental carbon sources in the case of co-metabolic degradation, or the maintenance of specific redox conditions for catabolism of individual compounds. This could be achieved by use of a multistage reactor with sequentially arranged reactor vessels allowing spatial separation of microbial associations that have developed to degrade the individual intermediary products, with the more labile compounds being catabolised in the initial reactors and the recalcitrant compounds being catabolised in the final reactors, forming the rate-limiting step of the overall catabolic process.

The third objective of the enrichment programme was to improve the tolerance of the acclimated microorganisms to inhibitory concentrations of Procion Red HE-7B. The results in Section 4.3.5

showed that prior exposure of the biomass to Procion Red HE-7B appeared to increase the resistance of the acclimated microorganisms to dye concentrations that were found to be inhibitory to unacclimated microorganisms, which was in agreement with the findings of Ogawa et al. (1988; 1989). These researchers reported that inhibition was caused by intercalation of dye compounds between DNA base pairs, so preventing enzymatic activities and cell replication (Ogawa et al., 1988). Intercalation can also cause frame-shift mutations upon replication of the distorted DNA double helix (Stanier et al.,1987) and this property may even be partially responsible for the development of microbial resistance when exposed to these dyes.

Intercalation as a mechanism of inhibition requires that the dye be suitable to: (a) pass through the cell membrane of the microorganisms; and (b) insert itself between the base pairs of DNA. Procion Red HE-7B is a large, highly sulphonated compound (MW = 1634 g/mol) therefore permeation of the dye through the microbial cell membranes and subsequent intercalation of the dye between DNA base pairs is thought to be unlikely as a mechanism of inhibition. Although it is possible that an alternative mechanism is responsible for the inhibitive effects observed with Procion Red HE-7B, the potential toxicity of the dye degradation products must not be overlooked. Decolourisation of Procion Red HE-7B occurred within 48 h of incubation for all concentrations of dye, subsequently liberating the aromatic amine degradation products. These degradation products are smaller than the dye compound and may be capable of penetrating the cell and inhibiting the microorganisms either by intercalation or some other intracellular mechanism. The microbial toxicity of aromatic degradation products of dyes has been reported by Chung (1983) and Ganesh et al. (1992). The latter noted inhibition of biomass in a waste-water system treating reactive dye waste water and suggested that this was caused by the products of dye degradation rather than the dye itself.

It must be noted that both acclimated and unacclimated serum bottles containing 20 and 50 mg/M of Procion Red HE-7B exhibited higher rates of gas production than the control bottles. This suggests that, at low concentrations, the presence of the dye in the anaerobic system is beneficial to the anaerobic microorganisms, possibly due to its role as a terminal electron acceptor. Rahman (1991) also reported that the addition of the reactive dye, Red B, to a biological culture capable of reducing the dye, appeared to enhance growth in the culture and proposed that the electron accepting nature of the dye was responsible for this.

4.4.2 Biological Decolourisation in an Anaerobic System

The first-order reactions for decolourisation of Procion Red HE-7B (Section 4.3.2) were not in accordance with the measured rates of decolourisation which were inversely proportional to the initial dye concentrations. In view of the results obtained with the toxicity assay, it is suggested that this was caused by increasingly inhibitive concentrations of Procion Red HE-7B degradation products in the system, resulting in decreasing decolourisation rates with increasing initial dye concentrations. Wuhrmann et al.(1980) also noted that with some dyes the rate of decolourisation decreased more

rapidly than predicted by a first-order reaction, when a large percentage of the dye had already been reduced. This was attributed to accumulation of toxic metabolic products in the medium. Although the decreasing rate of degradation of Procion Red HE-7B with increasing dye concentration did not directly correlate with the observation of Wuhrmann et al.(1980), it is probable that the toxicity of

First-order reactions for degradation of azo dyes by anaerobic microorganisms were reported by Larsen et al. (1976), Wuhrmann et al. (1980), Meschner and Wuhrmann (1982) and Kremer (1989). Pseudo first-orders for reduction of azobenzene in an anaerobic sediment-water system were reported by Weber and Wolfe (1987), although azo reduction was attributed to abiotic factors associated with the sediment and could not be directly compared to azo reduction mediated by anaerobic microorganisms. In all instances the site of azo reduction was assumed to be intracellular and the favoured primary rate-limiting factor for azo reduction was the rate of permeation of the dyes through the cell membrane (Larsen et al.,1976; Wuhrmann et al.1980; Meschner and Wuhrmann, 1982; and Yatome et al., 1991). This is particularly applicable with sulphonated azo dyes such as Procion Red HE-7B, as their hydrophilic nature is not conducive to cell permeation.

Permeabilisation of the biomass was, therefore, investigated to determine whether permeation of Procion Red HE-7B through the microbial cell membrane was the principal rate limiting factor for decolourisation. The results achieved, showing that permeabilisation of the biomass inhibited decolourisation of Procion Red HE-7B, were in contrast to the results reported by Meschner and Wuhrmann (1982) when using the same techniques. These researchers showed that, by permeabilising the cells of *Bacillus cereus,* the reduction rate of sulphonated dyes could be substantially increased. Furthermore, dyes not previously reduced by whole cells were reduced by the permeabilised cells.

It was, therefore, initially suggested that the contradictory results achieved with Procion Red HE-7B were a result of cell damage during the permeabilisation procedure. However, it was noted that gas production during pre-incubation of the biomass was identical for permeabilised and non-permeabilised cells i.e. that the metabolic state of the permeabilised biomass was similar to that of the non-permeabilised control cells, prior to Procion Red HE-7B addition. This suggested that any inhibition of the biomass occurred upon contact with Procion Red HE-7B and was confirmed by monitoring the metabolic state of the permeabilised cells following their exposure to the dye. The results showed that both permeabilised cells originally suspended in buffer and permeabilised cells originally suspended in the aqueous phase of the permeabilisation mixture, were inhibited by exposure to Procion Red HE-7B. Although the former group of microorganisms were able to resume metabolic activity (as measured by gas production) after 48 h of incubation, the latter did not resume metabolic activity during the 120 h incubation period. These results suggest that the permeabilisation process facilitated entry of the dye into microbial cells which were previously impermeable to the dye, thereby inhibiting the biomass. As suspending the biomass in the aqueous phase of the permeabilisation mixture was thought to increase the rate of transport of the dyes into the microbial cells (Meschner and Wuhrmann, 1982) this could explain why the permeabilised cells in this mixture were more inhibited than those in the buffer.

These results, therefore, suggest that Procion Red HE-7B was too large to permeate the cell membranes of the non-permeabilised anaerobic microorganisms, as found by Ogawa et al. (1981) with direct dyes, and that decolourisation of the dye in the standard assay system was extracellular. As permeability was not thought to be a rate-limiting factor for reduction of Procion Red HE-7B, the theories of Gingell and Walker (1971) who investigated azo reduction in a cell-free culture (i.e. without the constraints of permeability) and Dubin and Wright (1975) who proposed azo reduction was extracellular in nature, were investigated. It must be noted that these researchers reported zero-order reactions for the microbial reduction of azo dyes, which is in contrast to the first-order reaction measured with Procion Red HE-7B.

Gingell and Walker (1971) proposed that a soluble flavin acted as an electron shuttle between the dye and a reducing enzyme, the rate-limiting factor being generation of reduced flavin. Dubin and Wright (1975) proposed that the rate of generation of soluble flavin was indirectly governed by the redox potential of the dye to be reduced, as no reduction of the dye could occur until the concentration of the reduced flavin was such that the redox couple for the electron carrier (i.e. soluble flavin) approached that of the dye. The reducing power for this proposed redox cycle is supplied by the microbial metabolism, therefore, the metabolic state of the microorganisms is also proposed to be a rate-limiting factor in azo reduction.

Thus, as mentioned in Section 4.3.2, the following possible rate-limiting factors were investigated for decolourisation of Procion Red HE-7B : the metabolic state of the microbial population in the presence and absence of a suitable electron donating compound (glucose); the presence of additional electron acceptors (nitrate and sulphate); and the redox potential of the system.

Decolourisation of Procion Red HE-7B as a sole carbon source was found to occur at a reduced rate when compared with the rate measured in the presence of a supplemental labile carbon source (glucose). Enhancement of azo reduction through addition of an electron donor (specifically glucose) was also noted by researchers such as Haug et al. (1991) and Wuhrmann et al. (1980). Haug et al. (1991) postulated that there were two possible ways for glucose to enhance reduction of the azo dye, Mordant Yellow 3. The glucose could either act as a donor of reduction equivalents (via NADPH or FADH2) or its addition could result in more actively respiring cells, in this way rapidly depleting the medium of oxygen and enabling azo reductase to transfer reduction equivalents to the azo dye. The latter explanation was later disproved through a series of experiments which showed that removal of oxygen from the medium occurred at the same rate for those experimental vessel with or without glucose. This suggests that the presence of glucose enhances the reduction rate by increasing the rate of formation of reduction equivalents i.e. reduced flavin nucleotides which, according to Gingell and Walker (1971), was the rate limiting step in the microbial reduction of the azo dye Red-2G.

Therefore, the rate of decolourisation of Procion Red HE-7B appeared to be directly related to the level of metabolic activity of the anaerobic microorganisms. A similar conclusion was reached by Harmer and Bishop (1992) who found that decolourisation of the azo dye (Acid Orange 7) was closely related to the metabolic activity of the wastewater bacteria, with increasing concentrations of

easily assimilable COD in the treatment system resulting in increased decolourisation of the azo dye. Thus, a treatment system designed to anaerobically decolourise textile dyes must take into account the requirement of this system for supplemental labile carbon in order to maintain the metabolic activity of the microorganisms and, consequently, the formation of reduced flavin nucleotides to reduce and decolourise the dyes.

The presence of nitrate in the standard assay system inhibited decolourisation for a period of time proportional to the concentration of nitrate added. This suggested that nitrate (as the more thermodynamically favourable electron accceptor) was reduced in preference to Procion Red HE-7B and that only after all the nitrate (and possibly nitrite) had been reduced did decolourisation of Procion Red HE-7B commence. This is in agreement with the proposal of Dubin and Wright (1975) i.e. that the redox potential of the particular compound controls the rate of reduction of that compound. Inhibition of azo reduction by nitrate or nitrite was also reported by Wuhrmann et al. (1980), although in this case the principal rate limiting factor of azo reduction was thought to be permeation of the dyes through the microbial cell walls.

The presence of sulphate as an additional electron acceptor had no effect on the rate of decolourisation of Procion Red HE-7B in the standard assay system. It is, therefore, probable that the dye was reduced preferentially to the sulphate compounds, which indicates that Procion Red HE-7B was the more favourable electron acceptor. It is, therefore, proposed that the reduction potential of the dye falls between that of nitrate and sulphate.

The redox potential of the anaerobic digester, measured in the presence of Procion Red HE-7B, dye and nitrate, and dye and sulphate, shows that reduction of the dye is dependant on the redox potential of the anaerobic system. It is probable that decolourisation of Procion Red HE-7B will occur anywhere within a range of redox potentials but that the rate will be influenced by whether the reduction potential of the system is at the upper or lower level of this range. This was also reported by Gingell and Walker (1971), when using cell free extracts of *Streptococcus faecalis,* who stated that the azo dye (Red 2-G) was reduced at a rate depending on the redox potential of the system. Although the precise redox potential for decolourisation is not known, it can be concluded that strictly anaerobic conditions are condusive to decolourisation of Procion Red HE-7B.

These results must be considered when designing a wastewater treatment system for decolourisation of textile effluent. It was reported by Wuhrmann et al. (1980) that in a nitrifying sewage works supplemented with an anaerobic treatment step for denitrification, azo compounds were not decolourised until all nitrite was denitrified. This has serious implications if it is intended to combine the treatment of textile and nitrate-containing effluent, as it would not be possible to combine denitrification and dye reduction in a simultaneous process unless the detention time in the anaerobic step corresponded to the sum of the detention times of the two reactions involved.

4.4.3 Abiotic Decolourisation of Procion Red HE-7B in a Biological Anaerobic System

The final issue to be addressed in this section is the role of abiotic decolourisation in the anaerobic system. Abiotic decolourisation was investigated with respect to adsorption and decoloursiation in the mineral salts medium. Adsorption of Procion Red HE-7B did not occur instantaneously (as found by Wuhrmann et al., (1980), with cell of *B. cereus)* but came to equilibrium over a 6 h incubation period. This gradual attainment of equilibrium between adsorbed and soluble dye neccessitated a rate of decolourisation (adsorption) to be calculated to determine the extent of decolourisation that could be attributed to biological decolourisation alone. However, the following questions arose with respect to whether the adsorbed dye could be considered to be abiotically decolourised, or whether biological factors were responsible for subsequently decolourising this dye :

a) was the adsorbed dye subsequently biologically decolourised ?; and

b) could the adsorbed dye inhibit, or at least limit, the decolourisation of dye in solution?

The first question was addressed by attempting to remove the adsorbed dye from the cells of an actively decolourising microbial population, subsequent to decolourisation of Procion Red HE-7B. It was found that no adsorbed dye could be extracted from actively decolourising cells but that some dye could be extracted from heat-killed cells. This suggested that the adsorbed portion of the dye was subsequently decolourised (with active microorganisms) and, therefore, no distinction was made between abiotic decolourisation (adsorption) and biological decolourisation, in the standard assay system.

The second question was not answered with respect to Procion Red HE-7B although Wuhrmann et al.(1980) revealed that some types of dyes adsorbed to bacterial cells were subsequently degraded, whereas others did not show any signs of degradation over a 24 h period. However, dyes that were not degraded did not usually affect the reduction rate of dye in solution.

The proportion of Procion Red HE-7B adsorbed to the biomass was low, due to the extreme solubility of the dye. Adsorption was shown to be of the mono-layer type with the data fitting both the Freundlich and Langmuir isotherm equations. No conclusions could be reached regarding the isotherm equation best suited to Procion Red HE-7B adsorption with digester sludge as the adsorbent, without further experimentation. According to the literature, a Freundlich relationship would be expected for adsorption of azo dyes to biological sludge. Shaul et al. (1986) calculated adsorption isotherms for a number of acid azo dyes, using activated sludge as the adsorbent. The results showed that the Freundlich model was the most suitable in all cases, which agreed with the research of Dohanyos et al. (1978) (cited by Shaul et al.,1986) who applied three adsorption models to data for 22 textile dyes and found the Freundlich relationship to be the most suitable for 18 of these dyes, with activated sludge solids as the adsorbent. Wuhrmann et al. (1980) also related dye adsorption by *Bacillus cereus* to the Freundlich adsorption model and observed the same relationship. As the Langmuir isotherm assumes uniform adsorption sites, and the Freundlich isotherm accomodates the

presence of more than one type of adsorption site, it is reasonable that this isotherm should be more suitable for representing adsorption data for a heterogenous adsorbent such as biological sludge.

Finally, decolourisation of Procion Red HE-7B in the mineral salts medium could have been caused by interaction of the dye with ions in the medium, flocculation of the soluble dye or adsorption of the dye to a precipitate that was noted in the medium. This form of abiotic decolourisation was found to occur within 15 min of incubation and, therefore, all decolourisation occurring in the first 15 min was attributed to abiotic decolourisation, when calculating reduction rates for Procion Red HE-7B.

4.5 CONCLUSIONS

a) The enrichment programme did not increase the rate of decolourisation of the dye, or select for microorganisms capable of mineralising the dye in the anaerobic system.

b) Prior exposure of the biomass to Procion Red HE-7B increased the resistance of the microorganisms to inhibitory concentrations of Procion Red HE-7B.

c) Decolourisation of Procion Red HE-7B in an anaerobic system was found to be first-order with respect to dye concentration.

d) It is suggested that decolourisation of Procion Red HE-7B occurs in the extracellular environment.

e) Permeability is, therefore, not proposed to be the principal rate-limiting factor for decolourisation of Procion Red HE-7B.

f) The metabolic state of the microbial population is a rate-limiting factor in decolourisation of Procion Red HE-7B.

g) The presence of competitive electron acceptors is rate-limiting with respect to decolourisation of Procion Red HE-7B.

h) The redox potential of the system is thought to play a role in the rate of Procion Red HE-7B decolourisation.

i) Abiotic factors causing decolourisation in the anaerobic system were adsorption and decolourisation by the mineral salts medium.

j) Adsorption of Procion Red HE-7B to the anaerobic biomass was found to follow the pattern of monolayer adsorption and dye adsorbed to the biomass was proposed to be subsequently decolourised.

CHAPTER FIVE

FINISHING EFFLUENTS AND DECOLOURISATION OF PROCION RED HE-7B

5.1 INTRODUCTION

This chapter deals with a combination of two processes, namely, anaerobic digestion of organic-rich textile finishing effluents and decolourisation of the azo dye Procion Red HE-7B. Anaerobic digestion of textile finishing effluents (desizing, scouring and bleaching effluents) was investigated as an aid to decolourising azo dyes. Preliminary results from this study indicate that anaerobic digestion shows potential as both a source of energy for azo reduction and for substantial COD reduction of a textile mill effluent. An additional advantage of anaerobic digestion as a treatment process is the production of methane gas, which may replace some of the conventional energy sources in a textile factory, and carbon dioxide which may be used to neutralise the mill effluent before discharge to sewer.

In section 5.1.1 a general review of textile finishing processes is given. A more specific literature review on anaerobic treatment of textile finishing effluents is presented in section 5.1.2 and the advantages of process combination are outlined in section 5.1.3.

5.1.1 Textile Finishing Processes and Effluents

A cotton-finishing mill receives raw cotton cloth / yarn which is cleaned and prepared for dyeing and printing. The preparation involves three wet processes: desizing, scouring and bleaching which may be combined or performed separately. The advantage of combining these processes into a single stage operation is a substantial reduction in the number of textile padding and washing processes. Of these effluents, only those from the desizing and scouring processes have an organic load suitable for anaerobic treatment, although bleaching effluents must be taken into consideration as they are often combined with scouring effluents.

Desizing involves the removal of size which is applied to the individual warp yarns during weaving. The size coats the yarns with a protective film which resists the abrasive effects of the filling yarns (weft) which are positioned by the shuttle action of the weaving loom (Hart et al., 1983). The size must be removed prior to dyeing to achieve even dyeing of the cloth.

Natural sizing agents include starches and starch derivatives, cellulose derivatives and protein sizes (BASF, 1977). The biodegradability of the desizing effluent depends primarily on the type of size and the desizing process used.

Starch and starch-derived sizes are obtained from maize, corn, potato, tapioca and rice (Hart et al., 1983). They are insoluble in water and are removed with acid or alkali, or by enzymatic desizing. The biological degradability of the starch-derived sizes is influenced by chemical modification. In

general, the non-modified starches are more readily biodegradable, although all starch-based sizes can be described as biodegradable (Schluter, 1990). The resulting desizing effluent is therefore energy rich in terms of anaerobic digestion.

Carboxymethyl cellulose (CMC) is the most economically significant of the cellulose derivatives (Schluter, 1990). It is formed by treating cellulose with sodium hydroxide and monochloroacetic acid. Carboxymethyl cellulose size is water soluble and can be removed by a hot water wash. Carboxymethyl cellulose is incompletely biodegraded with a short residence time but complete degradation could be expected with increased residence times (Schluter, 1990).

Protein sizes are technically outdated as they are far less constant in quality and produce poorer sizing effects than synthetic sizes (BASF, 1977). The synthetic sizes vary in their chemical basis with the two most important groups being the polyvinyl alcohol's (PVA) and the polyacrylates. Synthetic sizes are considered relatively recalcitrant, although approximately 90% removal of PVA has been reported in an aerobic acclimated system (Hart et al., 1983 ; Porter, 1976). Schluter (1990) also reported that PVA degradation was found to be possible with acclimated microorganisms. Polyacrylate size shows little biological degradability although some removal through adsorption to the biomass has been demonstrated in an activated sludge system (Schluter, 1990).

Cotton desizing effluent is characterised by temperatures of 90 to 95 ^{o}C and contains detergents and auxiliaries (wetting, metal complexing and antifoam agents) in addition to the sizes and solid matter. The COD of the desizing effluent is dependent on the type of size removed, with starch based sizes giving rise to the highest COD values for the final effluent, while CMC size results in the lowest COD values, as the size deposit needed for starch sizes is twice as high as that for CMC size (Schluter, 1990).

Once the cotton has been desized it is **scoured** with hot alkali and detergent to remove natural impurities from the cotton together with the spinning oils (Hart et al., 1983). The substances to be removed in scouring (i.e. pectin's, waxes and proteins) are found mainly in the primary wall and cuticle, which has a thickness of approximately 1 % of the fibre diameter (BASF, 1977). The secondary wall, which constitutes over 90 % of the bulk of the mature fibre, consists of cellulose arranged in a complicated lamellae structure. Although natural cotton contains a relatively small proportion of impurities, these impurities are difficult to remove. The waxes have a high molecular weight, which makes their removal difficult and the proteins are situated in the central cavity of the fibre and are, therefore, relatively inaccessible to chemical attack. Prolonged boiling with sodium hydroxide solutions (up to 2% w/v) is adequate to solubilise all unwanted impurities (other than the natural colouring matter) which can then be washed away with water (Trotman, 1968). The cellulose portion of the cotton is not affected by this treatment providing that air is excluded.

The following changes are brought about by boiling with alkali (Groves et al., 1990) :

a) the pectin's and pectoses are converted to soluble pectic salts;

b) the proteins are degraded into soluble amino acids or ammonia;

c) the mineral matter is dissolved;

d) dirt is removed;

e) the saponifiable matter is hydrolysed to form soaps which in turn emulsify the unsaponifiable oils and retain the dirt particles in suspension and

f) the hydrophilic properties of the fibre are improved thereby enhancing the water absorptivity and the evenness of dye and chemical uptake.

Boiling with alkali is carried out in vessels known as kiers which may be either open (the liquor boils at atmospheric pressure) or closed (the liquor boils under pressure at temperatures higher than 100 °C). Because the scouring process is carried out in kiers, the resultant scouring effluent may be referred to as kier liquor.

Cotton scouring effluents contain cotton-derived organics which may be converted to chemical energy in an anaerobic digester. The wax component of the effluent is known to be recalcitrant although neutralisation of the effluent in the digester (by carbon dioxide produced as an end-product of anaerobic digestion) should facilitate physical removal of the wax compounds. Scour effluents are characterised by high temperatures and pH and contain additional organic compounds such as detergents (usually anionic) and auxiliaries.

After scouring, the cotton still retains some natural colouring compounds. These are removed by bleaching which destroys the colouring matter of fibres and associated impurities. Two categories of bleaches, namely, oxidising or reducing bleaches, can be used in combination with wetting, softening and stabilising agents. Cotton is generally bleached with oxidising chemicals such as sodium hypochlorite, sodium chlorite and hydrogen peroxide in a batch process. This process is often carried out by passing the cloth through a standing bowl containing the bleaching chemicals, after which the cloth is rotated on a beam for 2 to 8 h.

The characteristics of the desizing and scouring effluent (i.e. high pH, total solids and COD value) all contribute to increase the cost of discharge to sewer. It is, therefore, desirable to treat or pre-treat the effluent on site to reduce COD values, pH and total solids.

5.1.2 Treatment /

Traditionally, biological treatment of cotton-finishing effluent involved an aerobic process combined with a chemical treatment system (Athanasopoulos and Karadimitris, 1988a). The operating costs of these processes were high due to energy requirements of aeration, nutrient and chemical costs and the cost of sludge treatment and disposal. By treating this wastewater in an anaerobic digester the total cost of treatment could be reduced.

Anaerobic digestion is a process whereby complex organic compounds are broken down to methane, carbon dioxide and water by an association of microoganisms in the absence of molecular oxygen. Complex organic compounds are hydrolysed by extracellular enzymes then fermented to simple organic acids by a group of microorganisms collectively referred to as the acidogens. Some of these

acids are degraded by the methanogens to produce methane and carbon dioxide (McCarty, 1974). Anaerobic treatment processes are characterised by low sludge production, in contrast to aerobic processes and have low energy costs as no aeration is required. Mixing in the digester can be achieved by use of the gas produced in the process. In addition, heating of the reactors would not be required as wastewater streams from textile finishing usually have temperatures ranging from 60 to 80 ^{o}C.

Enrichment of microoganisms for anaerobic digestion of textile finishing effluents

Although scouring and desizing effluents contain a percentage of labile carbon, the presence of recalcitrant compounds, for example, sizes such as PVA and CMC and cotton-derived organics such as waxes and auxiliaries, combined with inhibitory conditions such as cation toxicity, necessitates the enrichment of a catabolic microbial population to ensure efficient attenuation of the effluent.

Enrichment involves the adaptation of a microbial community to degrade a previously recalcitrant compound, through prior exposure to that compound (Spain and Van Veld, 1983). Adaptation is defined as a change in the microbial community that increases the rate of transformation of a test compound as a result of prior exposure to that test compound (Spain and Van Veld, 1983). Mechanisms that bring about adaptation include gene transfer or mutation, production of inducible enzymes and population changes within the microbial community. The latter refers to a shift in the microbial population which may occur upon exposure to a test compound to favour those microorganisms capable of degrading the compound.

Anaerobic digestion of a mixed textile finishing effluent

The literature is limited with respect to degradation of cotton scouring and desizing effluents by adapted microbial associations. Most of the papers were published by the authors Athanasopoulos and Karadamitris (1988a, 1988b) and Athanasopoulos (1992). The first paper reports the successful adaptation of anaerobic microorganisms to degrade a textile finishing effluent comprised of streams from woven fabric finishing, knitted fabric finishing and yarn dyeing and finishing.

This effluent (described above) was characterised by high volumes with a relatively low organic load, thus maintenance of a high treatment rate over extended periods would prove difficult with respect to biomass washout. For this reason, fixed film anaerobic reactors were investigated for treatment of the mixed effluent. These reactors have a matrix present for holding microorganisms in the system, thereby preventing washout. The support matrix may consist of packing media that is either fixed in position or randomly packed, as in an anaerobic filter, or may consist of sand particles on which the biomass becomes attached (fluidised or expanded bed).

An anaerobic filter was chosen by Athanasopoulos and Karadamitris (1988a) for treatment of the above textile effluent. This consisted of a 60 M cylinder, jacketed for temperature control at 35 ^{o}C and packed with polypropylene pall rings. Initially the filter was fed with a solution of 6 % (w/v) cow manure solids to establish a suitable population of anaerobic microorganisms. After 20 d the feed was replaced with a glucose solution and the loading was increased to 1 kg COD/m^3 / d while progressively replacing the glucose solution with wastewater. After four months the feed was entirely

wastewater. Removal of COD for loadings up to 0,8 kg COD/m^3/d varied from 66 to 80 % and for higher loadings was approximately 50 %. The concentration of volatile fatty acids was reported to remain low (100 to 150 mg/M). Biogas production varied from 0,2 to 0,4 M/g of influent COD and was found to be 20 M/d for loading up to 1 kg COD/m^3/ d. Methane content of the biogas was found to range from 70 % to 80 % (v/v). When COD loading was increased to 1,3 kg/m^3/ d, biogas production decreased gradually and stopped within a month.

Scale-up of this laboratory process was investigated by Athanasopoulos and Karadamitris (1988b). A study was undertaken to determine the appropriate packing medium for optimum performance of a fixed film reactor treating textile finishing effluent. From the performance results of the test reactors, it was concluded that loose-fill medium could not be used in a full scale anaerobic reactor as short circuiting (channelling) occurs due to the random placement of the medium. In addition, it was shown that specific surface area of modular media had no significant effect on the performance of the reactor. Crossflow medium was recommended and it was suggested that selection of the appropriate cross flow medium should be based on the resistance to plugging.

In the final assessment, Athanasopoulos and Karadimitris (1988b) concluded that the investment cost for pre-treatment of cotton textiles finishing wastewater in an upflow filter would be high. However, the economic benefits of anaerobic digestion (as opposed to activated sludge treatment) were significant. It was estimated that treatment of the wastewater in an anaerobic system would yield 1 350 m^3/d of biogas, the energy of which was calculated to be equivalent to approximately 1 000 kg of fuel oil per day. This could be used to replace electricity or liquid petroleum gas in some textile mill processes. In addition, the flue gas from the biogas burner would be adequate to neutralise the entire mill effluent.

Athanasopoulos (1992) investigated the use of an anaerobic expanded bed reactor for treatment of the textile effluent described above. The reactor utilised quartz sand as the support medium. After an initial acclimation stage, the COD loading was gradually increased from 0,1 to 0,63 kg/m^3/d. At the lower COD loadings the removal was approximately 87 %, and this decreased to 50 % at the upper loading. For COD loading above this the COD removal was constant at 35 %. The volatile fatty acids decreased as the COD loading increased which is contrary to what would usually be expected with an unstable anaerobic digester. In most circumstances, digester failure occurs through an increase in volatile fatty acids (VFA) and concomitant decrease in pH, thus inhibiting the activity of the methanogens. However, in this case the acidification step appeared to be rate limiting which indicated that the bulk of the COD was recalcitrant. When the COD loading increased to 0,68 kg/m^3/d, the biogas production decreased gradually and ceased after a few days. This could have been due to inhibition of either the acidogens or methanogens

Promising results for the full-scale treatment of textile finishing effluent were given by Marte and Keller (1991) in a report on anaerobic pre-clarification of textile wastewater process streams (as part of the River Glatt rehabilitation project). They concluded that, with appropriate optimisation measures, approximately 80% degradation could be expected for desizing and preparation (scouring

and bleaching) streams. This meant an average lowering of the overall COD of approximately 50 to 60 %. With respect to printing pastes, alginate thickeners exhibited 90 % degradation, although semi-emulsion thickeners could not be anaerobically degraded.

Many textile effluents may be successfully treated by anaerobic digestion, although segregation of wastewater streams is imperative when designing a biological treatment system so that concentrated effluent streams can be treated without the additional volume of dilute wash-water streams. Segregation also allows the treatment system to target specific organic compounds, at designated stages in the process and allows the waste waters to be fed to the digester in such a way that

5.1.3 Anaerobic Digestion of Cotton Scouring Effluent as an Energy Source for Decolourisation of Procion Red HE-7B

As concluded in Chapter Four, Procion Red HE-7B decolourisation does not provide an adequate source of energy for the azo-reducing microorganisms. The process, therefore, requires additional substrate/s to maintain the population of anaerobic microorganisms in the treatment system. Laboratory experiments utilised glucose as the additional substrate for rate determining experiments. This would not be feasible for large-scale treatment and an alternative carbon source with the following characteristics would be required :

a) low cost, preferably a form of waste; and

b) readily available at the site of treatment.

As it is intended to decolourise dye-containing waste on-site in an anaerobic treatment system, it is desirable that the substrate for the digester is readily available at the factory. This substrate may be provided by textile effluents with high organic loads, such as those arising from scouring and desizing. These waste streams provide organic carbon for anaerobic microbial metabolism and indirectly aid decolourisation. Additional benefits of a combined process are :

a) the COD content of the desizing and scouring wastestreams will be substantially reduced, resulting in reduced effluent discharge costs;

b) the high pH (approximately 13,5) commonly associated with scouring effluent, is neutralised by carbon dioxide production in the digester, reducing the costs of acid previously used to neutralise effluent prior to discharge;

c) the colour of the final effluent will be reduced; and

d) energy will be produced in the form of methane which can either be used to heat the digester, making the waste treatment process self sufficient, or be utilised elsewhere in the factory if required.

Section (5.2.1) outlines the experimental plan and procedure followed during the enrichment programme. Enrichment of biomass to anaerobically digest textile finishing effluent was divided into two phases. Firstly, serum bottle acclimation studies were conducted with a kier liquor from Smith and Nephew (Pinetown) and secondly, acclimation studies were carried out in a laboratory-scale digester with a mixed cotton scouring and bleaching effluent (John Grant, Jacobs, Durban). The effluent from Smith and Nephew was a complex mixture of sizes, organics and auxiliary compounds emanating from kiering of varied fabrics. This made it difficult to predict the degradability of typical components of textile pre-treatment streams and, consequently, the effluent stream from John Grant was chosen as representative of a typical cotton scouring and bleaching effluent. This effluent emanated from the scouring and bleaching of cotton linters and, therefore, contained no sizing agents. The only organic compounds present in the John Grant effluent were those scoured from the cotton together with some auxiliaries from the scouring process. Hydrogen peroxide was the bleaching agent used. The carbon content and pH of these effluents is given in **Table 5.1.**

Effluent source	Organic carbon (mg/M)	Inorganic carbon (mg/M)	pH
Smith & Nephew	15 000 to 20 000	10 000 to 11 000	13.5
John Grant	2 500 to 3 600	1 320 to 1 800	12.2

Section 5.2.2 outlines the experimental plan and procedure followed to investigate the ability of the enriched biomass to decolourise Procion Red HE-7B. The effluent used for these tests was John Grant kier liquor and the inoculum was obtained from the semi-continuous enrichment digester.

5.2.1 Enrichment of Microbial Populations Capable of Anaerobically Digesting Cotton Scouring Effluent

Experimental outline and procedure for serum bottle studies

Preliminary serum bottles tests using Smith and Nephew kier liquor aimed to establish :

a) whether anaerobic microorganisms would survive in the inhibitory conditions of the effluent i.e. high concentrations of sodium hydroxide and presence of organic compounds that could be toxic to the microorganisms;

b)

c) the minimum period of time required for adaptation to occur.

The serum bottles (120 mM, Aldrich Chemical Company) were divided into two categories: controls which contained no effluent, i.e. the only available organic substrate was that in the digester sludge and bottles containing 10, 50 and 100 % effluent respectively.

Inoculum was obtained from Umbilo and Umzinto Waste-Water Treatment Works (WWTW). These WWTWs were chosen as a source of inoculum as they both receive and treat a significant percentage

of textile effluent. The inoculum contained residual organic material in addition to biomass. Enrichment bottles (120 mM) were filled with 70 mM of appropriately diluted kier liquor (10, 50 or 100 %, in triplicate) and 30 mM inoculum. The kier liquor had a pH of 13,5 **(Table 5.1)** but was lowered to within the range of 8,0 to 8,5 pH units before use. Control bottles contained 30 mM inoculum and 70 mM distilled water. The bottles were overgassed with oxygen-free nitrogen (Fedgas) for 15 min at a flow rate of 0,5 M/min to displace oxygen. After overgassing, the bottles were quickly sealed with butyl rubber caps and aluminium crimping seals (Aldrich Chemical Company) to prevent the ingression of oxygen. The bottles were incubated at 32 °C for 1 h, after which time the gas pressures inside the bottles were equilibrated to atmospheric pressure by inserting a hypodermic needle (fitted to a 50 mM glass syringe) through the rubber septa of the serum bottles. The bottles were incubated for 80 d and metabolic activity was monitored through measurement of digester gas production.

Experimental outline and procedure for John Grant enrichment study

The second enrichment programme, using John Grant cotton scouring and bleaching effluent, was performed on a larger scale. A round-bottomed reactor (5 M) and flange (Quickfit) constituted the enrichment vessel. The inoculum was a mixture of biomass from the initial serum bottles study, biomass from laboratory digesters seeded with biomass from Umbilo and Umzinto WWTWs which had been operated to exhaust the sludge of any residual organics, and biomass from the Umgeni river mangrove swamps. The latter two constituted the bulk of the inoculum and were mixed in a 1 : 1 (v/v) ratio. Inoculum from the mangrove swamps was obtained by removing anaerobic sediment from the bed of a saline river. The rationale behind using this inoculum for degradation of cotton scouring effluent was the presence of plant derived organics in the effluent, which are similar to plant matter present in the mangrove sediment. Furthermore, microorganisms in the mangrove sediment are able to tolerate the sediment salt concentrations (3,5 %) which should infer a competitive advantage with respect to cation toxicity.

A 30 % (v/v) mixed inoculum (1 500 mM) and 3 500 mM of 10 % (v/v) John Grant effluent, were added to the digester which was overgassed with oxygen-free nitrogen for 30 min at a flow rate of 0,5 M/min, to displace any oxygen present. The pH of the effluent was lowered to 8,35 by sparging with carbon dioxide before being added to the reactor, although all subsequent additions of effluent were not pH adjusted. The reactor was incubated (without mixing) in a water bath at 32 ($\pm$ 1) ^{o}C. A 3-way syringe valve (with Luer lock fittings) supplied by Aldrich Chemical Company was used to extract samples from the digester and introduce liquid into the digester, without oxygen ingression. Gas from the digester was transferred via silicone tubing to a gas trap consisting of an upturned measuring cylinder clamped in a beaker of citric acid-acidified sodium chloride solution (20 % NaCl and 0,5 % citric acid). Additional ports were available for insertion of pH electrodes etc.

The reactor was initially operated in batch mode with additional effluent added once gas production had ceased. This operational mode was used to predict a suitable residence time for a semi-continuous digester. An initial residence time of 50 d was chosen and the digester operated in semi-continuous

mode, with 400 mM of effluent added every fourth day. The digester was monitored by measuring DOC, digester gas production and pH.

Analytical techniques

The volume of gas produced in the serum bottles was measured using the method of Owen et al. (1979). A hypodermic needle attached to a glass syringe was inserted through the butyl rubber seals of the serum bottles and the bottles allowed to equilibrate to atmospheric pressure. Displacement of the ground glass plunger corresponded to the volume of gas produced. The readings were not corrected to standard temperature and pressure since the experiment was designed to compare the relative gas productions of the control and enrichment bottles only. Gas volume in the enrichment digester was measured by liquid displacement.

Dissolved organic carbon (DOC) was measured using a Beckmann Total Carbon Analyser. Samples (5 mM) were withdrawn from the digester and acidified to pH 2,0 with hydrochloric acid (conc.) followed by rapid sparging with nitrogen (10 min) to remove the inorganic carbon. The duration of sparging was calculated from a test in which a sample was sparged for 30 min, with samples injected into the total carbon channel of the instrument every 2 min. It was found that after approximately 6 min no change in total carbon value occurred, therefore, a sparging time of 10 min was chosen. The samples were centrifuged at 5 000 rpm for 10 min to remove suspended matter and each supernatant injected into the total carbon channel. The results were converted to mg of DOC / M by use of a potassium biphthalate calibration curve which is shown in **Fig 5.1**.

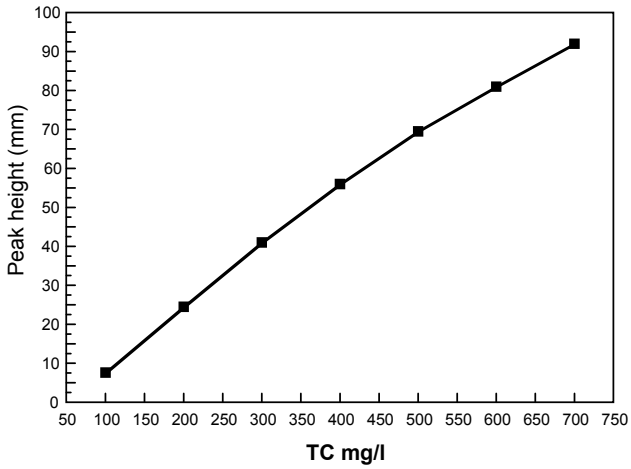

Fig 5.1 : Calibration curve for total carbon using potassium biphthalate standards.

5.2.2 Decolourisation of Procion Red HE-7B Combined with Anaerobic Digestion of Cotton Scouring Effluent

Experimental outline

The aim of these experiments were to determine :

a) whether anaerobic microorganisms capable of digesting scour effluent organics would also decolourise Procion Red HE-7B; and

b) whether the mechanism of decolourisation was analogous to that reported in Chapter Four.

Two experiments were performed to fulfill these aims.

The first experiment measured the rate of decolourisation of Procion Red HE-7B when added to anaerobic digesters (120 mM serum bottles) in which the microbial population was actively degrading John Grant scouring effluent at concentrations of either 50 or 100 % (v/v). Controls, consisting of uninoculated scouring effluent (50 or 100 % v/v), were prepared in serum bottles (120 mM) to assess the decolourising ability of uninoculated John Grant scouring effluent. Additional controls, consisting of sterilised John Grant scouring effluent (50 %), were prepared in serum bottles (120 mM) to determine whether any decolourisation observed in the unsterilised controls could be attributed to biological activity in the effluent.

The second experiment evaluated the effect of nitrate addition on rate of decolourisation of Procion Red HE-7B when Procion Red HE-7B (100 mg/M) and nitrate (3mM) were added to anaerobic digesters (120 mM serum bottles) in which the microbial population was actively degrading John Grant scouring effluent 50 % (v/v). The rate of decolourisation measured in these experimental

Experimental procedure

Experiments were performed in serum bottles (120 mM). Inoculum was withdrawn from the enrichment digester by means of a 3-way syringe valve (Aldrich Chemical Company) and centrifuged at 5 000 rpm. A mass of 5 g of inoculum (wet weight) and 100 mM of effluent (50 or 100 %, diluted with distilled water) were added to each experimental bottle. All serum bottles were overgassed with oxygen-free nitrogen for 15 min at a flow rate of 0.5 M/min, capped and sealed. The bottles were incubated for 40 h at 32 °C, during which time gas production and pH were monitored. After 40 h of incubation, Procion Red HE-7B stock solution was added to each bottle by hypodermic needle and syringe, to give an initial concentration of 100 mg/M dye. Sodium nitrate stock solution was also added to the appropriate bottles at this time to give an initial concentration of 3 mM. Samples (2,5 mM) were withdrawn from the assay bottles by syringe at hourly intervals. These samples were immediately centrifuged at 10 000 rpm to remove suspended particles.

Uninoculated control bottles (120 mM serum bottles) contained 100 mM of 50 or 100 % (v/v) unsterile John Grant scouring effluent, diluted with distilled water. Additional control bottles contained 100 mM of 50 % (v/v) John Grant scouring effluent which was sterilised by autoclaving at 121 °C for 15 min. The control bottles were overgassed with oxygen-free nitrogen for 15 min at a flow rate of 0,5 M/min, sealed and incubated for 40 h at 32 °C. After 40 h of incubation, Procion Red HE-7B stock solution was added to each bottle by hypodermic needle and syringe, to give an initial concentration of 100 mg/M dye. Samples (2,5 mM) were withdrawn from the assay bottles by syringe at hourly intervals. These samples were immediately centrifuged at 10 000 rpm to remove suspended particles.

Procion Red HE-7B stock solution

A 10 g/M stock solution of Procion Red HE-7B was prepared by dissolving 1 g of commercially available Procion Red HE-7B powder (ICI) in distilled water and making up to 100 mM in a volumetric flask.

Sodium nitrate stock solution

A stock solution of $NaNO_3$ was made up by dissolving 255 mg in distilled water and making up to 10 mM, to give a 300 mM solution; 1 mM of stock solution was added to each experimental bottle (by syringe) to give an initial nitrate concentration of 3 mM.

Analytical procedure

Decolourisation was monitored by measuring absorbance at 520 nm on a LKB Biochrom Ultraspec spectrophotometer (model 4050) and converting the readings to dye concentration using a calibration curve (Appendix C).

5.3 RESULTS

Section 5.3.1 presents results from serum bottles enrichment studies and data from monitoring the John Grant enrichment digester in both batch and semi-continuous mode. Section 5.3.2 presents results from the combination of Procion Red HE-7B decolourisation and anaerobic digestion of John Grant effluent. All experimental data is presented in Appendix G.

5.3.1 Enrichment of Microbial Populations to Tolerate and Degrade Cotton Scouring Effluent

Fig 5.2. shows the gas production (cumulative, not corrected for STP) monitored during incubation of digester sludge (microoganisms and residual organic matter) in serum bottles with 0 (control), 10, 50 and 100 % Smith and Nephew kier liquor. The results shown are averages of triplicate results (Appendix C). The volume of gas produced by the control bottles (containing no kier liquor) and the enrichment bottles (with kier liquor) was similar for the first 30 d of incubation indicating that gas production resulted from anaerobic digestion of the residual organic carbon in the digester sludge and not mineralisation of organic compounds in the kier liquor. However, at approximately 37 d of incubation the rate of gas production in the 10 % enrichments began to increase in excess of the controls. At this time the rate of gas production in the controls was low possibly due to a lack of available substrate and it is therefore probable that any gas produced in the 10 % bottles subsequent to the 37 d of incubation resulted from anaerobic digestion of organic substrate in the kier liquor. As gas production in the 50 and 100 % enrichments was similar to that of the controls throughout the 80 d incubation period, it is probable that degradation of the kier liquor organics did not occur in these bottles. The reasons for this are unclear but may involve inhibition of the anaerobic biomass by the increasing salinity of the 50 and 100 % (v/v) effluent.

Fig 5.3 shows digester gas production (cumulative, not corrected for STP) and DOC removal (cumulative) in the 5 M enrichment digester during the start-up period. This digester was run in batch mode until gas production ceased at which point 1 M of digester liquid was withdrawn and 1 M of

John Grant scouring effluent added. As the mangrove sediment (used as a source of inoculum for the enrichment digester) contained a high percentage of solid organic matter it may be speculated that degradation of these organics may have occurred in preference to the organics in the scouring effluent, as was found with the serum bottle enrichments. However, **Fig 5.3** shows that gas production was closely related to the added effluent concentration as measured by DOC in the digester, with gas production ceasing when DOC was low and increasing overnight subsequent to the addition of effluent. This indicated that organic compounds in the scouring effluent were being preferentially degraded.

Fig 5.4 shows a steady rate of DOC removal (cumulative) and digester gas production (cumulative) with the digester operating in semi-continuous mode. **Fig 5.5** is a plot of pH and DOC for the digester over the same time period. These results show a gradual increase in residual DOC, indicating incomplete removal of organic carbon in the scouring effluent. Frequent sampling between days 5 and 7 showed a 35 % decrease in DOC between the fifth and sixth day and a further 8 % decrease by the end of the seventh day. Although digester feeding of the digester was scheduled to occur every 4th day, it was decided to increase the residence time to facilitate a greater decrease in DOC although only 7 % DOC was removed by day 11. Over the next 8 d of operation, the residual DOC in the digester gradually increased. It is probable that the labile carbon sources were digested preferentially causing an accumulation of recalcitrant compounds in the digester.

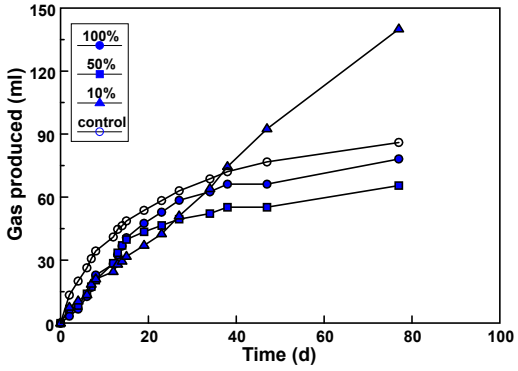

Fig 5.2 : Cumulative digester gas production (mM) from inoculum incubated with 0 % (control), 10 %, 50 % and 100 % Smith & Nephew kier liquor. Inoculum contains residual organics.

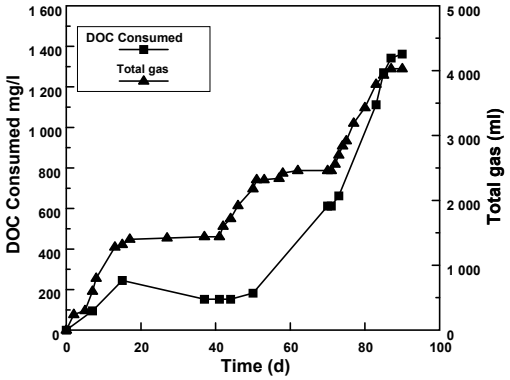

Fig 5.3 : DOC consumption and digester gas production shown for the 80 d start-up period. Digester in batch mode.

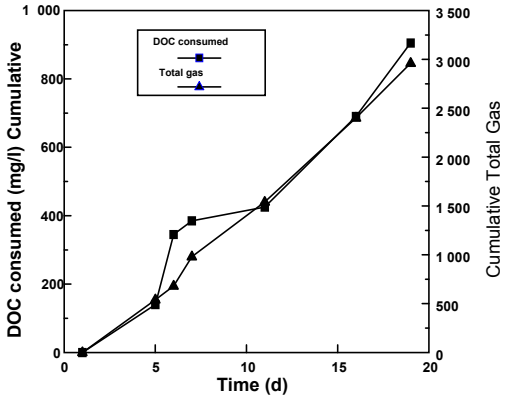

Fig 5.4 : DOC removal (cumulative) and digester gas production (cumulative) for the digester in semi-continuous mode, showing a constant rate of DOC removal.

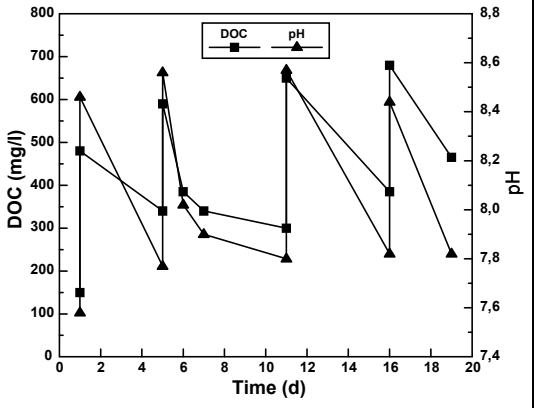

Fig 5.5 : DOC (mg/l) and pH for the digester in semi-continuous mode, showing gradual increase in residual DOC over time.

5.3.2 Decolourisation of Procion Red HE-7B Combined with Anaerobic Digestion of Cotton Scouring Effluent

Fig 5.6 shows decolourisation of Procion Red HE-7B in inoculated serum bottles with 50 % and 100 % John Grant scouring effluent and 1 g/M glucose, as carbon and energy sources for anaerobic digestion. The curves of ln C_t/C_o (where C_t is the dye concentration at time t and C_o is the dye concentration at time zero) versus time are linear and may, therefore, be said to be first order. The rate of decolourisation in 100 % scour effluent (k = - 0,573) was considerably higher that in the 50 % bottles (k = - 0,203). These decolourisation rates were comparable to those measured under standard assay conditions (k= - 0,441) where1 g/M of glucose was used as the additional carbon source. The increased rate of decolourisation in the inoculated 100 % scour effluent bottles was also noted in the uninoculated controls which exhibited a greater rate of decolourisation with 100 % effluent than with 50 % effluent **(Fig 5.7)**.

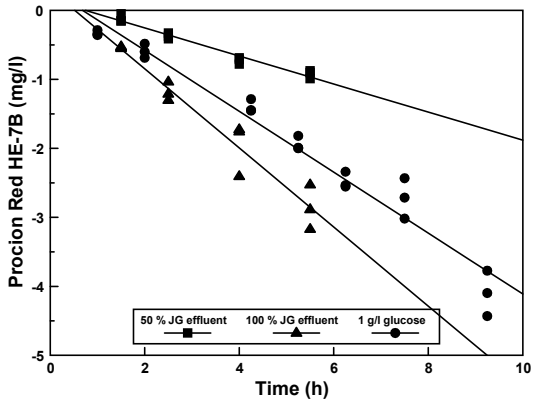

Fig 5.6 : Procion Red HE-7B decolourisation with John Grant scouring effluent (50 and 100 %) and glucose as carbon sources for anaerobic digestion.

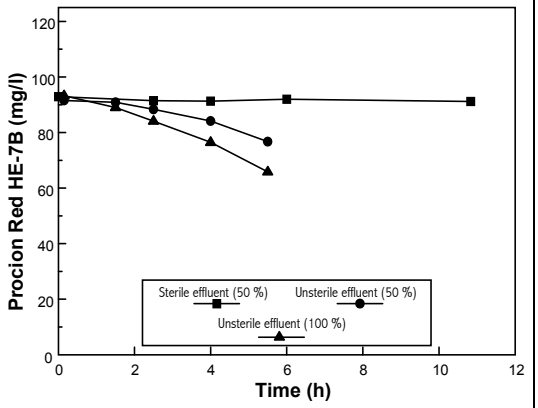

Fig 5.7 : Decolourisation of Procion Red HE-7B in uninoculated John Grant scouring effluent (sterile and non-sterile).

To determine whether the effluent was biologically active, Procion Red HE-7B was incubated with uninoculated scour effluent that was either sterilised by autoclaving, or remained unsterilised. The results are given in **Fig 5.7**. The sterilised bottles showed no significant colour loss, therefore decolourisation in the non-sterile bottles could be attributed to reducing conditions in the effluent, brought about by microorganisms in the effluent (i.e. other than the inoculum) that were actively catabolising the organics in the effluent and, thereby, decolourising the azo dye. Alternatively, decolourisation could have been mediated by a heat-labile compound (possibly a reducing agent) which was inactivated in the autoclaving process. The increased rates of decolourisation measured in the bottles with 100 % scouring effluent can, therefore, be attributed to a greater concentration of microorganims or chemical reducing agent in the effluent, or both.

Fig 5.8 shows gas production in the serum bottles (containing 50 % scour effluent) prior to dye addition. The pH readings for the bottles prior to dye addition and after decolourisation, are presented in **Table 5.2.** The initial pH values (8,1) were lowered due to carbon dioxide production during the 40 h pre-incubation period. No marked pH change occured during decolourisation and addition of nitrate had no significant effect on the pH of the system.

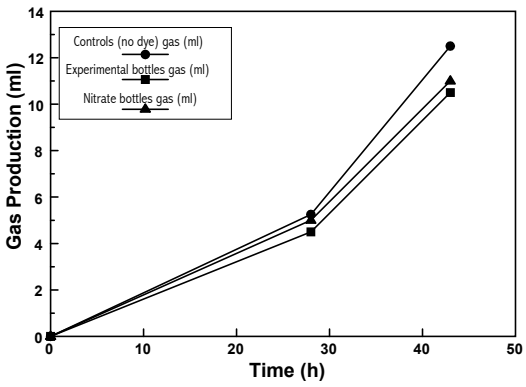

Fig 5.8 : Gas production in bottles incubated with 50 % John Grant scouring effluent prior to the addition of Procion Red HE-7B.

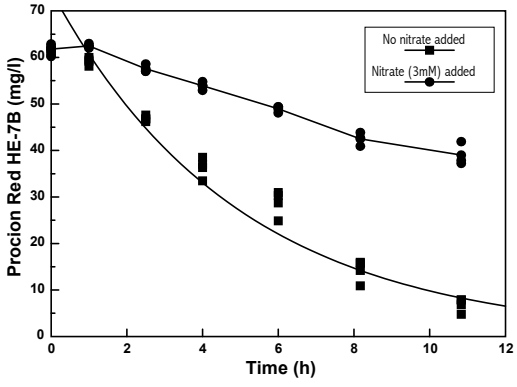

Fig 5.9 : Decolourisation of Procion Red HE-7B in the presence and absence of 3 mM nitrate, incubated with 50 % John Grant scouring effluent.

The effect of nitrate on the decolourisation process was assessed to determine whether the mechanism of decolourisation was electrochemical, as concluded in previous experiments (refer to Chapter Four).

Fig 5.9 shows that the presence of nitrate resulted in a significantly reduced rate of decolourisation, suggesting that nitrate (and possibly nitrite) reduction must occur before decolourisation of Procion Red HE-7B can take place.

Table 5.2 : pH values during the pre-incubation of enriched inoculum in 50 % John Grant effluent and after the decolourisation of Procion Red HE-7B.

Time (h)	pH (control, no dye)	pH (dye only)	pH (nitrate and dye)
0	8.1	8.1	8.1
28	7,2 to 7,4	7,0 to 7,2	6,9 to 7,4
43[a]	7,0 to 7,1	6,9 to 7,0	6,9 to 7,2
55[b]	6,8 to 7,0	6,5 to 6,8	6,9 to 7,2
79[c]	6,8 to 7,1	6,5 to 6,6	6,9 to 7,2

[a] Dye added at 43 h
[b] Decolourisation complete (dye only)
[c] Decolourisation complete (nitrate and dye).

5.4 DISCUSSION

Adaptation of microorganisms to anaerobically degrade the organic component of two textile finishing streams, namely, a mixed kier liquor (Smith and Nephew) and a kier liquor from scouring and bleaching of cotton linters (John Grant), was achieved. In both cases, additional carbon sources were present with the enrichment inoculum, either as residual organics in the digester sludge or organics in the mangrove sediment. For the Smith and Nephew kier liquor enrichments, the additional carbon was exhausted before degradation of the kier liquor organics was initiated suggesting that the organic compounds in the digester sludge were more labile than those in the kier liquor. In contrast, the biomass in the John Grant enrichment digester showed a preference for the dissolved organic carbon in the effluent suggesting that the carbon sources in the John Grant scouring effluent were more accessible to the microorganisms than that present in the mangrove sediment. When the digester was operated semi-continuously, 35 % of the dissolved organic carbon was degraded in the 24 h period following addition of the effluent to the digester, although approximately 50 % of the total DOC was not degraded in the allotted residence time (50 d). This suggested that a percentage of the organic carbon in the John Grant scouring effluent was readily degradable, but that residual DOC required an extended residence time to be mineralised. Consequently, the more recalcitrant compounds accumulated in the digester, increasing DOC values in the final effluent. This problem must be addressed before scale-up of the process can be considered.

A prolonged enrichment programme may result in microorganisms capable of degrading these compounds. However, it is unlikely that efficient DOC removal would be achieved in a single-stage digester unless an extended residence time was introduced or the digester was operated as a batch reactor, as digestion of labile carbon sources would occur preferentially. A multi-stage anaerobic digester, allowing spatial separation of microbial populations adapted to degrade various components of the effluent would enable different residence times to be implemented for optimum removal of DOC. This multi-stage anaerobic digester would consist of initial reactors, operating with short residence times for degradation of the labile carbon sources and subsequent reactors, with extended residence times to ensure mineralisation of the effluent.

Decolourisation of azo dyes in anaerobic digesters utilising textile finishing effluent as a feed source has shown promising results. Decolourisation of Procion Red HE-7B in an anaerobic digestion process using John Grant kier liquor as a carbon source was found to occur at a rate comparable to that measured under standard assay conditions (Chapter Four). Azo reduction was found to be first order with respect to dye concentration and to be inhibited by the presence of nitrate. A similar result was obtained in experiments using glucose as an auxiliary carbon source (Chapter Four). In addition, the inoculum had not been exposed to Procion Red HE-7B prior to these experiments which indicates that decolourisation of Procion Red HE-7B occurs through a non-specific electrochemical reduction mechanism in which the dye is utilised as a terminal electron acceptor by the microbial population and is consequently reduced and decolourised. An advantages of non-specific decolourisation is that a digester operating at a suitable redox potential with an alternative source of carbon (other than the dyes) should be capable of reducing and decolourising a wide range of azo dyes. A disadvantage of this non-specific mechanism of decolourisation is that azo reduction produces compounds (which may be broadly classed as sulphonated aromatic amines) which are unlikely to be mineralised in the anaerobic digestion process. Some aromatic amines have been found to be mutagenic (Chung, 1982) although, as reported in Section 4.4.1, mutagenicity tests for compounds closely resembling the degradation products of reactive textile dyes, for example, 4-amino-1-naphthalene-sulphonic acid, 1-amino-2-naphthol, anthranillic acid and sulphanilic acid, did not find these compounds to be mutagenic. It is interesting to note that the above-mentioned compounds are sulphonated and therefore extremely water soluble. As microbial cell walls are relatively impermeable to sulphonated azo compounds (Meschner and Wuhrmann, 1982) this may effect the mutagenic properties of these compounds, rendering the sulphonated compounds less mutagenic to the test bacteria than compounds that can readily enter the microbial cells. It is, however, possible that high concentrations of these aromatic compounds could be inhibitory to the microorganisms in a biological treatment system which would be significant in a combined treatment system.

Design of an anaerobic digester combining treatment of organic rich textile finishing effluent with decolourisation of azo dyes must therefore take into account the different residence times required for efficient degradation of organic compounds which vary in their ease of biodegradation and the minimum residence time required for decolourisation of azo dyes to prevent accumulation of dye breakdown products in the digester. The latter would be relatively short (maximum of 2 d for a 200 mg/M solution) in comparison to that required for biodegradation of scour effluent organic compounds or recalcitrant sizing agents. Again, a multi-stage digester may be required, the initial stages of which would include a high rate digestion process, possibly utilising starch or starch derived sizes as a feed source, combined with decolourisation of dye-containing streams. A fixed film reactor may be necessary to enable a high flow rate through the system while retaining the biomass in the reactor. The type of support medium used would be important as the dyes would probably adsorb to a medium such as activated carbon. This could be advantageous or disadvantageous depending on the process. The final stages of the process would consist of reactors with residence times suited to

degradation of targeted organic compounds, the design of which would depend on the residence times required.

5.5 CONCLUSIONS

a) Anaerobic digestion of textile finishing effluents, such as those emanating from scouring and desizing processes, can be achieved following enrichment of an adapted microbial population.

b) The varied compositions of these effluent streams could necessitate spatial separation of specific microbial populations in a multi-stage digester to ensure efficient removal of organic carbon.

c) Decolourisation of azo dyes in these digesters probably occurs through a non-specific electrochemical reduction mechanism.

d) Products resulting from a zo reduction are unlikely to be mineralised in the digestion process. These products may be inhibitory to the microorganisms if allowed to accumulate, necessitating a high rate anaerobic digestion process for decolourisation of textile dyes.

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APPENDIX A

CLASSIFICATION OF DYES

In general dyes consist of two basic components: the *chromogen* (electron acceptor) and the *auxochrome* (electron donor) which regulates solubility and dyeing. The chromogen is an aromatic body containing a colour-giving group called a *chromophore*. The chromophore causes colour by altering absorption bands in the visible region. Some common chromophores are presented in **Table A.1**.

Table A.1 : The structure of some common dye chromophores

nitroso	NO or (=N-OH)
nitro	NO_2 or (=N-OOH)
azo group	-N=N-
ethylene group	-C=C-
carbonyl group	-C=O-
carbon-nitrogen group	-C=NH; CH=N-
carbon-sulphur groups	-C=S; -C-S-S-C-

Dyes may be classified according to their chromophoric structure or alternatively, by their application. Neither system of classification is satisfactory by itself as the same chromophoric system may be present in dyes differing widely in usage and application, since the presence or absence of solubilising groups, proton-accepting groups, long-chain alkyl groups, etc., are the factors which determine dyeing characteristics and suitability for a particular purpose (Abrahart, 1977).

A.1 THE COLOUR INDEX

The following is an excerpt from Burkinshaw S.M (1990).

The colour index is the internationally accepted catalogue of dyes and pigments. The first edition, published in 1924, used chemical constitution as the basis for classification of dyes and pigments. The second (1951) and third (1971) and the current revised third editions (1976; 1981 and 1987) classify colourants according to :

a) *Constitution*, for the chemist requiring information on chemical structure, preparative methods, etc.

b) *Application*, for the user seeking information on commercial products, dyeing properties, etc.

Table A.2 gives the Colour Index (C.I) classification of colorants, according to application and constitution.

Table A.2 : C.I. classification of colourants	
Application	**Constitution**
Acid dyes	Nitroso
Azoic colouring matters	Nitro
Basic dyes	Azo
Developers	Azoic
Direct dyes	Stilbene
Disperse dyes	Carotenoid
Fluorescent brightening agents	Diphenylmethane
Food dyes	Triaryl methane
Ingrain dyes	Xanthene
Leather dyes	Acridine
Mordant dyes	Quinoline
Natural dyes	Methine
Oxidation bases	Thiazole
Pigments	Indamine
Reactive dyes	Indophenol
Reducing agents	Azine
Solvent dyes	Oxazine
Sulphur dyes	Thiazine
Vat dyes	Sulphur
	Lactone
	Aminoketone
	Hydroxyketone
	Anthraquinone
	Indigoid
	Phthalocyanine
	Natural organic colouring matters
	Oxidiation bases
	Inorganic colouring matters

The revised third edition of the Colour Index consists of several volumes :

Volumes 1 to 3 classify colourants according to application type. Each colouring matter within each of the nineteen classes is ascribed a Colour Index Generic Name according to hue, e.g., CI Acid Red 11, CI Reactive Yellow 16, etc.

Volume 4 divides colourants

Volume 5 includes :

a) An index of commercial names in alphabetical order. Since colourants are given a variety of commercial names which may disguise the fact that one colourant is sold under different names by different manufacturers, for each commercial product the CI Generic Name and CI Constitution Number is provided (if the colourant structure has been declared) so as to permit equivalent products to be identified.

b) An index of CI Generic Names, each entry including the corresponding commercial name to allow the identification of equivalent products.

c) An index of manufacturers of colourants.

Volume 6 is the first (1976) supplement to volumes 1 to 4.

Volume 7 is the second (1982) suplement to volumes 1 to 4 and a supplement to volume 6.

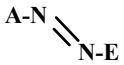
Volume 8 is the third (1987) supplement to volumes 1 to 4 and a supplement to volume 7.

A.2 DYES IN THE TEXTILE INDUSTRY

Textile dyes are usually classified according to application and will be described accordingly in this section, however, as the azo chromophore is the single most important chromophore represented throughout the application classes of textile dyes, the chemically classified group of azo dyes will also be described briefly.

Azo dyes (Gregory, 1990)

These dyes are the most important class, accounting for over 50 % of all commercial dyes. Azo dyes contain at least one azo group (-N=N-) (monoazo) but can contain two (disazo), three (trisazo), or more rarely, four azo groups (polyazo). The azo group is attached to two radicals of which at least one, but usually both, are aromatic.



In the monoazo dyes, which are the most important group, the A radical contains electron-accepting groups and the E radical contains electron-donating groups, particularly hydroxy and amino groups. If the azo dyes contain only aromatic radicals they are known as carbocyclic or homocyclic azo dyes. If they contain one or more heterocyclic radicals, they are know as heterocyclic azo dyes.

Classification according to application

Acid dyes (Burkinshaw, 1990)

The majority of these dyes are soluble in water, solubility being conferred by the presence of sulphonic acid groups on the dye, usually in the form of a sodium salt ($-SO_3Na$). The dyes are insoluble in acid and therefore acidic conditions are used for dyeing to achieve good exhaustion in dyeing of wool and nylon. Generally, the lower the natural affinity of the dyes for the fibers, the more acidic the dyeing conditions, and visa versa. Process chemicals (dyeing auxillaries) that are associated with acid dyeing are: sulphuric acid, acetic acid, sodium sulphate and surfactants.

There are nine chemical classes of nonmetallised acid dye of which three are the most important :

a) **Azo.** This class constitutes by far the largest number of acid dyes, and includes the majority of red, yellow, orange, brown and black hues. The dyes range from relatively simple monoazo

dyes that generally possess low affinity for wool and nylon, to larger molecular weight dyes to which high affinity is imparted by the presence of alkyl chains.

b) **Anthraquinoid.** These acid dyes provide a range of mostly bright blues, violets, and greens and have good all round fastness properties on wool and nylon fibres.

c) **Triphenylmethane.** These dyes provide brilliant violets, blues and greens of relatively low all-round fastness on wool and nylon.

Azoic Dyes (Burkinshaw, 1990)

An azoic colouring matter is a water-insoluble azo compound produced *in situ* in textile fibres by the interaction of a diazo component with a coupling component. Azoics are principally used for dyeing cellulosic fibres on which they provide a wide range of bright hues, excluding greens and bright blues. Navies and blacks may also be achieved with azoic dyeing. Auxiliary chemical associated with azoic dyeing are: metal salts, formaldehyde, sodium hydroxide, sodium nitrate and acids.

Basic Dyes (Burkinshaw, 1990)

These are amino and substituted amino compounds that are soluble in acid and become insoluble when alkali is added. They are used to dye acrylics or can be used with a mordant dye for dyeing wool and cotton. Auxiliary chemicals associated with basic dyeing are acetic acid and softening agents.

Direct Dyes (Burkinshaw, 1990)

Direct dyes can dye cotton and viscose rayon directly from a neutral dyebath containing sodium chloride, which decreases the solubility of the dyes. Direct dyes are relatively inexpensive and provide a wide range of hues, however, they are characterised by poor fastness especially with respect to washing,, a property that can be improved by aftertreatments. Auxiliary chemicals associated with direct dyeing are: sodium salts, fixing agents and metal salts (copper or chromates). Fibre-reactive dyes have largely replaced direct dyes for dyeing of cotton. Direct dyes belong to several chemical classes, the following are the most important :

a) **Azo.** The majority of direct dyes are of the disazo, trisazo and polyazo types.

b) **Phthalocyanine.** These bright blue and turquoise dyes possess high fastness to light but low fastness to wet treatments on cotton.

c) **Stilbene.** This is a relatively small but important group of mostly red, yellow and orange dyes.

Disperse Dyes (Burkinshaw, 1990)

A disperse dye is defined as *a substantially water-insoluble dye having substantivity for one or more hydrophobic fibres, for example, cellulose acetate, and usually applied from fine aqueous dispersion.* In addition to acetate fibres, the dyes are used on polyester, polyamide and acrylic fibres. Auxiliary

chemicals associated with disperse dyeing are: carrier, sodium hydroxide and sodium hydrosulphite. Disperse dyes belong to the azo, anthraquinone, nitrodiphenylamine and styryl groups.

Fibre Reactive Dyes (Godefroy, 1993)

Reactive dyes are coloured components capable of forming a covalent bond between the dye molecule and the fibre. Fibre reactive dyes have been developed for wool and polyamide but the major success in this field has been the application to cotton and cotton blends. Auxiliary chemical associated with reactive dyeing are: sodium chloride, sodium hydroxide and ethylene diamine.

The structure of a fibre-reactive dye is essentially divided into two parts, the chromogen and the reactive system. It is convenient to consider them separately as many chromogens are common to several dye ranges and only the reactive systems differ.

The reactive system is the component of the dye that reacts with the fibre. Reactive systems can be divided into :

a) **Heterocyclic reactive systems.** These heterocyclic ring structures all contain nitrogen atoms and are usually based on the s-triazine, pyrimidine and pyrimazone ring structures. The most commonly used displaceable substituent is chlorine, but success has been achieved with fluorine, quaternary amines and methyl sulphonyl groups.

   The monchloro-s-triazine dyes contain one monochloro-s-triazine reactive group and are characterised by fairly low exhaustion. This is improved by the incorporation of two monochloro-s-triazinyl rings into the dye molecule, thus providing two opportunities for reaction with the fibre and improving the exhaustion process. An example of this type of dye is Procion Red HE-7B. There are many other types of heterocyclic reactive systems but these will not be covered in this appendix.

   These reactive systems react with ionised hydroxyl groups on the cellulose substrate by a nucleophillic substitution mechanism. Alkaline conditions are required for the ionisation of the fibre and, therefore, hydroxyl ions are present and compete with the ionised cellulose as a nucleophillic reagent. This results in hydrolysed dyes which can no longer react with the cellulose fibre and accounts for the low levels of exhaustion achieved with some reactive dyes.

b) **Reactive systems based on nucleophillic addition.** These reactive systems interact with the cellulose fibre by means of a nucleophillic addition mechanism.

The chromophores used for reactive dyes are the following :

a) **Azo.** These may be divided into those dyes which have aromatic radicals based on benzene and naphthalene ring structures to give yellow, red, blue and green shades, and those azo dyes where the radicals contain heterocyclic compounds. When the heterocyclic coupling agents are indoles, pyrazolones and pyridones, yellow to orange shades are achieved, and when heterocyclic diazo compounds with either sulphur or sulphur and nitrogen are used, strong shades of blue to green are obtained.

b) **Anthraquinone.** These dyes give bright colours with good fastness and are used for strong shades from blue to green.

c) **Phthalocyanine.** These are used for the production of turquoise hues which cannot be prepared from azo or anthraquinone dyes.

Mordant Dyes (Burkinshaw, 1990)

The term mordant dye refers to a dye which is applied to a fibre in conjunction with a metallic mordant (often chromium). Characteristically, mordant dyes contain groups (e.g., -OH, -COOH) which are capable of forming a stable coordination complex with a chromium ion inside the fibre. The formation of this large molecular weight complex results in an often dramatic increase in fastness of the dyeing to light and wet treatments. Mordant dyes give fast, full, but generally dull shades on wool and nylon fibres. Auxiliary chemicals associated with mordant dyeing are: chromium and other metal salts, acetic acid and sodium sulphate.

The following chromophores are associated with mordant dyes:

a) **Azo.** This group, consisting mainly of monoazo dyes, comprises the majority of mordant dyes and, with the exception of bright greens, blues and violets, provides a comprehensive range of shades on wool and nylon. These are characterised by very high fastness to light and wet treatments.

b) **Anthraquinone**. Provides mostly blues, reds and browns.

c) **Triphenylmethane.** Provides mostly bright violets and blues and are characterised by moderate fastness to light wool and nylon.

d) **Xanthene.** This group contributes a very small number of brilliant, mostly red ,dyes.

Sulphur Dyes (Burkinshaw, 1990)

Sulphur dyes are used for the dyeing of cellulosic fibres in medium to deep shades of generally dull brown, black, olive, blue, green, maroon and khaki hues. These dyes are chemically complex and for the most part are of unknown structure, the majority of which are prepared by thionation of various aromatic intermediates. Auxiliary chemicals associated with sulphur dyeing are: sodium sulphide and other salts, and acetic acid.

Vat dyes (Burkinshaw, 1990)

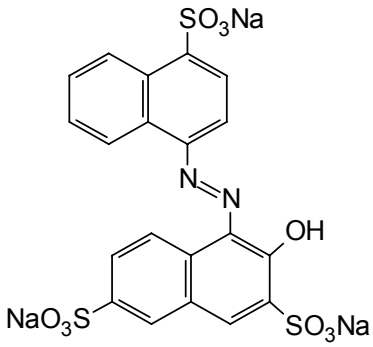
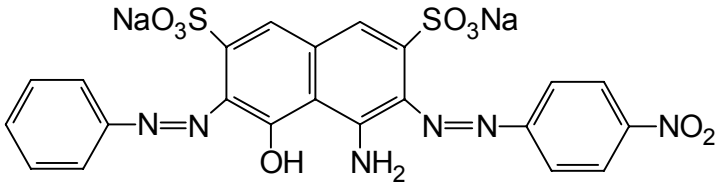
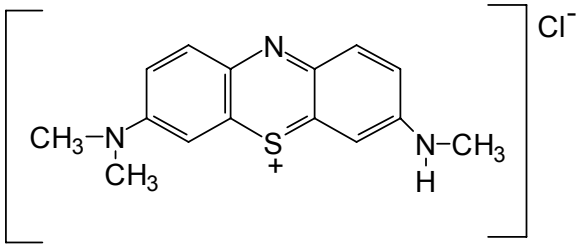
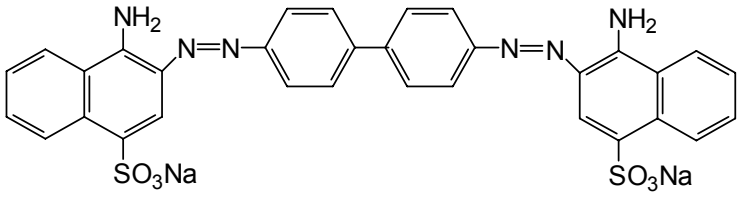
These are water-insoluble dyes which contain at least two conjugated carbonyl groups that enable the dye to be converted, by means of reduction under alkaline conditions, into the corresponding water-soluble ionised *leuco compound.* It is in this form that the dye is adsorbed by the substrate. Subsequent oxidation of the leuco compound *in situ* regenerates the parent insoluble vat dye within the fibre. The dyed material is then soaped in order to develop the true hue and optimum fastness properties of the dyeing. The principle use of vat dyes is in the dyeing of cellulosic fibres on which they provide a very wide range of hues that are generally of outstanding all-round fastness. Auxiliary

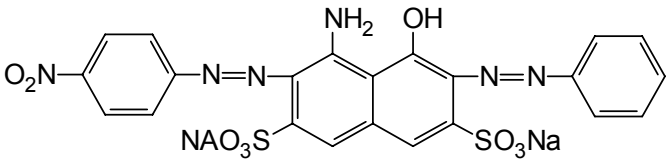
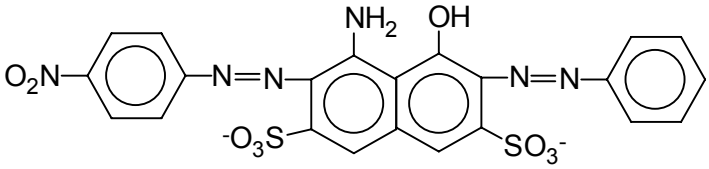
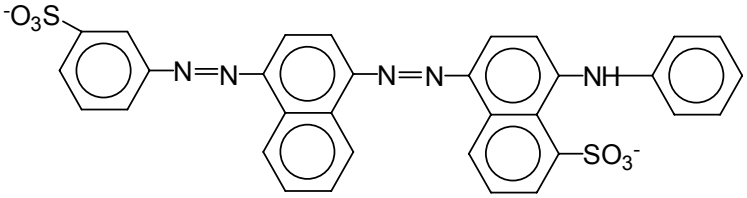
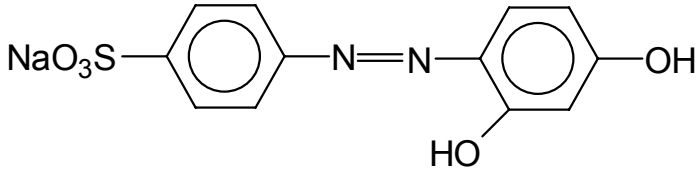
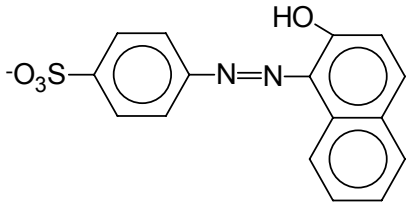
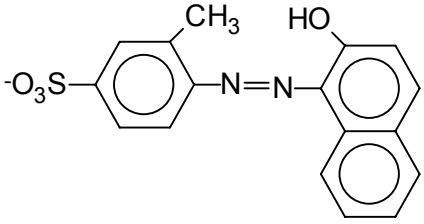
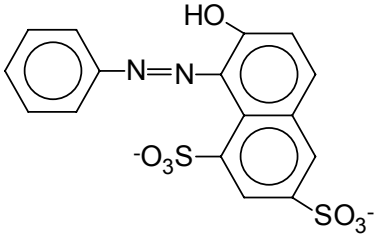
chemicals associated with vat dyeing are: sodium hydroxide, sodium hydrosulphite and other salts, and sufractants. The chemical classes of vat dyes may be divided into Indigoid and Thioindigoid, and Anthraquinone which is the largest and most important class of vat dyes.

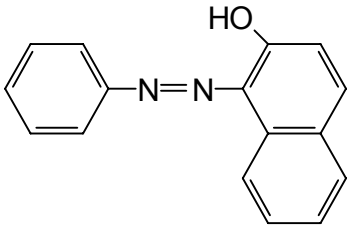
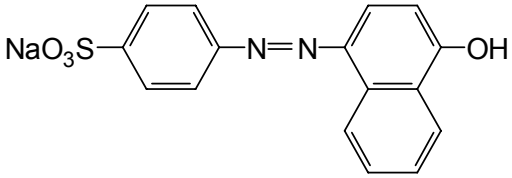
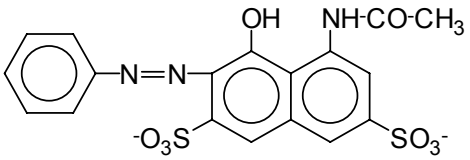
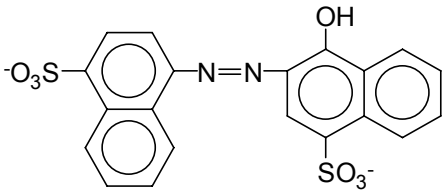
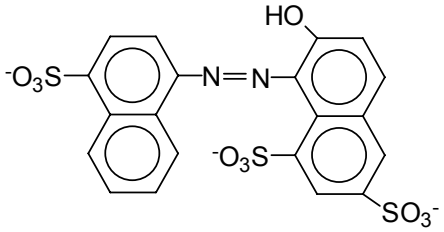
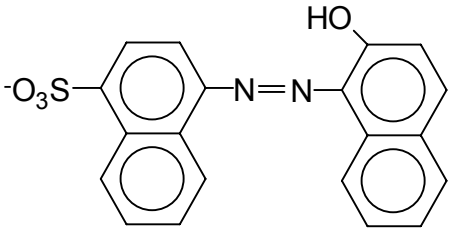
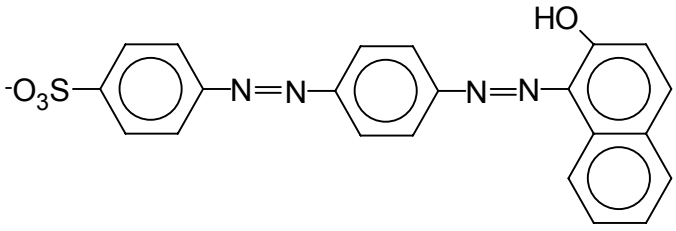
APPENDIX B

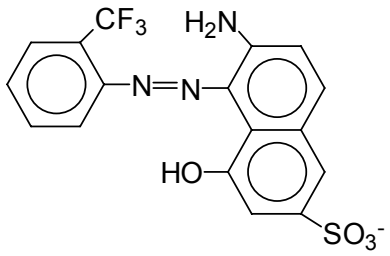
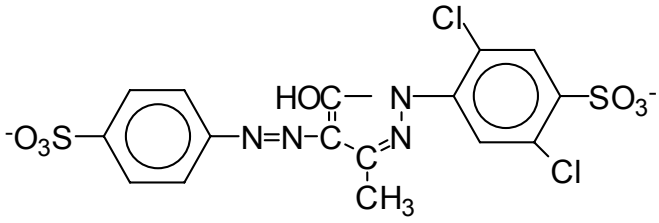
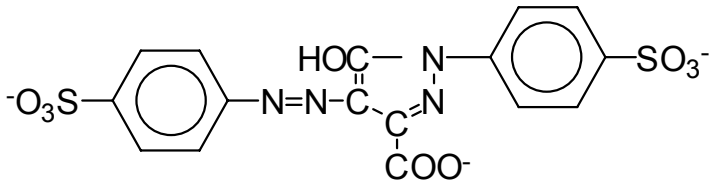
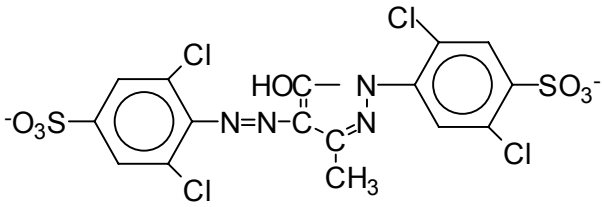
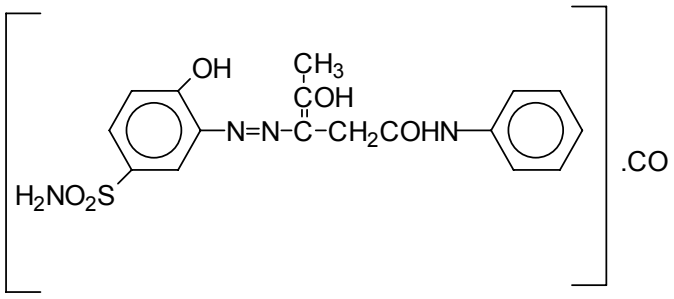
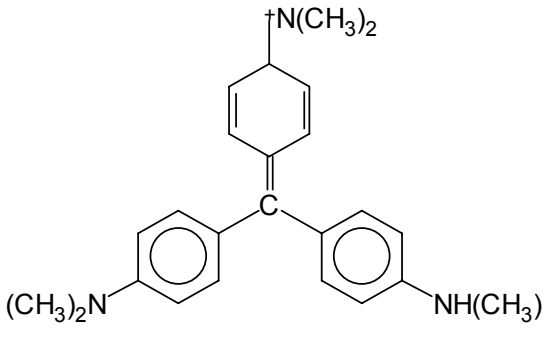
LIST OF DYES REFERRED TO IN LITERATURE REVIEW

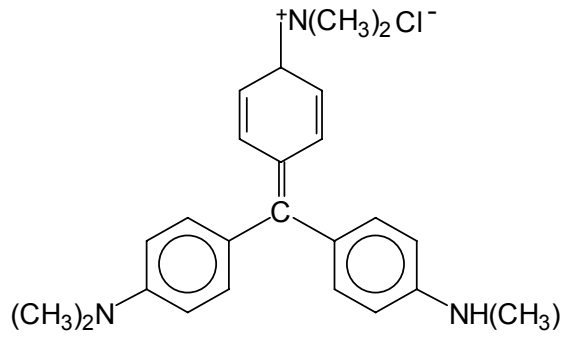
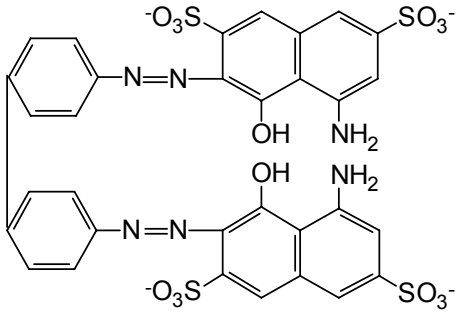
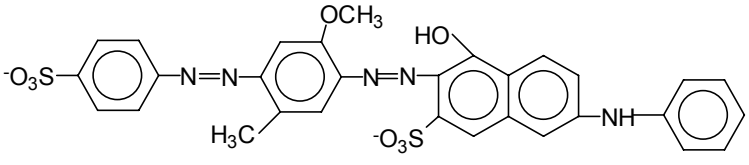
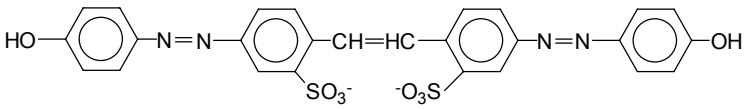
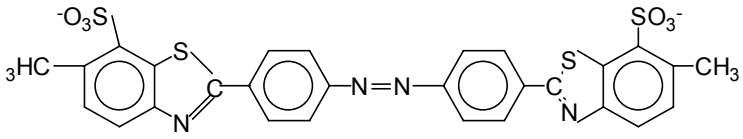
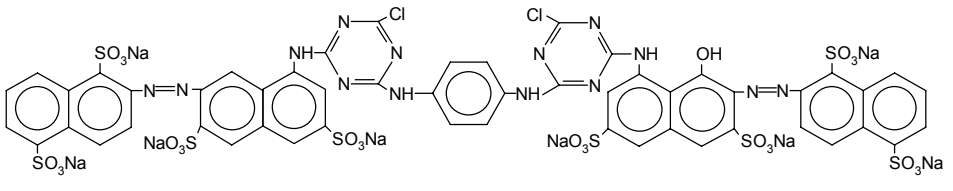
Appendix B lists the dyes discussed in the literature reviews in Chapters Two and Three. Dyes referred to by the Colour Index classification numbers are listed under CI ---, in alphabetical order. All other dyes are listed in alphabetical order.

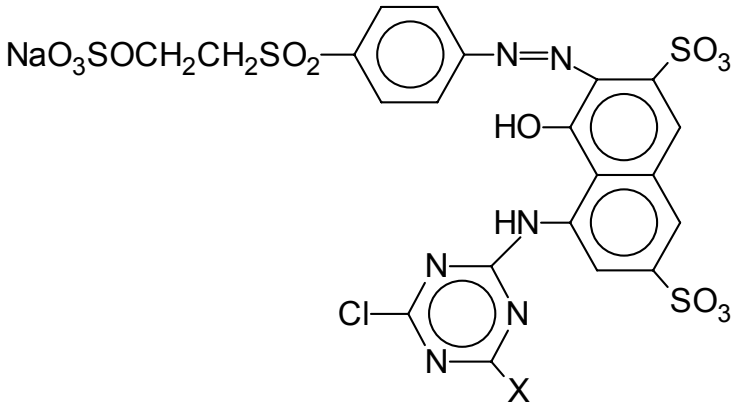
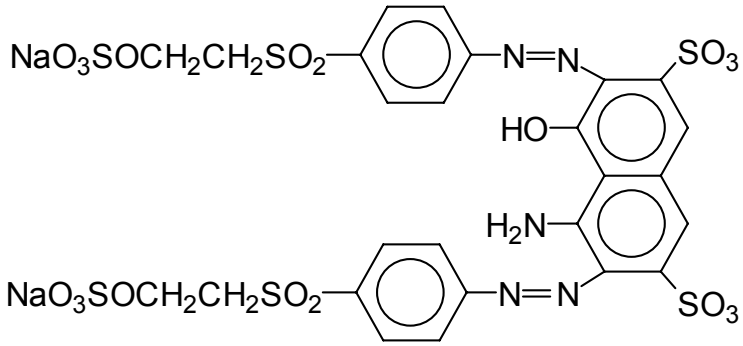
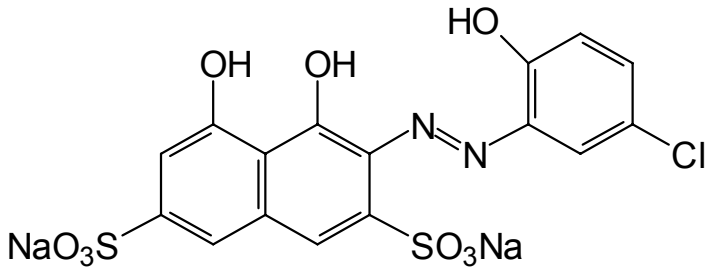
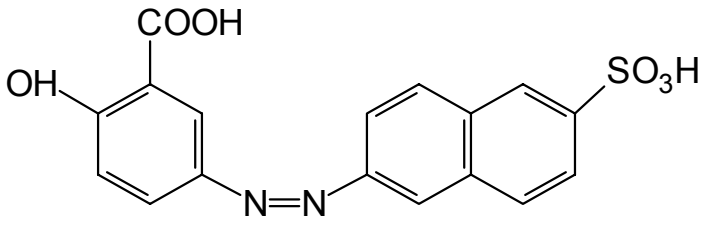
Dye name	Description	Structure
Allura Red	Azo dye	Structure not given in paper
Amaranth	Monoazo dye	
Amido Black	Disazo dye	
Azure B	Heterocyclic dye	
Congo Red	Disazo dye	

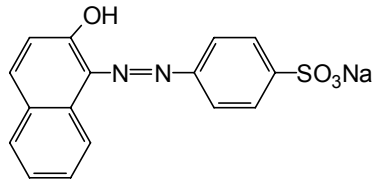
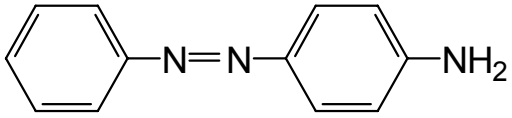
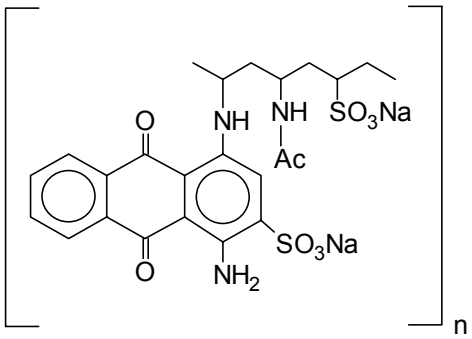
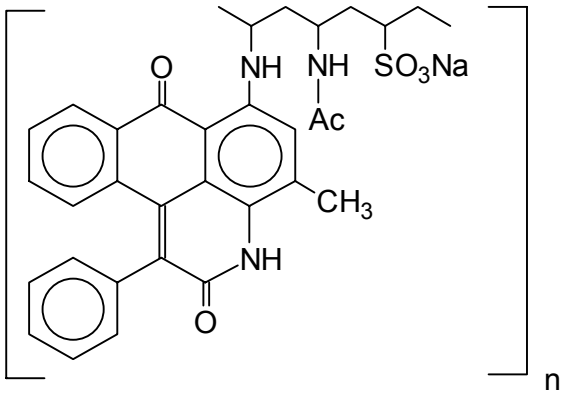
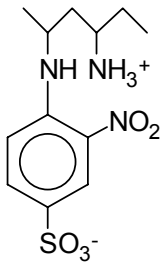
10 B (H/C)	Disazo dye	
CI Acid Black 1	Disazo dye	
CI Acid Blue 80	Anthraquinone dye	Structure not given in paper
CI Acid Blue 113	Disazo dye	
CI Acid Orange 6	Monoazo dye	
CI Acid Orange 7	Monoazo dye	
CI Acid Orange 8	Monoazo dye	
CI Acid Orange 10	Monoazo dye	

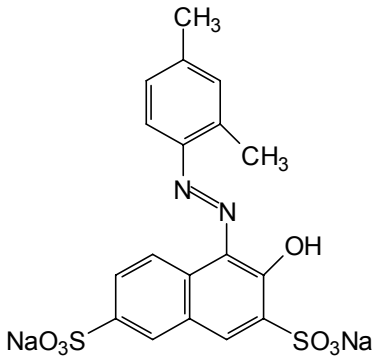
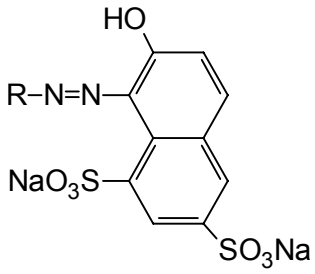
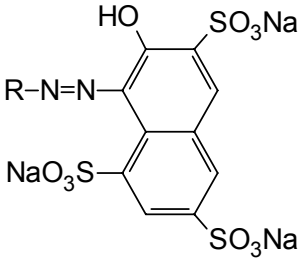
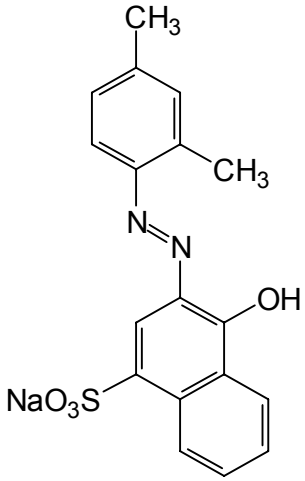
CI Acid Orange 12	Monoazo dye	
CI Acid Orange 20	Monoazo dye	
CI Acid Red 1	Monoazo dye	
CI Acid Red 14	Monoazo dye	
CI Acid Red 18	Monoazo dye	
CI Acid Red 88	Monoazo dye	
CI Acid Red 151	Disazo dye	

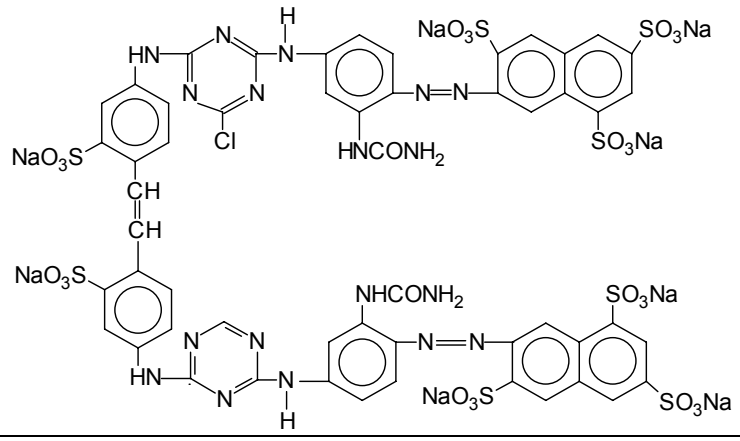
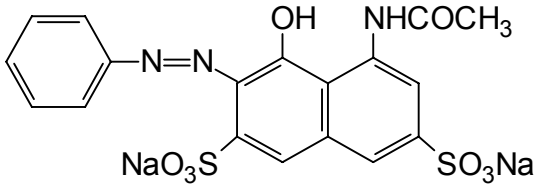
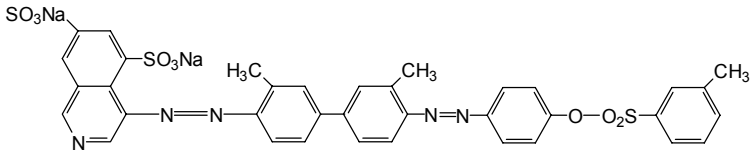
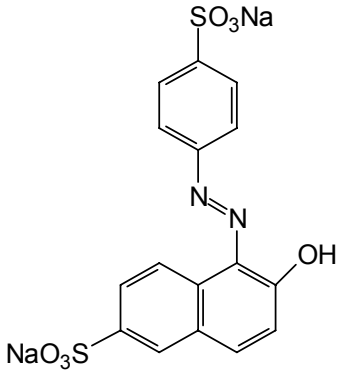
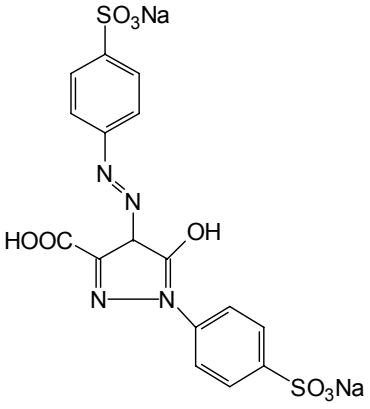
CI Acid Red 337	Disazo dye	
CI Acid Yellow 17	Monoazo dye	
CI Acid Yellow 23	Monoazo dye	
CI Acid Yellow 49	Monoazo dye	
CI Acid Yellow 151	Monoazo dye	
CI Basic Violet 1	Triphenylmethane dye	

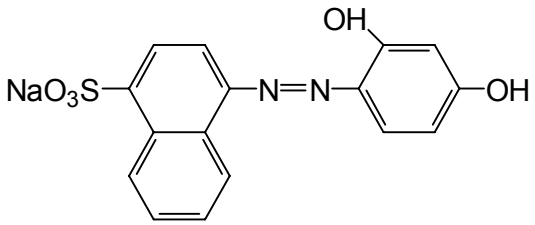
CI Basic Violet 3	Triphenylmethane dye	
CI Direct Blue 6	Disazo dye	
CI Direct Red 7	Disazo dye	Structure not given in paper
CI Direct Violet 9	Disazo dye	
CI Direct Yellow 4	Disazo dye	
CI Direct Yellow 28	Monoazo dye	
CI Reactive Red 141		

CI Reactive Red 198	Reactive monoazo dye	
CI Reactive Red X-3B	Azo dye	Structure not given in paper
CI Reactive Black 5	Reactive azo dye Commercial name is Remazol Black GFA	
Eriochrome Blue SE	Azo Dye	
Fast Red E		Structure not given in paper
Methyl Orange	Azo dye	Structure not given in paper
Mordant Yellow 3	Monoazo dye	

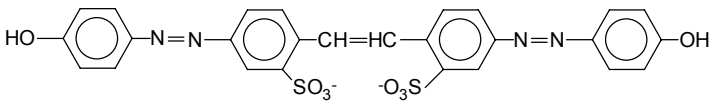
Navy 106	Mixture of 3 reactive dyes Remazol Golden yellow RNL, Remazol red RB and Remazol Black GFA.	Remazol Golden Yellow - Structure not given in paper
		Remazol Red RB (see CI Reactive Red 198)
		Remazol Black GFA (see CI Reactive Black 5)
Orange II	Azo dye	
p-Aminoazobenzen		
Poly B	Polymeric dye	
Poly R	Polymeric dye	
Poly Y	Polymeric dye	

Ponceau 3R	Monoazo dye	
Ponceau 4R	Monoazo dye	
Ponceau 6R	Monoazo dye	
Ponceau SX	Monoazo dye	
Procion Red G	Reactive azo dye manufactured by ICI	Structure not given in paper

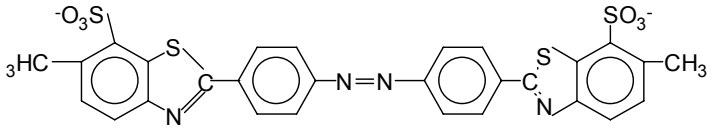
Reactofix Golden Yellow	Reactive azo dye	
Red 2G	Monoazo dye	
RS (H/C)	Disazo dye	
Sunset Yellow	Monoazo dye	
Tartrazine	Monoazo dye	

Tropeolin O	Monoazo dye	

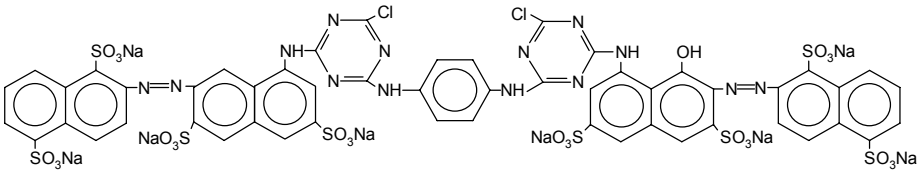
CI Direct Yellow 4



CI Direct Yellow 28



CI Reactive Red 141



Reactive Black 5

Navy 106 Mixture of 3 reactive dyes Remazol yellow, Remazol red and Remazol blue.

APPENDIX C

STANDARD ASSAY CONDITIONS FOR MEASUREMENT OF PROCION RED HE-7B DECOLOURISATION

C.1 INTRODUCTION

A number of experiments were made in order to determine the most satisfactory conditions for measurement of Procion Red HE-7B decolourisation rates. The rationale behind some of the conditions prescribed for the standard assay, as described in Section C.2, are briefly described in this introductory section.

Although decolourisation of Procion Red HE-7B was found to occur when no supplemental carbon source was added, this was a slow method and the results were difficult to replicate. The literature also supported the addition of a supplemental carbon source in order to obtain reliable estimates of dye reduction rates (Wuhrmann et al., 1980). It was, therefore, decided to measure all decolourisation of Procion Red HE-7B in the presence of a supplemental carbon source, namely, glucose. Further experimentation was done to determine an optimum glucose concentration for measurement of Procion Red HE-7B decolourisation and, from these results, it was decided that glucose should not be limiting at any stage in the decolourisation process.

Although precautions were taken to minimise oxygen contamination during preparation of the assay bottles, it was impossible to exclude all oxygen from the system. This resulted in a lag phase of approximately 15h prior to the onset of decolourisation. It was decided to minimise the lag phase by pre-incubating the assay bottles overnight (18 h) in the presence of glucose.

To determine the concentration of Procion Red HE-7B to be used in the standard assay, varying concentrations of the dye were investigated. It was found that, for initial concentrations below 100 mg/M, it was difficult to measure the rate of decolourisation accurately over the entire degradation period, as the majority of the readings were close to the lower limits of the analytical technique. For this reason, a standard dye concentration of 100 mg/M, recommended by the ETAD (method no. 105) for measurement of primary degradability of textile dyes in an anaerobic asssay system, was adopted as the reference dye concentration for measurement of decolourisation in the standard assay system.

C.2 EXPERIMENTATION

The experimentation section begins with a list of the materials required for the standard assay. Following this are the sections describing experimental and analytical procedures for the standard assay.

C.2.1 Materials

Mineral salts medium

A defined medium consisting of trace elements, minerals and vitamins is prepared according to Owen et al. (1979) with some modifications. The stock solutions for preparation of the medium are presented in **Table C.1,** and the method for preparation of the medium is presented in **Table C.2.**

Table C.1 : Stock solutions for preparation of mineral salts medium.

Stock solution	Composition	Concentration (mM)
S(1)	$(NH_4)_2HPO_4$	26.7000
S(2)	$CaCl_2.H_2O$	16.7000
	NH_4Cl	26.6000
	$MgCl_2.6H_2O$	120.0000
	KCl	86.7000
	$MnCl_2.4H_2O$	1.3300
	$CoCl_2.6H_2O$	2.0000
	H_3BO_3	0.3800
	$CuCl_2.2H_2O$	0.1800
	$Na_2MoO_4.2H_2O$	0.1700
	$ZnCl_2$	0.1400
S(3)	$FeCl_2.4H_2O$	370.0000
S(4)	Biotin	0.0020
	Folic acid	0.0020
	Riboflavin	0.0050
	Thiamin	0.0005
	Pantothenic acid	0.0005
	B_{12}	0.0001

Table C.2 : Instructions for preparation of defined medium

Step	Instruction	Volume (mM)	Mass (g)
1	Add 1M of distilled water to a 2 M volumetric flask		
2	Add the following to the volumetric flask :		
	Stock solution S(1)	5.4	

	Stock solution S(2)	27.0	
	Stock solution S(3)	1.8	
	Stock solution S(4)	18.0	
3	Add $NaHCO_3$ as powder		8.4
4	Add glucose as powder		2.0
4	Make up to volume with distilled water		

Procion Red HE-7B stock solution

The commercially available preparation of Procion Red HE-7B (ICI) is used in these experiments. A stock solution is prepared by adding 500 mg of Procion Red HE-7B powder to a 100 mM volumetric flask and making up to volume with distilled water, to give a concentration of 5 000 mg/M. A volume of 2 mM is added to each assay bottle to give an approximate concentration of 10 mg Procion Red HE-7B per 100 mM, i.e. 100 mg/M.

Inoculum

Inoculum for all assay experiments is obtained from the Procion Red HE-7B enrichment digester which contains acclimated biomass that has been exhausted of residual organic substrate. This biomass was incubated in the presence of Procion Red HE-7B for four months during which time the residual organic substrate in the digester sludge provided an additional carbon source for metabolic activity (Section 4.1.1).

Reaction vessels

Experiments are performed in serum bottles (120 mM, Aldrich Chemical Company) which are sealed with butyl rubber stoppers and aluminium crimp seals.

C.2.2 Experimental Procedure

A 30 % (v/v) inoculum is used per serum bottle which is equivalent to 30 mM of digester sludge (from the laboratory Procion Red HE-7B enrichment digester) in a total liquid volume of 100 mM, in a 120 mM serum bottle. The inoculum is centrifuged at 5 000 rpm for 20 min to remove excess liquid which may contain dye and/or degradation products. The centrate is decanted and the biomass pellet (from 30 mM of digester sludge) resuspended in 100 mM of mineral salts medium. The sludge/medium mixture is decanted into a serum bottle (120 mM, Aldrich Chemical Company).

Assay bottles are over-gassed with oxygen-free nitrogen (Fedgas) at a flow rate of 0.5mM/min for 15 min and sealed with butyl rubber stoppers and aluminium crimp seals. The assay bottles are pre-incubated for 18 h in a waterbath at 32 ^{o}C (without shaking). Upon completion of pre-incubation 2 mM of Procion Red HE-7B stock solution is added to each assay bottle by means of a hypodermic needle and glass syringe (2 mM) to give an initial dye concentration of 100 mg/M.

C.2.3 Analytical Procedure

Samples (2 mM) are withdrawn from the assay bottles by a hypodermic needle and syringe which is inserted through the butyl rubber septa of the assay bottles. Prior to sample removal, 3 mM of oxygen-free nitrogen is expelled into the assay bottle to maintain the pressure inside the assay bottles with the decreasing liquid volume. The samples are immediately centrifuged at 6 500 rpm for 25 min to remove any suspended material. The absorbance of the supernatant is read at the maximum wavelength of absorbance for Procion Red HE-7B (520 nm) using quartz microcuvettes (vol = 1.5 mM; path length = 10 mm, Zeiss) and a LKB Biochrom Ultraspec (model 4050). The absorbance figures are converted to the equivalent dye concentrations using a calibration curve of absorbance (520 nm) versus Procion Red HE-7B concentration **(Fig C.1)**

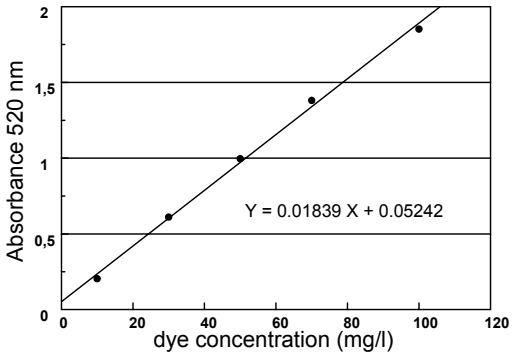


Fig C.1 : Calibration curve of Procion Red HE-7B (mg/M) versus absorbance measured at 520 nm.

APPENDIX D

DATA FOR SECTIONS 4.3.1 AND 4.3.2

D.1 INTRODUCTION

Appendix D contains experimental data for section 4.3.1: : *comparison of decolourisation rates for acclimated and unacclimated biomass* and section 4.3.2: *biological decolourisation of Procion Red HE-7B*.

D.2 EXPERIMENTAL DATA : COMPARISON OF DECOLOURISATION RATES FOR PROCION RED HE-7B BY ACCLIMATED AND UNACCLIMATED BIOMASS.

Table D.1 : Data from experiment to compare rates of decolourisation of Procion Red HE-7B by acclimated and unacclimated biomass.

	Procion Red HE-7B (mg/M)					
Time (h)	**Acclimated biomass**			**Unacclimated biomass**		
0.00	56.42	57.39	57.61	51.41	53.15	53.04
1.00	45.70	46.08	45.81	40.10	40.86	40.32
2.00	43.37	44.13	42.22	32.65	32.11	33.58
3.50	33.58	31.62	31.02	18.74	17.97	17.27
4.50	23.36	22.49	21.51	13.46	13.24	13.08
6.00	14.98	16.18	13.03	7.70	9.00	8.57
8.00	6.34	6.01	6.23	5.03	3.78	5.03
	$\ln(C_t/C_o)$					
	Acclimated biomass			**Unacclimated biomass**		
1.00	-0.21	0.22	0.23	-0.25	-0.26	-0.27
2.00	-0.26	-0.26	-0.31	-0.45	-0.50	-0.46
3.50	-0.52	-0.60	-0.62	-1.01	-1.08	-1.12
4.50	-0.88	-0.94	-0.99	-1.34	-1.39	-1.40
6.00	-1.33	-1.27	-1.49	-1.90	-1.78	-1.82
8.00	-2.19	-2.26	-2.22	-2.32	-2.64	-2.36
	R-Square = 0,954 (*y = -0,289x + 0,272*)			R-Square = 0,989 (*y = -0,318x + 0,083*)		

D.3 BIOLOGICAL DECOLOURISATION OF PROCION RED HE-7B

D.3.1 The Order of Procion Red HE-7B Decolourisation with Respect to Dye Concentration

The data in Table D.2 is presented in Fig's 4.5 and 4.6, Section 4.3.2, Chapter Four.

Table D.2 : Experimental data used to determine the order of Procion Red HE-7B decolourisation, with respect to dye concentration.

Time	**Procion Red HE-7B (mg/M)**								
	Initial concentration 100 mg/M			**Initial concentration 150 mg/M**			**Initial concentration 200 mg/M**		
	100 ,00	100,00	100,00	150,00	150,00	150,00	200,00	200,00	200,00
0.25	64.03	62.02	62.89	89.31	84.04	89.96	124.90	127.00	122.40
1.00	45.38	43.37	47.17	71.20	63.27	65.71	95.00	92.00	100.54
2.00	35.16	31.19	38.74	56.47	54.08	54.89	79.90	78.60	82.95
4.25	15.09	14.49	17.38	27.87	22.81	25.31	48.86	42.82	51.14
5.25	8.73	8.40	10.20	16.18	17.16	15.04	27.00	26.46	38.36
6.25	4.98	4.92	6.07	10.36	11.61	11.45	26.89	18.03	25.59
7.50	3.13	4.11	5.52	6.94	8.40	9.17	19.17	13.84	21.62
9.25	1.06	0.74	1.44	3.13	5.74	3.95	14.22	9.66	16.02
10.25				3.40	4.60	3.51	8.30	7.92	11.29
12.00				1.83	2.91	2.53	6.94	6.01	8.79
$\ln (C_t/C_o)$									
	Initial dye concentration (mg/M)								
	100,00	**100,00**	**100,00**	**150,00**	**150,00**	**150,00**	**200,00**	**200.00**	**200.00**
0.25	-0.340	-0.360	-0.290	-0.230	-0.280	-0.310	-0.270	-0.320	-0.200
1.00	-0.600	-0.690	-0.480	-0.460	-0.440	-0.490	-0.450	-0.480	-0.390
2.00	-1.450	-1.450	-1.290	-1.160	-1.300	-1.270	-0.940	-1.090	-0.870
4.25	-1.990	-2.000	-1.820	-1.710	-1.590	-1.790	-1.530	-1.570	-1.160
5.25	-2.550	-2.530	-2.340	-2.150	-1.980	-2.060	-1.540	-1.950	-1.570
6.25	-3.020	-2.710	-2.430	-2.560	-2.300	-2.280	-1.870	-2.220	-1.730
7.50	-4.100	-4.430	-3.770	-3.350	-2.680	-3.130	-2.170	-2.580	-2.030
9.25				-3.270	-2.910	-3.240	-2.710	-2.780	-2.380
10.25				-3.890	-3.360	-3.570	-2.890	-3.050	-2.630
12.00							-3.700	-3.900	-3.240
	R-Square = 0,959			**R-Square = 0,975**			**R-Square = 0,962**		
	y = -0,441x + 0,301			***y = -0,316x + 0,04***			***y = -0,252x + 0,003***		

D.3.2 Procion Red HE-7B Decolourisation with Permeabilised Cells

Table D.3 : Experimental data for decolourisation of Procion Red HE-7B by permeabilised and non-permeabilised microorganisms

	Procion Red HE-7B (mg/M)										
Time (h)	**NP**	**NP**	**NP**	**PB**	**PB**	**PB**	**PB AVG**	**P**	**P**	**P**	**P AVG**
0.08	84.14	86.70	92.52			87.57	87.57		85.12	90.34	87.73
1.00	83.66	86.70	89.42	89.36	85.34	86.21	86.97		82.84	84.69	83.77
3.00	71.26	75.50	80.18	81.70	82.95	83.44	82.70	81.43	80.72	83.60	81.92
5.00	51.41	56.25	59.24	79.74	80.88	82.13	80.92	80.12	79.52	80.23	79.96
7.00	33.25	41.35	42.06	78.00	79.96	81.86	79.94	78.54	78.44	79.85	78.94
9.00	25.59	31.84	31.73	78.22	79.96	80.67	79.62	78.16	79.47	78.93	78.85
13.00	19.99	21.67	21.51								
17.00	7.53	9.11	10.25								
25.00				76.04	77.95	79.74	77.91	75.66	75.17	75.77	75.53

NP = non-permeabilised biomass

PB = permeabilised biomass suspended in phosphate buffer

P = permeabilised biomass suspended in the aqueous phase of the permeabilisation solution

The experimental data presented in Table D.3 is presented in Fig 4.7, Section 4.3.2, Chapter Four.

D.3.3 Rate of Decolourisation of Procion Red HE-7B as a Sole Carbon Source

Table D.4 : Experimental data for decolourisation of Procion Red HE-7B as a sole carbon source.

	Procion Red HE-7B (mg/M)						
Time	**Controls (glucose)**			**Time**	**Experimental bottles (no additional carbon source)**		
0.25	66.83	64.81	65.68	0.25	80.50	81.32	77.13
1.00	48.18	46.16	49.97	2.00	81.05	83.33	80.45
2.00	37.95	33.98	41.54	22.00	72.84	70.99	71.91
4.25	17.89	17.29	20.17	30.00	55.82	56.03	54.35
5.25	11.53	11.20	13.00	48.00	57.88	56.52	58.37
6.25	7.78	7.72	8.86	77.00	45.49	38.58	42.88
7.50	5.93	6.91	8.32	125.00	22.16	26.67	23.41
9.25	3.86	3.54	4.24	144.00	19.22	19.12	17.70
				168.00	13.19	11.88	15.09
				192.00	9.60	5.03	10.63
				235.00	8.57	2.53	9.27
	ln (C_t/C_o)						
Time	**Control**			**Time**	**Experimental bottles (no additional carbon source)**		
1.00	-0.330	-0.340	-0.270	2.00	0.040	0.020	0.040
2.00	-0.570	-0.650	-0.460	22.00	-0.100	-0.140	-0.070
4.25	-1.320	-1.320	-1.180	30.00	-0.370	-0.370	-0.350
5.25	-1.760	-1.760	-1.620	48.00	-0.330	-0.360	-0.280
6.25	-2.150	-2.130	-2.000	77.00	-0.570	-0.750	-0.590
7.50	-2.420	-2.240	-2.070	125.00	-1.290	-1.110	-1.190
9.25	-2.850	-2.910	-2.740	144.00	-1.430	-1.450	-1.470
				168.00	-1.810	-1.920	-1.630
				192.00	-2.130	-2.780	-1.980
				235.00	-2.240	-3.470	-2.120
	R-Square = 0,959				**R-Square = 0,924**		
	y = -0,441x + 0,301				***y = -0,011x + 0,141***		

The data in Table D.4 is presented in Fig 4.8, Section 4.3.2, Chapter Four.

D.3.4 Rate of Decolourisation of Procion Red HE-7B in the Presence of Additional Electron Acceptors

Table D.5 : Data for measurement of Procion Red HE-7B decolourisation in the presence of 1, 5 and 10 mM nitrate.

	Procion Red HE-7B (mg/M)								
Time (h)	**Control (no nitrate)**			**Avg**	**Time (h)**	**1 mM nitrate**			**Avg**
0.25	56.42	57.39	57.61	57.14	0.25	54.78	55.06	51.36	53.73
1.00	45.70	46.08	45.81	45.86	1.00	45.87	46.63	45.49	45.99
2.00	39.61	39.02	38.69	39.11	2.00	45.43	45.32	45.54	45.43
3.50	33.58	31.62	31.02	32.07	3.00	45.27	45.54	46.08	45.63
4.50	23.36	22.49	21.51	22.45	4.00	37.98	38.47	36.24	37.56
6.00	14.98	16.18	13.03	14.73	5.00	33.04	33.80	28.79	31.88
8.00	6.34	6.01	6.23	6.19	6.00	29.23	28.74	23.57	27.18
10.00	3.13	2.42	3.73	3.09	7.00	22.16	23.08	16.67	20.64
					9.25	13.90	15.36	9.93	13.06
					13.00	2.86	3.18	2.48	2.84
Time (h)	**5 mM Nitrate**			**Avg**	**Time (h)**	**10 mM Nitrate**			**Avg**
0.25	53.37	52.07	52.88	52.77	0.25	55.00	53.97	57.61	55.53
1.00	47.82	45.92	45.81	46.52	1.00	49.07	49.35	50.60	49.67
2.00	46.19	45.70	45.87	45.92	2.00	47.55	48.69	49.89	48.71
8.00	42.99	43.75	42.33	43.02	3.50	46.57	48.20	48.97	47.91
9.00	43.42	40.81	42.12	42.12	4.50	44.78	45.27	45.76	45.27
10.00	40.92	37.87	37.66	38.82	6.00	43.37	43.64	45.00	44.00
11.00	33.74	26.78	27.71	29.41	8.00	45.11	43.26	44.62	44.33
12.00	29.07	21.02	22.49	24.19	22.00	45.59	42.93	44.40	44.31
13.00	24.28	18.03	19.12	20.48	23.00	39.56	37.66	38.85	38.69
15.00	14.60	14.11	13.46	14.06	24.00	33.14	33.20	31.73	32.69
17.00	11.18	8.08	7.86	9.04	26.00	17.81	18.35	16.18	17.45
19.00	8.89	5.58	4.27	6.25	27.00	16.18	16.23	14.39	15.60
20.50	5.52	2.70	2.53	3.58	29.00	9.71	9.11	7.75	8.86
					31.00	4.87	5.09	4.16	4.71
					33.00	3.24	3.13	3.35	3.24

Table D.6 : Data for measurement of the rates of decolourisation of Procion Red HE-7B in the presence of nitrate.

	$\ln(C_t/C_o)$						
Time	**Control**			**Time**	**1 mM nitrate**		
1.00	-0.21	-0.22	-0.23	4.00	-0.18	-0.17	-0.23
2.00	-0.35	-0.39	-0.40	5.00	-0.32	-0.30	-0.46
3.50	-0.52	-0.60	-0.62	6.00	-0.44	-0.46	-0.66
4.50	-0.88	-0.94	-0.99	7.00	-0.71	-0.68	-1.01
6.00	-1.33	-1.27	-1.49	9.25	-1.18	-1.09	-1.53
8.00	-2.19	-2.26	-2.22	13.00	-2.76	-2.66	-2.91
10.00	-2.89	-3.16	-2.74				
	R-Square = 0,964				**R-Square = 0,928**		
	y = -0,309x + 0,31				***y = -0,244x + 0,88***		
Time (h)	**5 mM nitrate**			**Time (h)**	**10 mM nitrate**		
10.00	-0.06	-0.07	-0.11	23.00	-0.14	-0.13	-0.13
11.00	-0.25	-0.42	-0.42	24.00	-0.32	-0.26	-0.34
12.00	-0.40	-0.66	-0.63	26.00	-0.94	-0.85	-1.01
13.00	-0.58	-0.82	-0.79	27.00	-1.04	-0.97	-1.13
15.00	-1.09	-1.06	-1.14	29.00	-1.55	-1.55	-1.75
17.00	-1.36	-1.62	-1.68	31.00	-2.24	-2.13	-2.37
19.00	-1.59	-1.99	-2.29	33.00	-2.64	-2.62	-2.58
20.50	-2.06	-2.72	-2.81				
	R-Square = 0,943				**R-Square = 0,989**		
	y = -0,218x + 2,09				***y = 0,256x + 5,79***		

Data from Table D.5 and D.6 are presented in Fig's 4.9 and 4.10, Section 4.3.2, Chapter Four.

Table D.7 : Data for measurement of Procion Red HE-7B decolourisation in the presence of sulphate									
	Procion Red HE-7B (mg/M)								
Time (h)	**Control (no sulphate)**			**5 mM sulphate**			**10 mM sulphate**		
0.25	56.96	54.19	56.74	56.14	59.41	56.09	60.60	56.96	59.24
1.00	42.88	43.80	45.00	45.05	44.73	43.42	45.87	45.27	45.54
2.00	39.45	40.27	42.17	41.41	40.32	38.64	41.19	39.56	40.27
3.00	35.97	38.04	35.92	38.04	35.43	34.88	37.11	35.43	35.43
4.00	30.75	31.51	33.96	35.10	31.95	31.51	34.12	31.73	31.95
6.00	24.82	26.89	28.90	29.77	28.14	26.95	29.88	29.45	29.77
10.00	9.11	10.20	10.20	12.37	11.29	12.37	12.37	12.37	10.74
13.00	5.31	7.32	6.66	4.76	6.39	5.85	6.94	5.31	4.22
	ln Ct/Co								
Time	**Control (no sulphate)**			**5 mM sulphate**			**10 mM sulphate**		
1.00	-0.23	-0.21	-0.23	-0.22	-0.28	-0.26	-0.28	-0.23	-0.26
2.00	-0.31	-0.30	-0.30	-0.30	-0.39	-0.37	-0.39	-0.36	-0.39
3.00	-0.40	-0.35	-0.46	-0.39	-0.52	-0.47	-0.49	-0.47	-0.51
4.00	-0.56	-0.54	-0.51	-0.47	-0.62	-0.58	-0.57	-0.59	-0.62
6.00	-0.77	-0.70	-0.67	-0.63	-0.75	-0.73	-0.71	-0.66	-0.69
10.00	-1.77	-1.67	-1.72	-1.51	-1.66	-1.51	-1.59	-1.53	-1.71
13.00	-2.31	-2.00	-2.14	-2.47	-2.23	-2.26	-2.17	-2.37	-2.64
	R-Square = 0,945			**R-Square = 0,944**			**R-Square = 0,949**		
	y = -0,208x + 0,114			***y = -0,207x + 0,077***			***y = -0,208x + 0,067***		

Data from Table D.7 are presented in Fig 4.11, Section 4.3.2, Chapter Four.

D.3.5 The Role of Redox Potential in the Microbial Decolourisation of Procion Red HE-7B

The raw data for this experiment has not been included as each figure (4.12, 4.13 and 4.14) has in excess of 500 data points.

Appendix E

DATA FROM SECTION 4.3.3

Appendix E contains data for Section 4.3.3 : *The abiotic decolourisation of Procion Red HE-7B*.

E.1 ADSORPTION OF PROCION RED HE-7B IN MINERAL SALTS MEDIUM AND SALINE SOLUTION

Table E.1 : Data from experiment to determine the extent of adsorption of Procion Red HE-7B to a sludge adsorbent, measured in mineral salts medium and saline solution.

	Procion Red HE-7B (mg/M)						
Time (h)	**Adsorption in saline solution**			**Time (h)**	**Adsorption in mineral salts medium**		
0.00	100.00	100.00	100.00	0.00	100.00	100.00	100.00
0.25	94.91	96.05	95.48	0.25	63.81	67.56	64.30
1.00	88.44	89.53	88.98	0.50	60.44	63.43	61.25
2.00	83.93	85.34	84.63	0.75	58.70	59.84	58.26
3.00	80.83	82.51	81.67	1.00	58.43	61.58	58.70
4.00	76.75	78.98	77.86	2.00	51.52	52.23	52.61
5.00	76.42	77.95	77.19	3.00	44.51	46.79	46.90
6.00	75.17	77.84	76.51	4.00	43.09	46.19	46.63
7.00	75.88	74.74	75.31	5.00	43.37	46.68	46.25
8.00	78.11	74.58	76.34	6.00	43.20	46.79	46.14
24.00	77.40	75.12	76.26				

The data in Table E.1 is presented in Fig 4.15, Section 4.3.3, Chapter Four.

E.2 PROCION RED HE-7B ADSORPTION ISOTHERM

Table E.2 : Representation of Procion Red HE-7B adsorption data by the Freundlich isotherm

c (mg/ M)	**x (mg/g)**			**x avg**	**ln c**	**ln x**		
5,36	0.87	0.84	0.92	0.88	1.68	- 0,14	- 0,17	- 0,08
10.61	1.31		1.18	1.25	2.36	0.27		0.17
16.07	1.65	1.71	1.63	1.66	2.78	0.50	0.54	0.49
20.64	1.81	1.86	1.76	1.81	3.03	0.59	0.62	0.57
25.79	1.81	1.73	2.00	1.85	3.25	0.59	0.55	0.69
						R-Square = 0,955		
						y = 0,504x + 0,96		

Data from Table E.2 is presented in Fig's 4.16 and 4.17, Section 4.3.3, Chapter Four.

Table E.3 : Adsorption data for Procion Red HE-7B with anaerobic biomass as the adsorbent, represented by the Langmuir isotherm.

c/x			**c (mg/M)**
3.09	3.19	2.91	5.36

4.04		4.49	10.61
4.86	4.71	4.94	16.07
5.70	5.54	5.87	20.64
7.12	7.44	6.46	25.79
R-Square = 0,957 ***y = 0,184x + 2,06***			

E.3 DECOLOURISATION OF PROCION RED HE-7B IN MINERAL SALTS MEDIUM

Table E.4. : Data from experiment to measure the extent of decolourisation caused by incubation of Procion Red HE-7B in sterile mineral salts medium.

	Procion Red HE-7B (mg/M)											
Time (h)	**Initial concentration 100 mg/M**			**Avg**	**Initial concentration 150 mg/M**			**Avg**	**Initial concentration 200 mg/M**			**Avg**
0.00	100	100	100	100	150	150	150	150	200	200	200	200
0.25	53.37	55.00	54.12	54.16	82.24	83.06	84.13	83.14	111.53	112.50	111.25	111.76
0.50	53.37	55.16	54.03	54.18	81.48	82.08	82.54	82.03	107.61	109.46	108.53	108.53
0.75	53.59	55.16	54.57	54.44	82.40	81.70	80.60	81.57	110.11	112.29	110.87	111.09
1.00	52.99	55.16	53.29	53.81	81.75	80.99	80.23	80.99	104.57	107.72	105.47	105.92
2.00	50.11	52.07	52.76	51.64	78.27	78.11	79.57	78.65	104.89	107.72	106.35	106.32
4.00	50.98	53.59	52.89	52.48	77.73	79.85	78.30	78.63	107.61	108.15	106.20	107.32
8.00	48.97	51.20	50.90	50.35	75.99	75.23	76.25	75.82	103.04	106.96	104.76	104.92
10.00	47.77	51.03	49.67	49.49	75.55	76.26	76.10	75.97	99.35	105.44	102.50	102.43

Appendix F

DATA FOR SECTIONS 4.3.4 AND 4.3.5

Appendix F contains calculations and experimental data from Section 4.3.4 : *Identification and fate of Procion Red HE-7B degradation products in an anaerobic system* and Section 4.3.5 : *Inhibitory effects of Procion Red HE-7B on an anaerobic microbial population*.

F.1 THEORETICAL GAS VOLUME CALCULATIONS

The Buswell equation (Tarvin and Buswell, 1934) was used to calculate the theoretical gas volumes for mineralisation of Procion Red HE-7B.

$$C_nH_aO_b + \left[n - \frac{a}{4} - \frac{b}{2}\right]H_2O \rightarrow \left[\frac{n}{2} - \frac{a}{8} + \frac{b}{4}\right]CO_2 + \left[\frac{n}{2} + \frac{a}{8} - \frac{b}{4}\right]CH_4$$

$$C_{46}H_{22}O_{25} + \left[46 - \frac{22}{4} - \frac{25}{2}\right]H_2O \rightarrow \left[\frac{46}{2} - \frac{22}{8} + \frac{25}{4}\right]CO_2 + \left[\frac{46}{2} + \frac{22}{8} - \frac{25}{4}\right]CH_4$$

Therefore **1 mole Procion Red HE-7B** yields **26,5 mole CO_2** and **19,5 mole CH_4**.

<u>To calculate the volume of gas (CH_4 + CO_2) expected for mineralisation of 1 mole of Procion Red HE-7B :</u>

$$V = \frac{nRT}{P}$$

CO_2 : V = 26,5 x 37,22 x 305 / 101325

V = 2,96896 kM CO_2

CH_4 : V = 19,5 x 35,71 x 305 / 101325

V = 2,09608 kM CH_4

It was neccessary to correct these volumes for solubility :

2,96896 kM CO_2 x 0,35 = 1,039136 kM CO_2

2,09608 kM CH_4 x 0,95 = 1,991276 kM CH_4

Therefore, a total volume of **3,03412 kM** digester gas would be expected from an anaerobic digester mineralising 1 mole of Procion Red HE-7B.

The molecular weight of Procion Red HE-7B = **1634 g/mol**.

<u>Therefore the number of moles in each of the assay bottles was :</u>

20 mg/M = 2 mg per assay bottle (100 mM) = 1,22399 x 10^{-6} moles

50 mg/M = 5 mg per assay bottle (100 mM) = 3,05997 x 10^{-6} moles

100 mg/M = 10 mg per assay bottle (100 mM) = 6,11995 x 10^{-6} moles

200 mg/M = 20 mg per assay bottle (100 mM) = 1,22399 x 10^{-5} moles

500 mg/M = 50 mg per assay bottle (100 mM) = 3,05997 x 10^{-5} moles

Table F.1 : Theoretical gas volumes for mineralisation of Procion Red HE-7B in assay bottles.					
	Procion Red HE-7B (mg/M)				
	20 mg/M	**50 mg/M**	**100 mg/M**	**200 mg/M**	**500 mg/M**
	Mineralisation of entire dye molecule				
Methane (mM)	2.43	6.05	12.17	24.33	60.83
Carbon dioxide (mM)	1.27	3.18	6.35	12.70	31.75
Total volume (mM)	**3.70**	**9.26**	**18.52**	**37.03**	**92.58**

F.2 EXPERIMENTAL DATA FROM THE ANAEROBIC TOXICITY ASSAY

Table F.2 : Digester gas volumes for assay bottles containing acclimated biomass										
	Time (d)									
	1	**2**	**4**	**8**	**13**	**17**	**23**	**30**	**38**	**52**
	Digester gas (mM)									
Controls	24.00	31.00	36.50	44.00	70.00	102.00	147.00	168.00	174.00	178.00
	28.00	36.00	43.00	50.50	76.50	111.00	158.00	179.00	185.50	189.50
	28.00	35.00	41.50	49.50	77.50	111.50	158.50	179.50	186.00	190.00
Avg	**26.70**	**34.00**	**40.30**	**48.00**	**74.70**	**108.20**	**154.50**	**175.50**	**181.80**	**185.80**
20 mg/M	22.00	29.50	36.00	45.00	71.00	105.50	152.50	172.50	178.00	182.00
	22.00	31.00	39.50	48.50	73.50	107.50	154.50	175.50	182.50	186.50
	21.50	30.00	38.50	47.50	72.50	110.00	156.00	176.00	182.00	186.00
Avg	**22.00**	**30.30**	**37.80**	**46.80**	**72.30**	**106.50**	**153.50**	**174.00**	**180.30**	**184.30**
50 mg/M	26.00	33.50	41.50	50.50	76.50	113.50	158.50	180.50	187.50	191.50
	24.00	31.00	38.50	47.00	71.00	108.50	155.00	176.00	182.00	186.00
	20.00	28.00	36.50	45.50	70.50	106.50	152.50	174.50	181.50	186.50
Avg	**23.30**	**30.80**	**38.80**	**47.70**	**72.70**	**109.50**	**155.30**	**177.00**	**183.70**	**188.00**
100 mg/M	19.00	28.50	35.50	44.00	71.00	106.00	150.00	172.00	179.50	183.50
	22.00	30.20	38.20	46.70	68.70	105.70	138.70	160.70	166.70	170.70
	23.00	32.00	40.00	48.50	72.50	109.50	155.50	177.50	184.50	188.50
Avg	**21.30**	**30.20**	**37.90**	**46.40**	**70.70**	**107.10**	**148.10**	**170.10**	**176.90**	**180.90**
200 mg/M	25.00	32.00	38.50	46.00	66.00	96.00	144.00	170.00	178.00	182.00
	27.00	34.00	41.00	48.90	68.90	99.90	149.90	175.90	182.40	186.40
	28.00	35.50	42.00	49.00	69.00	100.00	151.00	178.00	186.00	191.00
Avg	**26.70**	**33.80**	**40.50**	**48.00**	**68.00**	**98.60**	**148.30**	**174.60**	**182.10**	**186.50**
500 mg/M	19.00	22.00	27.50	35.00	57.00	84.00	134.00	161.00	169.00	174.00
	26.00	35.00	40.50	47.50	67.50	93.50	147.50	173.50	181.50	186.50
	25.00	32.00	38.00	45.00	65.00	90.00	143.00	173.00	181.00	186.00
Avg	**23.30**	**29.70**	**35.30**	**42.50**	**63.20**	**89.20**	**141.50**	**169.20**	**177.20**	**182.20**

Data in Table F.2 is presented in Fig 4.22 and Fig 4.24, Section 4.2.5, Chapter Four.

Table F.3 : Digester gas volumes for assay bottles with unacclimated biomass										
	Time (d)									
	1	**2**	**4**	**8**	**13**	**17**	**23**	**30**	**38**	**52**
	Digester gas (mM)									
Controls	21.00	27.00	33.00	37.50	43.50	50.50	68.50	88.50	125.50	205.50
	19.00	26.50	32.50	36.50	43.50	53.50	86.50	124.50	150.50	176.50
	19.50	27.50	33.50	37.50	43.50	51.00	69.00	89.00	127.00	177.00
Avg	**19.83**	**27.00**	**33.00**	**37.17**	**43.50**	**51.67**	**74.67**	**100.67**	**134.33**	**186.33**
20 mg/M	19.00	25.00	32.00	37.00	43.00	51.00	70.00	102.00	142.00	176.00
	18.50	24.50	28.00	33.00	39.00	46.50	64.50	83.50	121.50	171.50
	20.00	24.50	29.00	33.50	40.50	48.50	66.50	88.50	136.50	176.50
Avg	**19.17**	**24.67**	**29.67**	**34.50**	**40.83**	**48.67**	**67.00**	**91.33**	**133.33**	**174.67**
50 mg/M	19.00	40.00	50.00	55.00	61.00	67.00	84.00	103.00	142.00	196.00
	19.00	42.00	50.50	56.50	62.50	68.50	86.00	108.00	155.00	195.00
	20.00	45.50	54.50	60.00	67.00	78.00	106.00	149.00	178.00	218.00
Avg	**19.33**	**42.50**	**51.67**	**57.17**	**63.50**	**71.17**	**92.00**	**120.00**	**158.33**	**203.00**
100 mg/M	22.00	29.00	34.00	38.00	43.50	49.50	62.50	79.50	103.50	161.50
	23.00	30.00	35.00	39.00	44.00	50.00	63.00	81.00	103.00	161.00
	23.00	29.50	34.50	39.50	44.50	50.00	62.00	79.00	107.00	165.00
Avg	**22.67**	**29.50**	**34.50**	**38.83**	**44.00**	**49.83**	**62.50**	**79.83**	**104.50**	**162.50**
200 mg/M	22.00	30.00	36.00	40.50	45.50	51.50	65.50	81.50	115.50	175.50
	21.50	27.50	33.50	38.50	44.00	49.00	62.00	78.00	108.00	166.00
	22.00	28.00	34.00	39.00	43.50	48.50	60.50	75.50	94.50	168.50
Avg	**21.83**	**28.50**	**34.50**	**39.33**	**44.33**	**49.67**	**62.67**	**78.33**	**106.00**	**170.00**
500 mg/M	21.50	29.00	35.00	40.00	59.00	82.00	120.00	145.00	167.00	171.00
	20.00	26.00	32.00	36.00	40.00	44.00	52.00	68.00	86.00	144.00
	20.00	26.00	31.80	35.80	40.30	44.30	52.30	76.30	120.30	170.30
Avg	**20.50**	**27.00**	**32.93**	**37.27**	**46.43**	**56.77**	**74.77**	**96.43**	**124.43**	**161.77**

Data in Table F.3 is presented in Fig 4.23 and Fig 4.25, Section4.3.5, Chapter Four.

Table F.4 : Methane production rates for acclimated biomass in the anaerobic toxicity assay.				
	Rate of gas production (mM/d)			
	Acclimated	**R-Square values**	**Unacclimated**	**R-Square values**
Control	7.198	0.981	3.920	0.885
20 mg/M	7.296	0.987	4.008	0.948
50 mg/M	7.353	0.984	4.154	0.843
100 mg/M	6.951	0.979	2.604	0.990
200 mg/M	6.801	0.973	2.657	0.938
500 mg/M	6.645	0.954	3.217	0.837

The data in Table F.4 was used to calculate the maximum rate ratios in Table 4.3, Section 4.3.5, Chapter Four.

Table F.5 : Methane production (%) values for acclimated and unacclimated biomass, during the toxicity assay												
	Time (d)											
Sample	**2 days**				**8 days**				**3 d**ays			
	accl	**Avg**	**unac**	**Avg**	**accl**	**Avg**	**unac**	**Avg**	**accl**	**Avg**	**unac**	**Avg**
Control	20.18	19.20	6.79	6.88	53.99	52.34	33.36	33.27	52.34	52.79	57.06	54.75
	17.80		6.92		52.16		33.17		53.49		55.67	
	19.62		6.93		50.86				52.55		51.52	
20 mg/l					47.49	46.41	30.45	30.89	53.92	54.36	50.32	50.07
					45.94		30.72		55.21		49.93	
					45.80		31.50		53.94		49.95	
50 mg/l					49.74	49.22	28.18	28.83	53.21	53.73	43.53	43.23
					48.92		28.81		54.33		42.59	
					49.01		29.51		53.64		43.57	
100 mg/l					47.72	46.81	37.09	37.26	54.67	54.14	42.75	42.53
					45.84		37.74		54.19		42.35	
					46.86		36.94		53.55		42.49	
200 mg/l					51.96	50.84	32.63	32.64	56.93	55.81	39.01	39.47
					50.88		32.67		55.83		39.68	
					49.68		32.63		54.67		39.72	
500 mg/l	13.26	13.85	5.15	5.68	48.57	48.34	31.69	31.75	55.67	56.27	53.92	53.57
	13.57		5.19		48.10		31.80		56.39		53.67	
	13.13		6.04						56.74		53.13	
	14.47		6.01									
	14.82		6.01									

Data from Table F.5 is presented in Fig's 4.26 and 4.27, Section 4.3.5, Chapter Four.

APPENDIX G

CHAPTER FIVE : EXPERIMENTAL DATA

G.1 DATA FROM ENRICHMENT SCHEMES

Table G.1 contains total gas production data from the serum bottle enrichment study, which utilised Smith and Nephew kier liquor as the substrate. The averages of the triplicate results in this table are presented in Fig 5.2, Chapter 5. Samples labelled 'C' are control samples which contained only inoculum and distilled water. Samples labelled 10 %, 50 % and 100 % are enrichment bottles which contained inoculum and effluent in 10, 50 or 100 % (v/v) concentrations, respectively.

Table G.1 : Gas production for Smith and Nephew serum bottle enrichments

	Sample												
Time (d)	**C AVG**	**10 % (1)**	**10 % (2)**	**10 % (3)**	**AVG (2&3)**	**50 % (1)**	**50 % (2)**	**50 % (3)**	**AVG**	**100% (1)**	**100% (2)**	**100% (3)**	**AVG**
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2.0	13.0	14.0	7.0	8.0	7.5	8.0	8.0	4.0	6.7	5.0	2.0	3.0	3.3
4.0	20.3	25.0	9.0	12.0	10.5	13.0	8.0	4.0	8.3	6.0	6.0	8.0	6.7
6.0	26.3	36.0	11.0	16.0	13.5	20.0	12.0	10.0	14.0	12.0	12.0	14.0	12.7
7.0	30.7	46.0	16.0	21.0	18.5	24.0	15.0	13.0	17.3	16.0	17.0	18.0	17.0
8.0	34.3	54.0	18.0	24.0	21.0	27.5	17.5	16.5	20.5	26.0	21.0	21.5	22.8
12.0	41.0	72.0	20.0	29.0	24.5	34.5	24.5	26.5	28.5	34.0	25.0	25.5	28.2
13.0	44.7	80.0	23.0	33.0	28.0	39.5	28.5	32.5	33.5	38.0	30.0	29.5	32.5
14.0	46.3	85.5	24.0	35.0	29.5	43.5	32.0	35.5	37.0	41.5	35.0	33.5	36.7
15.0	48.7	91.5	26.0	37.5	31.8	47.0	35.0	37.5	39.8	44.5	40.0	37.5	40.7
19.0	53.7	109.5	29.5	44.5	37.0	52.0	39.0	39.5	43.5	50.0	47.0	45.5	47.5
23.0	58.3	123.5	31.5	53.5	42.5	56.0	42.0	41.5	46.5	54.0	53.0	51.5	52.8
27.0	63.0	135.5	37.5	64.5	51.0	58.0	45.0	45.5	49.5	61.0	59.0	55.5	58.5
34.0	68.7	147.5	49.5	78.5	64.0	60.0	47.0	49.5	52.2	65.0	63.0	59.5	62.5
38.0	72.1	153.5	58.5	90.5	74.5	62.0	51.0	52.5	55.2	68.0	67.0	63.5	66.2
47.0	76.8	161.5	70.5	114.5	92.5	62.0	51.0	52.5	55.2	68.0	67.0	63.5	66.2
77.0	86.0	173.5	125.5	154.5	140.0	71.0	59.0	66.5	65.5	74.0	81.0	79.5	78.2

Table G.2 : Data for the John Grant enrichment digester during batch operation.

Time (d)	pH	Total Gas (mM) cumulative	Total Organic Carbon (mg/M)	Notes
0.00	7,3	0.00	360.00	fed 10 % scour efffluent
0.00	8.2			
2.00		240.00		
5.00		300.00		
7.00	8.1	600.00	265.00	
8.00		800.00		
13.00		1 280		
15.00	7.2	1 320	115.00	
17.00		1 400		
27.00	7.2	1 420		
37.00		1 440	208.00	
41.00		1 440	600.00	Re-fed with 10 % effluent
42.00		1 600		
44.00		1 720	560.00	
46.00		1 920		
50.00		2 180	530.00	
51.00		2 320		
53.00		2 320		
57.00		2 340		Overgassed digester with nitrogen.
58.00		2 420		
62.00		2 460		
71.00	7,5 / 8,9	2 460	100 / 900	Re-fed digester 1M of conc effluent.
72.00		2 560		
73.00	8.5	2 700	850.00	
74.00		2 840		
75.00		2 920		
77.00		3 190		
80.00		3 430		
83.00	7.6	3 790	400.00	
85.00	7.6	3 930	242.00	
87.00	7.6	3 990	170.00	
90.00	7.6	4 030	150.00	

Cumulative gas production and cumulative dissolved organic carbon (data from **Table G.2)** are plotted in **Fig 5.3 (Chapter Five).**

Table G.3 : Data from John Grant digester operating in semi-continuous mode

Time (d)	pH	Total gas (mM)	Total organic carbon (mg/M)	Notes
0.00	7,5 / 8,5	4 030	1,0/ 4,8	Digester fed 400 mM John Grant Scouring efluent.
1.00		4 110		
4.00	7.8	4 570	340.00	
4.00	8.6		590.00	Digester fed
5.00	8.0	4 710	385.00	
6.00	7.9	5 010	340.00	
7.00		5 170		
10.00	7,8	5 570	300	
10.00	8.6		650.00	Digester fed
15.00	7,8	6 430	385.00	
15.00	8.4		680.00	Digester fed
18.00	7.8	6 990	465.00	
19.00		7 150		
22.00		7 450		

Data from **Table G.3** are presented in **Fig 5.4** and **5.5 (Chapter Five)**.

G.2 COMBINATION OF ANAEROBIC DIGESTION OF JOHN GRANT SCOURING EFFLUENT AND DECOLOURISATION OF PROCION RED HE-7B

Table G.4 : Data from experiment to determine the order and rate of decolourisation of Procion Red HE-7B in 50 and 100 % John Grant scouring effluent (inoculated).

	Procion Red HE-7B concentration (mg/M)					
Time (h)	**50(1)**	**50(2)**	**50(3)**	**100 (1)**	**100 (2)**	**100(3)**
0.0	61.0	62.2	61.1	61.0	63.2	63.3
1.5	52.4	53.4	58.1	36.1	36.9	36.5
2.5	40.5	42.9	44.0	16.6	22.5	18.9
4.0	29.9	28.6	30.7	10.5	11.3	5.7
5.5	25.5	23.1	24.8	2.6	5.1	3.5
	ln Ct/Co					
Time (h)	**50(1)**	**50(2)**	**50(3)**	**100 (1)**	**100 (2)**	**100(3)**
1.5	-0.1528	-0.1527	-0.0493	-0.5252	-0.5381	-0.5500
2.5	-0.4108	-0.3715	-0.3280	-1.3016	-1.0353	-1.2102
4.0	-0.7129	-0.7789	-0.6869	-1.7591	-1.7259	-2.4054
5.5	-0.8744	-0.9901	-0.9012	-3.1711	-2.5258	-2.8847

Data from **Table G.4** are presented in **Fig 5.6 (Chapter Five)**.

Table G.5 : Decolourisation data from sterilised and non-sterilised John Grant scouring effluent (uninoculated).

Time (h)	**Sterilised bottles**	**Time (h)**	**Non-sterilised bottles**	
	50 %		**50 %**	**100 %**
	Procion Red HE-7B (mg/M)		**Procion Red HE-7B (mg/M)**	
1.0	92.9	0.2	91.6	93.3
4.0	91.5	1.5	90.9	89.0
6.0	91.3	2.5	88.4	84.1
8.2	92.0	4.0	84.2	76.5
10.8	91.2	5.5	76.7	65.8

The data in **Table G.5** are presented in **Fig 5.7, Chapter Five**.

Table G.6 : Decrease in Procion Red HE-7B concentration (mg/M) in inoculated serum bottles containing 100 mg/M of dye (D 1 to 3), and inoculated serum bottles containing 100 mg/M of dye and 3 mM nitrate (N 1 to 3). John Grant scouring effluent (50 %) was the substrate for these experiments.

Time (h)	**Procion Red HE-7B (mg/M)**					
	D (1)	**D (2)**	**D (3)**	**N (1)**	**N (2)**	**N (3)**
0.0	61.3	62.0	60.8	60.2	62.3	62.9
1.0	60.0	58.1	59.5	62.0	63.0	62.4
2.5	46.7	46.2	47.6	58.6	57.1	57.0
4.0	37.0	33.5	38.5	54.8	54.0	52.9
6.0	30.3	24.9	31.0	49.4	49.4	48.1
8.2	15.7	10.9	16.0	43.9	42.7	40.9
10.8	7.9	4.8	7.9	41.9	37.9	37.2

Data from **Table G.6** are presented in **Fig 5.9, Chapter Five**.