MICROBIAL DEGRADATION OF TEXTILE DYES TO SAFE END-PRODUCTS

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Abstract

Discharges from textile dyehouses often include large amounts of reactive azo dyes. These are recalcitrant to conventional wastewater treatment processes thus resulting in coloured waterways. Azo dyes can be decolourised biologically in bioreactors via fission of the azo (-N=N-) bonds to produce aromatic amines, but these are known to be toxic and/or genotoxic. This investigation concentrates on the toxicity and genotoxicity produced by decolourisation of a simulated textile effluent (STE) containing the reactive azo dye C.I. Reactive Black 5 in its hydrolysed form (RBOH). Two bioreactors were used, a batch-fill type Sequencing Batch Reactor (SBR) and continuous flow type Hybrid Anaerobic Baffled Reactor (HABR), operated under both anaerobic and anaerobic-aerobic conditions. The microflora of each bioreactor was studied in order to elucidate the interrelations between toxicity and/or genotoxicity, decolourisation efficacy and the microbial population.

Initially, the rapid bacterial toxicity and genotoxicity assays chosen were validated for their suitability before applying them to assessment of the SBR and HABR. High levels of decolourisation were observed in both bioreactors under all conditions (86-94%). Under anaerobic conditions, high levels of toxicity were observed in both bioreactors (EC_{50} values 0.24-0.46% of original concentration), whilst the toxicity was only significantly decreased (EC_{50} 16.89-17.80%) after anaerobic-aerobic operation in the HABR due to mineralisation of aromatic amines. No significant genotoxicity was observed.

Microflora numbers and composition were assessed using Fluorescent *in situ* Hybridisation (FISH) and Denaturing Gradient Gel Electrophoresis (DGGE). It was concluded that both the SBR and HABR operated under anaerobic conditions could support a relatively diverse, thriving, stable bacterial and archaeal population, but that increasing bacterial diversity was encouraged by the compartmentalisation of the HABR. Operation under anaerobic-aerobic conditions showed greatest divergence and appearance of unique species adapted to the aerobic environment in the HABR, due to the different conditions that could be provided by a compartmentalised bioreactor. The predominant species identified by culturing and sequencing under both anaerobic and anaerobic-aerobic conditions were *Clostridium* and *Bacteroides* spp. in the SBR and *Staphylococcus* and *Bacteroides* spp. in the HABR. The archaeal population of the SBR was predominated by *Methanosaeta* sp. under anaerobic-aerobic conditions, and *Actinomadura* sp. was only observed in aerated samples. Several bacterial species were

found to be unique to the aerobic sections of the HABR, and some were identified as *Nocardia* and *Rhodococcus* spp..

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Abbreviations

16S rRNA	small subunit ribosomal RNA
BOD	biological oxygen demand
CMC	carboxymethyl cellulose
COD	chemical oxygen demand
CSLM	Confocal Scanning Laser Microscope
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	deoxyribonucleic acid
DO	dissolved oxygen
FISH	Fluorescent *in situ* Hybridisation
HABR	Hybrid Anaerobic Baffled Reactor
HRT	hydraulic retention time
IR	infra red
O2	Orange II (C.I. Acid Orange 7)
OD	optical density
OLR	organic loading rate
PC	Procion Crimson H-EXL
PCOH	hydrolysed Procion Crimson H-EXL
PCR	Polymerase Chain Reaction
PMFA	Postgates Medium F Agar
PN	Procion Navy H-EXL
PNOH	hydrolysed Procion Navy H-EXL
PVOH	polyvinyl alcohol
PY	Procion Yellow H-EXL (C.I. Reactive Yellow 138:1)
PYOH	hydrolysed Procion Yellow H-EXL
RABIT	Rapid Automated Bacterial Impedance Technique
RB	C.I. Reactive Black 5
RBC	Rotating Biological Contactor
RBOH	hydrolysed C.I. Reactive Black 5
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-Polymerase Chain Reaction
SAA	Schaedler Anaerobe Agar
SAB	Schaedler Anaerobe Broth
SBR	Sequencing Batch Reactor
STE	simulated textile effluent
SY	Sunset Yellow FCF (C.I. Food Yellow 3)
TDS	total dissolved solids
TNA	total nucleic acid
TOC	total organic carbon
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
UASB	Upflow Anaerobic Sludge Blanket (reactor)
UV	ultra violet

Chapter 1: Introduction

1.1 Textile dyes

1.1.1 Classification and usage

Textile dyes are one of the most prevalent types of chemicals in use today; over 100 000 commercially available dyes exist (Banat *et al.* 1996). Dyes form the bulk of substances manufactured in or imported into Great Britain in quantities exceeding 1 tonne per year, with over 7×10^5 tonnes of dyestuff produced annually in the UK (Banat *et al.* 1996, Rajaguru *et al.* 2000). Worldwide, nearly 3×10^5 tonnes of textile dyes are discharged per annum (Willetts & Ashbolt 2000). Dyes are generally small molecules comprising two key components; the chromophore, which is an aromatic group responsible for the colour, and the auxochrome, which is a functional group that binds the dye to the fibre (Correia *et al.* 1994). They are classified into categories based upon either their chromophoric structure or their practical application in the dyehouse (Carliell 1993).

The Colour Index, devised by the Society of Dyers and Colourists in 1924, is the internationally accepted catalogue that collates data on all of the recognised current dyes (O'Neill *et al.* 1999). It lists 28 categories based upon chromophoric structure including nitroso, azo, quinoline and sulphur (Carliell 1993), and 19 categories based upon application including acid, basic, vat, sulphur, disperse, pigment, solvent, direct, mordant and reactive (Easton 1995). Each dye is assigned a generic name determined by its application characteristics, such as Reactive Black (RB), and then a constitution number based on its chemical structure where it is declared, such as C.I. 20505 (O'Neill *et al.* 1999). The same dye may be sold under different commercial names, which is why the Colour Index assigns a generic name. Different categories of dyes can be used with many fibre types, such as cellulosic (e.g. cotton), wool, nylon, acetate, polyamide, polyester, polyacrylic and leather (Colour Index International 1987, Correia *et al.* 1994).

1.1.2 The occurrence of textile dyes in water courses

The textile industry discharges its effluent, which contain significant quantities of dyes from the colouring process, to municipal wastewater treatment plants (Cripps *et al.* 1990, Pierce 1994). However, it is known that textile dyes are not readily degraded in conventional wastewater treatment plants (Kulla 1981, Cripps *et al.* 1990, Easton 1995, O'Neill *et al.* 1999). Biodegradation is rendered difficult firstly due to the complex aromatic structure of dye molecules (Banat *et al.* 1996, Hao *et al.* 2000) and

chemical properties such as high water solubility and high molecular weight, which inhibit permeation through biological cell membranes (Willmott *et al.* 1998). Secondly, textile dyes are designed to have a high degree of chemical and photolytic stability (Willmott *et al.* 1998) as they need to resist oxidative breakdown attributable to sunlight, water, oxidative detergents (Easton 1995, Waters 1995), bleach and oxidative processes of microbes utilising perspiration for growth (O'Neill *et al.* 1999). Therefore dyes are extremely resistant to oxidative degradation, which is the main method of biological breakdown under the aerobic conditions prevailing in wastewater treatment plants.

Although dyes are not biodegraded in wastewater treatment plants, there are two major mechanisms for dye removal. The first is precipitation from the effluent during primary settling of sludge (Pierce 1994). This mechanism eliminates water insoluble dyes, such as those in the disperse, vat and sulphur categories, and involves reactions of calcium and magnesium salts with anionic and cationic dyes respectively (Baughman & Perenich 1988). The second mechanism is adsorption to the sludge biomass, also known as bioelimination. This is effective in eliminating many dyes whether water soluble or insoluble, particularly those in the disperse, basic, acid and direct categories (Pierce 1994, Willmott *et al.* 1998, Hao *et al.* 2000). However, the reactive types of dye are not removed during wastewater treatment processes, and are then passed to the point of discharge for the treated effluent, which are natural watercourses.

1.1.3 The fate of textile dyes in water courses

Once dyes enter natural watercourses, they are rarely further broken down by the degradative systems commonly occurring in aquatic environments. Microbial processes such as bioconcentration and hydrolytic reactions are rarely significant, as biodegradation is unlikely to occur in the less severe conditions of the natural environment if it did not occur in the rigorous conditions of a wastewater treatment plant (Baughman & Perenich 1988). Photodegradation by the UV component of natural light is ineffective due to the photolytic stability inherent in the dyes (Pierce 1994). Therefore, dyes are extremely stable and remain in the environment for a considerable time, as shown by the fact that the half-life of hydrolysed C.I. Reactive Blue 19 is about 46 years (Hao *et al.* 2000), and as such are considered xenobiotics.

1.2 Reactive azo dyes

1.2.1 Chemical structure

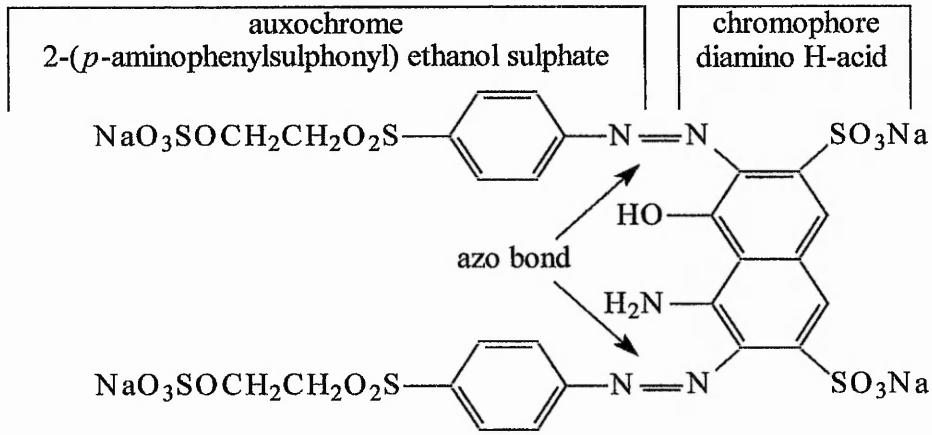
Among the categories of dyes classified by chromophoric structure, azo is undoubtedly the largest class manufactured today. Azo dyes account for 60-70% of all textile dyestuffs produced (Carliell *et al.* 1998, O'Neill *et al.* 1999), with at least 3000 kinds used for various purposes in textiles, papermaking, cosmetics, pharmaceutical and food industries (Chung 2000). Although they exhibit great structural variety (Cripps *et al.* 1990), all azo dyes are characterised by the presence of nitrogen-nitrogen double bonds (-N=N-), known as azo bonds, within the chromophore (Hao *et al.* 2000). These azo bonds join the chromophore with radicals that form the auxochrome, usually aromatic amines (Chung 2000), and molecules containing one, two, three or more bonds are known as mono-, dis-, tris- or polyazo dyes (Colour Index International 1987).

Dyes are also classified in terms of their practical application in the dyehouse (see section 1.1.1). In these terms, a class of dyes exists known as reactive dyes. This class is characterised by the method of binding of the dye to the textile fibre, which occurs via formation of a covalent bond between the reactive site in the auxochrome of the dye and the functional group on the fibre (Carliell 1993, Hao *et al.* 2000). The bonds form under the influence of high temperature and pH (O'Neill *et al.* 1999), and are produced by mechanisms known as nucleophilic substitution or nucleophilic addition (Colour Index International 1987, Carliell 1993). An example of a reactive group that binds by nucleophilic substitution is monochlorotriazinyl (e.g. Procion dyes), and by nucleophilic addition is vinylsulphone (e.g. RB) (Colour Index International 1987).

The first reactive dyes for cellulosic fibres, such as cotton, appeared in 1956 and are still used widely in a variety of printing and dyeing processes (Colour Index International 1987). Reactive dyes are highly water-soluble, due to the presence of sulphonic acid groups (Nörtemann *et al.* 1986), anionic (Correia *et al.* 1994), have a small molecular size to facilitate rapid dyeing (Colour Index International 1987) and whose use results in fabrics with excellent wet fastness due to the formation of covalent bonds (Hao *et al.* 2000). The most common bond joining the chromophore and auxochrome of reactive textile dyes is azo (Carliell *et al.* 1998, Hao *et al.* 2000). The structure of C.I. Reactive Black 5 (RB), a commonly used reactive azo dye, can be seen in Figure 1.1. It is important to note that commercially supplied reactive dyes will only contain 25-60% pure colour, the remainder being comprised of other substances such as salt, diluents such as sodium lignosulphonate, moisture, and small quantities of other

dyes (O'Neill *et al.* 1999). Therefore any studies undertaken using a commercially supplied dye will need to take into account effects that may be caused by the impurities present in the product.

Figure 1.1: The structure of C.I. Reactive Black 5 (C.I. 20505)



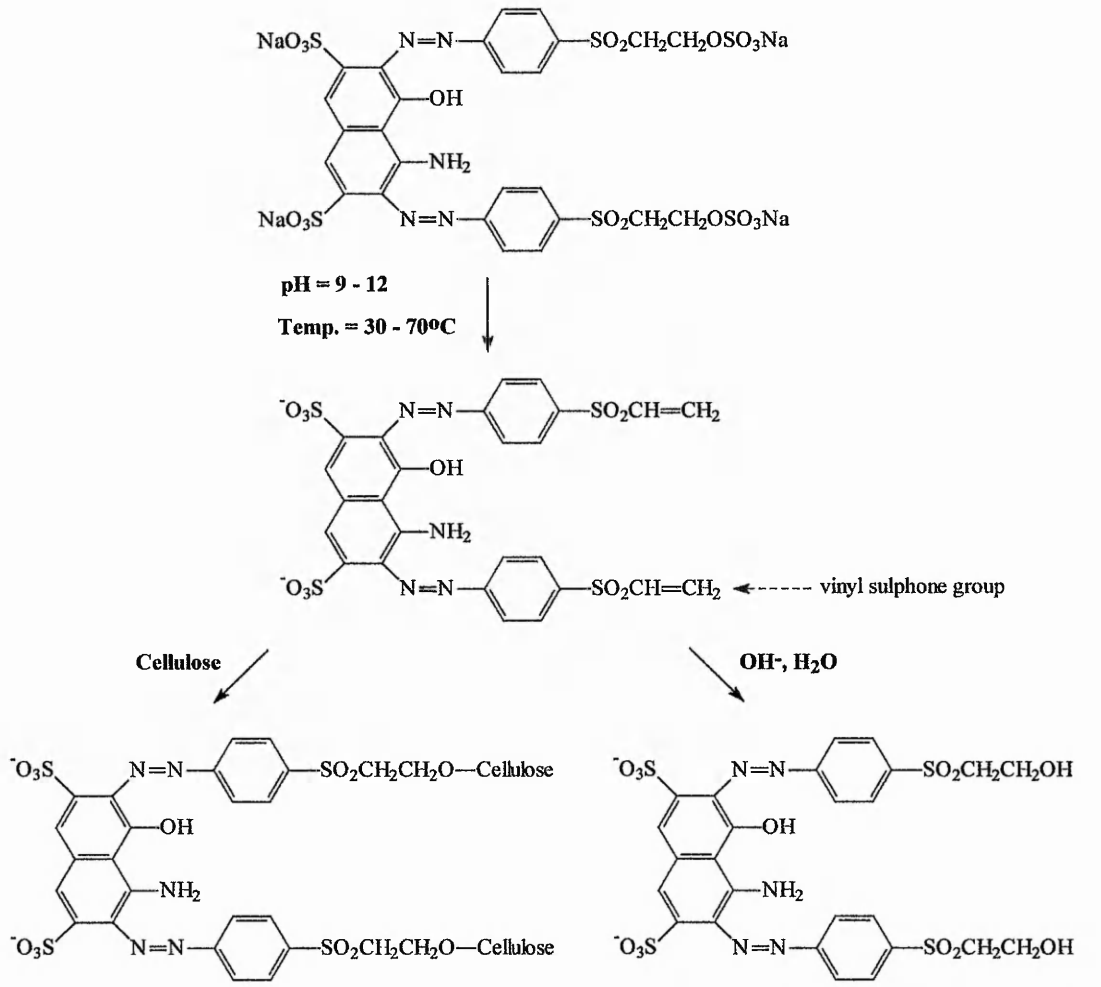
C.I. Reactive Black 5

(Adapted from Colour Index International 1987)

1.2.2 The environmental significance of reactive dyes

In the textile dyeing industry there are significant amounts of dye present in wastewater effluents, and this is especially true for reactive dyes (Carliell *et al.* 1998, Hao *et al.* 2000). The reason for this involves the mechanism by which the dye is bound to the fibre during dyeing. Under the hot, alkaline conditions of a dyebath, the auxochrome of the dye undergoes a structural change to produce the vinyl sulphone ($-SO_2-CH=CH_2$) group, which is the active form that binds the fibre (Hao *et al.* 2000). However, this active form is highly unstable and has a high affinity for hydroxyl ions formed from the water present, and so often undergoes hydrolysis before binding to the fibre can occur (Weber & Stickney 1993). This process is represented diagrammatically in Figure 1.2. The hydrolysed dyes, which are now highly stable, no longer have any affinity for the fibre (Colour Index International 1987, Carliell 1993, Hao *et al.* 2000) and so are lost to waste in the effluent.

Figure 1.2: Hydrolysis of the reactive azo dye C.I. Reactive Black 5 during dyeing



(Adapted from Weber & Stickney 1993, Hao *et al.* 2000)

Because of the hydrolysis mechanisms that occur during dyeing, reactive dyes exhibit low levels of binding (fixation) to the material. The average level of fixation of reactive dyes to cotton is between 70 and 80% (Pierce 1994, Waters 1995), but can vary dramatically from 50 to 90% with the higher levels applicable to dyes with two or more reactive groups (Easton 1995, O'Neill *et al.* 1999). Thus, reactive dyes will be lost to the effluent of textile dyehouses at between 10 and 50% of the input (Carliell *et al.* 1994). The pollution potential of reactive dyes is exacerbated by the fact that they are now one of the most commonly used types of dye (Southern 1995), with RB being the most frequently utilised dye globally. As an example, analysis of the wastewater of two Severn Trent catchment areas, Loughborough and Wanlip, in 1994 revealed that reactive dyes constituted 61 and 67% respectively of the total classes of dye present (Churchley 1995, O'Neill *et al.* 1999). Actual concentrations of reactive dyes in dyehouse effluents have been reported to range from 60-250 mgL^{-1} on average with

shock loading of 800-1400 mgL^{-1} (Pierce 1994, O'Neill *et al.* 1999), which equates to an estimated highest concentration in the receiving river of 1.6 mgL^{-1} depending on river levels (Hobbs 1989).

1.2.3 The recalcitrant nature of reactive azo dyes

Reactive azo dyes are not eliminated to any great extent in the aerobic sewage treatment plants that most dyehouses release their effluent to. There are two main reasons for this. Firstly, they are not readily biodegraded by activated sludge. The sulphonated naphthalenes, that are common features of reactive azo dyes, confer water solubility, and thus they are not susceptible to microbial attack by unadapted populations (Nörtemann *et al.* 1986, Cripps *et al.* 1990, Chung 2000, Hao *et al.* 2000). Secondly the process of bioelimination (adsorption to cells), which is an important method of pollutant removal in wastewater treatment, is ineffective for reactive dyes. This class shows an average adsorption level to the microbial biomass of an activated sludge reactor of only 10-30% (Pierce 1994, Waters 1995). These levels are unaffected by the number of sulphonated groups present on the dye, or by the ease of hydrolysis during the dyeing process (Hitz *et. al.* 1978, Willmott *et al.* 1998). Therefore, approximately 70-90% of the reactive azo dyes entering wastewater treatment plants will pass through unchanged and be discharged to a river (Hobbs 1989, Hao *et al.* 2000).

1.3 Decolourisation of textile dyes

1.3.1 Textile dyes as pollutants

Effluent streams from the textile dyeing industry can contain high concentrations of textile dyes, which pass into natural watercourses. To assess the environmental impact of the release of these dyes, the hazards posed to the environment and man must be determined (Hobbs 1989). Several studies on the toxicity of textile dyes have shown that they exhibit low toxicity to mammals and aquatic organisms such as fish and microbes (Davis *et al.* 1994, Chung 2000). In a study by Brown and co-workers in 1981, over 200 dyes of the classes reactive, direct, disperse, acid, vat, mordant, pigment and solvent were deemed non-toxic to microorganisms found commonly in wastewater treatment plants (inhibitory concentrations all >100 mgL^{-1}), with only basic dyes being inhibitory to the populations. It has also been observed that reactive dyes have the lowest toxicities of all the classes (Hao *et al.* 2000). There is also little evidence of bioaccumulation in natural watercourses (O'Neill *et al.* 1999). However, the definition of environmental

pollutants is "chemicals of natural or synthetic origin that are released by mans activity into the environment where they have an undesirable effect on the environment or on man via the environment" (Leisinger 1983). Under section 1[4] of the Environmental Protection Act 1990, the undesirable effect not only includes harm to the health of living organisms or other interference with the ecological systems of which they form part, but also in the case of man includes offence caused to any of his senses or harm to his property (Litten 1995).

Colour in natural water courses is one of the first types of contaminant to be recognised (Banat *et al.* 1996), and it is known that the human eye can detect reactive dyes at concentrations as low as 0.005 mgL^{-1} in clear river water (Pierce 1994, Willmott *et al.* 1998). The average dye concentrations in rivers occur at over 200 times higher than this detection limit. Improved quality of rivers has lead to decreased turbidity and hence increased visibility of coloured pollutants. Subsequently, colour in water courses was the subject of an increasing number of public complaints in the late 1980s and early 1990s (GraveletBlondin *et al.* 1997). In 1992 a national survey of colour pollution was conducted, which identified colour as the subject of over 500 complaints to the National Rivers Authority (NRA) (Pierce 1994), which constituted about 5% of total complaints (O'Neill *et al.* 1999). These complaints, which affected over 600 km of river, were centred mainly in the Severn Trent region (Willmott *et al.* 1998). It is therefore clear that, although textile dyes cannot be considered pollutants in terms of adverse effects on the health of organisms, they are considerable pollutants in aesthetic terms.

1.3.2 The requirement for decolourisation

In order to protect natural water courses from pollution, consent limits on all water quality criteria known as River Quality Objectives (RQOs) are set by the Environment Agency (EA) in England and Wales. The EA was formed in 1995 following the amalgamation of the NRA and Her Majesty's Inspectorate of Pollution (HMIP), and the Scottish Environment Protection Agency (SEPA) in Scotland (Willmott *et al.* 1998). An example of the data used by the EA to determine RQOs for colour in three rivers in the Severn Trent catchment area can be seen in Table 1.1.

Table 1.1: Absorbance values used to determine river quality objectives in three Severn Trent catchments

Wavelength (nm)	Standard for River (absorbance at λ)		
	Churnet	Erewash	Bentley Brook
400	0.025	0.025	0.027
450	0.015	0.015	0.016
500	0.012	0.012	0.014
550	0.010	0.010	0.012
600	0.008	0.008	0.010
650	0.005	0.005	0.008
700	0.003	0.003	0.004

(Pierce, 1994, Waters 1995)

As a result of the high number of complaints about colour in 1992, the colour consent limits imposed on sewage treatment plants were made more stringent in order to continually achieve the RQOs (Pierce 1994, O'Neill *et al.* 1999). Colour consent limits do not deal in absolute values, as equal concentrations of different dyes may visibly yield different hues (Willmott *et al.* 1998). Also, the intensity of a colour is pH dependent and usually increases as the pH is raised, and the pH of waste effluents can vary from as low as 2 to as high as 12 (Hao *et al.* 2000). Therefore, it was concluded by the NRA that absorbance measurements are the most suitable approach for determining colour in wastewaters (Willmott *et al.* 1998).

Colour consent limits are expressed as absorbance values of a sample, passed through a 0.45 μm filter measured in a 1 cm path length cell, measured over the 400-700 nm range of wavelengths at 50 nm intervals (Willmott *et al.* 1998, O'Neill *et al.* 1999). Table 1.2 shows the colour consent limits for three Severn Trent sewage treatment plants and values for a 'typical' sewage treatment plant. Firstly it must be noted that the colour consent limits are higher than the standards used to determine the RQOs. This is because the dilution factors of the receiving waters are taken into account when setting colour consents on sewage treatment plants (Pierce 1994, Willmott *et al.* 1998). Also, the limits imposed on the Severn Trent sewage treatment plants are lower than the 'typical' limit, which is due to the fact that there are many textile works in this area and so each must be more limited to prevent pollution of the receiving water (O'Neill *et al.* 1999). Concentrations of dyes as low as 0.1 mgL^{-1} may give an absorbance of 0.05 at λ_{max}, and thus the consent limits shown in Table 1.2 will equate to dye concentrations of 0.1-2 mgL^{-1} (Pierce 1994, Hao *et al.* 2000). As dyes will be present in sewage treatment effluents at an average of 55-225 mgL^{-1}, with shock loading

concentrations of RB of up to 1400 mgL^{-1}, compliance with the colour consent limits may require the removal of up to 99.9% of the colour (Pierce 1994, O'Neill *et al.* 1999).

Table 1.2: Colour consent limits on three Severn Trent sewage treatment plants and a 'typical' plant

Wavelength (nm)	Colour Consent Limit (absorbance at λ)			
	Leek	Wanlip	Pinxton	'Typical'
400	0.060	-	-	0.115
450	0.040	-	-	0.085
500	0.035	0.020	0.028	0.065
550	0.025	0.021	0.025	0.055
600	0.025	0.012	0.024	0.040
650	0.015	0.012	0.017	0.028
700	-	-	-	0.013

(O'Neill *et al.* 1999)

Different processes for colour removal typically include physical, chemical, electrical and biological schemes. However, the textile industry generates large volumes of effluent which are extremely variable in colour, chemical composition and pollution load dictated by production processes and fashion cycles, as dyeing is usually carried out in batchwise fashion (Willmott *et al.* 1998, Esteves *et al.* 2000). There are different auxiliary chemicals associated with dyeing processes, such as salts, acids and bases, oxidising and reducing agents, buffers, carriers (e.g. phenyl phenols, chlorinated benzenes), starches, surfactants and other finishing agents (e.g. polyvinyl alcohol), and the amount of and types used depends on the type of dye used and the mode of application (Correia *et al.* 1994). Due to this, textile effluents have very high and variable biological and chemical oxygen demand (BOD and COD), low BOD/COD ratios due to the difficulty in biodegradation of dyes, high levels of total dissolved solids (TDS) and high concentrations of heavy metals (e.g. cadmium, copper) (Correia *et al.* 1994, Hao *et al.* 2000). Therefore, a single, universally applicable end-of-pipe solution to decolourise dyehouse wastewater effluents is unlikely (Willmott *et al.* 1998), which renders dye decolourisation methods extremely difficult and costly (Hao *et al.* 2000). The major methods of dye decolourisation that are currently employed to treat textile wastewaters, divided into physical and chemical (physico-chemical) and biological methods, will be discussed.

1.3.3 Physico-chemical methods

There have been several physico-chemical methods reported to be effective at decolourising wastewater containing textile dyes over the past two decades. However,

few of these methods have been taken up by the textile industry due to several limitations inherent to physico-chemical techniques. The most important of these general limitations is that no one technique is able to decolourise effluents containing mixtures of numerous types of dyes (Banat *et al.* 1996). Additionally, dyehouses will vary the types of dye used dependant on application processes and fashion (Willmott *et al.* 1998). This, combined with the fact that most commercially supplied dyes only contain 25-60% of the dye it is marketed as (O'Neill *et al.* 1999), explains why dyehouses would be extremely reluctant to install a decolourisation process that could not efficiently decolourise a large number of dye types. The second general limitation is the fact that physico-chemical methods rarely decrease COD or BOD levels. Therefore, in order to achieve the desired goal of a fully treated effluent, additional biological treatment is also required which will further increase the costs of decolourisation (e.g. chemical coagulation followed by aerobic biological oxidation, powdered activated carbon-activated sludge (PACT)) (Carliell 1996, Hao *et al.* 2000).

Physical methods include the use of membranes to filter out dye molecules (Banat *et al.* 1996). Different membrane units can be selected to retain solutes of different molecular weights (e.g. 100-1K for ultrafiltration, 15-0.5K for nanofiltration, <1K for reverse osmosis), and so the most appropriate unit for retention of dyes can be selected (Hao *et al.* 2000). The major limitation of this method is blocking of the units, which also require regeneration or replacement (Banat *et al.* 1996).

Another type of physical method is adsorption of the dye to a suitable medium. The most commonly used type of adsorbent is activated carbon, either in granulated or powder form, and an example of an industrial application of this technique is called the Katox treatment which utilises activated carbon and air (Banat *et al.* 1996). However, activated carbon is expensive, requires regeneration, is not suitable for some dye classes (e.g. water insoluble dyes) and cannot decolourise raw wastewater due to competition between the dye molecules and other organic/inorganic particles (Kuo 1992, Hao *et al.* 2000). Low-cost alternatives have been considered (e.g. chitosan fibre which is a shellfish waste product, flyash which is a power generators waste product, silica gel, bauxite, peat and wood) (Kennerley 2000), but it has been discovered that a particular adsorbent is only applicable to a particular class of dyes dependant on ionic charge (Hao *et al.* 2000). The major limitation of this method is problem caused by the requirement to dispose of sludge or exhausted active compounds, especially in the case of activated carbon adsorption (Davis *et al.* 1994, Southern 1995).

Another type of physical method are electrochemical techniques, which are relatively new in terms of textile waste treatment, and involve passing a current through electrodes to result in the different chemical reactions of electrooxidation, electrocoagulation, electroreduction and electroflotation (Banat *et al.* 1996). Electrooxidation occurs at the anode where chlorine and ozone gases are evolved, and the anode then dissolves into metal ions which act as coagulation agents for electrocoagulation. Electroreduction occurs at the cathode where hydrogen gas is produced, the bubbles of which act as a flotation agent to carry the suspended particles to the surface in electroflotation (Hao *et al.* 2000). Electrochemical techniques have been shown to be effective at decolourising reactive dyes at levels of >90% using dye concentrations of up to 5000 mgL^{-1}, and those dyes with vinylsulphone reactive groups appear to be removed predominantly by electrocoagulation (Walsh *et al.* 1994). However, the major limitation of this type of decolourisation method is the high running-cost of maintaining the reactor (Banat *et al.* 1996).

Chemical methods include coagulation of the dye with an inert medium, also known as flocculation. Flocculation can be achieved using various inorganic and organic salts, such as iron ($Fe(II)/Ca(OH)_2$), aluminium or lime, and then combined with flotation (e.g. electroflotation) or mechanical separation (e.g. membrane filtration) (Banat *et al.* 1996, Kennerley 2000). Flocculation will remove insoluble dyes (e.g. disperse) but only to a maximum of 50%, and it does not work well for highly water-soluble dyes (e.g. reactive) (Hao *et al.* 2000). Also, as flocculation is merely the physical removal of the dye material from the effluent, a large amount of sludge is generated which still presents major problems of disposal (Kuo 1992, Davis *et al.* 1994).

One type of chemical decolourisation method that has been the focus of a significant amount of recent research is a group known as Advanced Oxidation Processes (AOPs). AOPs are based on the generation of highly reactive hydroxyl radicals (·OH) which react with dye molecules and destroy chromophoric ring structure, thus eliminating the colour. AOPs include ozonation, chlorination (e.g. sodium hypochlorite), photocatalytic degradation (e.g. UV with or without catalysis such as titanium dioxide or zinc oxide) and oxidation (e.g. hydrogen peroxide) (Davis *et al.* 1994, Banat *et al.* 1996). The combination of iron coagulation with hydrogen peroxide oxidation (e.g. $FeII/H_2O_2$ and $FeIII/H_2O_2$), known as a Fenton's reaction, is commonly employed (Hao *et al.* 2000). Studies by Bandara and co-workers in 1997 (cited in Hao *et al.* 2000) showed that The Fenton's reaction could completely degrade the simple azo dye C.I. Acid Orange 7 (O2) using the following reactions:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2\cdot + H^+$$

$$C_6H_{11}N_2NaO_4S + \cdot OH + 37/2O_2 \rightarrow$$

$$16CO_2 + 3H_2O + 1/2NO_3^- + 1/2NH_4^+ + 1/2N_2 + NaHSO_4^- + H^+$$

(Hao *et al.* 2000)

Where $C_6H_{11}N_2NaO_4S$ is O2

Fenton reactions have been shown to decolourise reactive azo, disperse, direct, acid and basic dyes at concentrations of 300 mgL^{-1} by an average of 97%, with simultaneous reduction of the COD by approximately 90% (Kuo 1992, Ince & Tezcanli 1999). However, these reactions only occur below pH 3.5, and textile wastewater is often highly alkaline (Hao *et al.* 2000). Titanium dioxide-mediated photocatalytic degradation has been shown to be effective at decolourisation of reactive azo dyes. For example, concentrations of RB and C.I. Reactive Blue 19 typically found in dyehouse effluents were completely decolourised and mineralised (demonstrated by a reduction in COD) within approximately 1 hour (Arslan & Balcioglu 1999, Peralta-Zamora *et al.* 1999, Poulios & Tsachpinis 1999), and other studies on reactive azo dyes have reported 86-90% colour removal within 30 minutes, with simultaneous reduction of the COD by approximately 83% (Davis *et al.* 1994, Hu & Wang 1999).

Ozonation has been used to effectively decolourise a number of different dye classes, including reactive azo dyes, with a very short reaction time (Churchley 1995, Peralta-Zamora *et al.* 1999). However, ozonation is ineffective for disperse dyes, does not mineralise dyes and requires the costly on-site generation of ozone which is unstable and hazardous (Kuo 1992, Davis *et al.* 1994). General limitations of AOPs include high cost plant requirements and operating expenses, but the major focus of concern is the fact that incomplete mineralisation of the dyes will result in the generation of the substituent aromatic amines, which may be toxic or carcinogenic (Banat *et al.* 1996, Hao *et al.* 2000). The implications of decolourisation with reference to the toxicity and carcinogenicity of aromatic amines are discussed in sections 1.4.1 and 1.4.2.

1.3.4 Biological methods

Many types of microorganism have been assessed for their ability to degrade or eliminate textile dyes. One type that has received considerable attention with regard to dye decolourisation are fungi, including *Phanerochaete chrysosporium*, *Neurospora crassa*, *Schizophyllum commune*, *Pycnoporus cinnabarinus*, *Myrothecium verrucaria*, *Phlebia tremellosa*, *Aspergillus sojae*, *Trametes versicolor*, *Coriolus versicolor*, *Mycelia*

sterilia, *Flavobacterium*, *Aspergillus*, *Trichoderma* and *Ganoderma* spp. (Banat *et al.* 1996, Hao *et al.* 2000). The mechanism by which fungi decolourise dyes is by the action of peroxidases, which produce oxygen radicals that destroy the bonds within the dye molecules by oxidation (Cao *et al.* 1993). Depending on the efficacy and quantity of oxygen radical production, decolourisation will be achieved either by partial oxidation which destroys the conjugated bonding system in the chromophore, or total oxidation in which mineralisation of the molecule takes place (Southern 1995).

Studies have shown that the use of fungi achieves high levels of decolourisation of dyes, such as that of Cripps and co-workers in 1990 where four azo dyes were decolourised by over 90% by *Phanerochaete chrysosporium* within 24 h, and that of Kirby an co-workers in 2000 where eight textile dyes at 200 mgL^{-1} were decolourised by the *Phlebia tremellosa* by >96% within 14 days. However, it should be noted that some studies have observed that reduction of colour may be due to adsorption of the dye to the chitin of the cell wall as opposed to oxidation of the molecule (Carliell 1993).

Therefore, many studies have concentrated on the use of crude peroxidase preparations for decolourisation. However, although the process is rapid, it seems that good levels of decolourisation cannot be achieved by crude peroxidases, with only 30-40% colour removal of azo dyes within 20 min (Cripps *et al.* 1990, Peralta-Zamora *et al.* 1999). Results have also been contradictory, with some groups reporting that lignin peroxidase achieves good decolourisation whilst manganese peroxidase has no activity, and others reporting the opposite (Hao *et al.* 2000). For example, a study by Ollikka and co-workers in 1993 using *P. chrysosporium* observed decolourisation of azo dyes at >75% within 15 min, and suggested this was due to the action of lignin peroxidase, whilst studies by Paszczynski and co-workers using the same organism have suggested the decolourisation of azo dyes to be due to the action of manganese peroxidase (Paszczynski & Crawford 1991, Paszczynski *et al.* 1992).

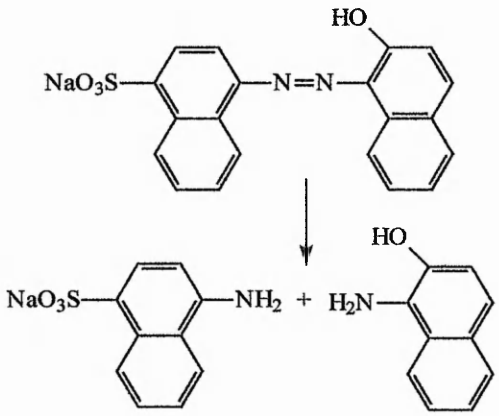
Other types of microorganism that have been studied for decolourisation of azo dyes amongst others include actinomycetes (e.g. *Streptomyces chromofuscus*; Paszczynski *et al.* 1992 and Pasti-Grigsby *et al.* 1996), yeast (e.g. *Candida zeylanoides* and *C. curvata*; Kakuta *et al.* 1998*b* and Hao *et al.* 2000) and algae (e.g. *Chlorella* sp. and *Oscillatoria* sp.; Cao *et al.* 1993 and Banat *et al.* 1996). However, the number of these types of studies have been very limited.

The other main focus of dye decolourisation has been the use of bacteria. It has been observed that dyes can be removed from effluents by adsorption to microbial cells (Carliell 1993). However, the level of adsorption is entirely dependent of the chemical

class of the dye. As the surface of cells is negatively charged (anionic), nonionic (e.g. disperse) and cationic (e.g. basic) dyes are well absorbed. However, anionic dyes (e.g. the water soluble reactives) are electrostatically repulsed and not absorbed to the biomass (Churchley *et al.* 2000). Studies have demonstrated the low levels of absorption of anionic dyes, for example the use of three types of anionic water soluble dye (reactive, acid and direct) at ~25 mgL^{-1} which were absorbed to a biomass isolated from a wastewater treatment plant under aerated conditions at only 20, 35 and 12% respectively within 3 h (Churchley *et al.* 2000). It should be noted that the reactive dye was tested in hydrolysed form, as would be found in a typical dyehouse effluent. An earlier study by Hu in 1992 showed that eleven reactive dyes at 100 mgL^{-1} were adsorbed to the cell walls of bacteria isolated from activated sludge at efficiencies ranging from 13-94%, but these higher levels of adsorption may have been due to very high molecular weights of some of the compounds (Shaul *et al.* 1991). It has also been noted that azo dyes are very rarely adsorbed to biomass, as they are often highly sulphonated, making them anionic, and thus are repulsed by the biomass (Shaul *et al.* 1991).

The major mechanism by which bacteria decolourise azo dyes is by reduction of the azo linkage under anaerobic conditions to produce the constituent aromatic amines, which are colourless (Cripps *et al.* 1990, Zaoyan *et al.* 1992). This reaction is demonstrated simply in Figure 1.3, using the acid azo dye C.I. Acid Red 88. A detailed discussion of the mechanism can be found in section 1.3.5. The ability of anaerobic microbes to decolourise azo dyes is well established, and most of the studies on dye decolourisation have concentrated on anaerobic bacterial species (Carliell *et al.* 1998, Hao *et al.* 2000). Bacteria used have included facultative or obligate anaerobes, such as *Bacillus*, *Clostridium*, *Aeromonas*, *Plesiomonas* and *Achromobacter* spp., *Alcaligenes faecalis*, *Enterococcus faecalis* and *Comomonas acidovorans*, and mixed cultures of often unidentified species (Gingell & Walker 1971, Banat *et al.* 1996, Hao *et al.* 2000).

Figure 1.3: Biodegradation of C.I. Reactive Red 88 via reduction of the azo bond



(Churchley *et al.* 2000)

Examples of mixed culture studies have been more numerous. In 1987, Brown and Hamburger studied the decolourisation of 16 azo dyes with an anaerobic sludge and observed 44-100% colour removal in periods ranging from 7-56 days. Carliell and co-workers in 1994 used an anaerobic sludge to decolourise 13 reactive azo dyes by 70-97% in periods ranging from 1-32 h. In 1996, Nigam and co-workers used a mixed microbial consortium, identified as consisting predominantly of *A. faecalis* and *C. acidovorans*, to decolourise eight reactive azo dyes by 67-89% within 24 h (Nigam *et al.* 1996*a*, Nigam *et al.* 1996*b*). More recently, a study by Beydilli and co-workers in 1998 observed 73-98% decolourisation of six reactive azo dyes within 64 h using an anaerobic sludge.

It has been noted that most azo dye decolourisation occurs anaerobically by reductive fission of the azo group. However, the absence of oxygen hinders mineralisation of the breakdown products that are formed, and it has been well documented that azo dye breakdown products, which are the constituent aromatic amines, build up in anaerobic systems (Kulla *et al.* 1983, Rafii *et al.* 1990, Zaoyan *et al.* 1992, Banat *et al.* 1996, Chung 2000). Thus, anaerobic decolourisation is incomplete with respect to total treatment of textile effluents. The implications of this incomplete breakdown are discussed further in sections 1.4.1 and 1.4.2.

A further problem which may be encountered when employing anaerobic decolourisation processes to textile wastewaters containing reactive dyes is the presence of high concentrations of salts. Salts such as sodium nitrate, sodium sulphate, sodium carbonate and sodium hydroxide are used in the reactive dyeing process to catalyse the formation of the covalent bond between the dye and the fibre, at typical concentrations of 20-100 gL^{-1} (Delée *et al.* 1998). However, the presence of nitrate and sulphate salts

could hinder the process of decolourisation under anaerobic conditions due to the competitive use of these salts as electron acceptors. It is believed that the mechanism of dye decolourisation under anaerobic conditions proceeds via the use of the dye as an electron acceptor, which is discussed further in section 1.3.5. A study by Carliell and co-workers in 1998 found that the presence of nitrate delays decolourisation of a reactive azo dye in direct proportion to the concentration present, whilst sulphate had no discernible effect up to 10 mM, and so it was proposed that the order of reduction of the compounds is nitrate > dye > sulphate. This study highlights yet another of the many aspects that need to be considered when an anaerobic biological decolourisation strategy is proposed for a textile dyehouse.

As has been noted, organisms that are capable of dye decolourisation under anaerobic conditions will not mineralise those dyes to carbon dioxide and water. However, under aerobic conditions, simple aromatic compounds can be completely mineralised by the oxygenases of many organisms via hydroxylation and ring-opening (Brilon *et al.* 1981*b*, Kulla *et al.* 1983, Zaoyan *et al.* 1992). A number of facultatively anaerobic and aerobic bacteria have been utilised to decolourise dyes under aerobic conditions, including *Pseudomonas*, *Rhodococcus*, *Alcaligenes* and *Caulobacter* spp., *Streptomyces chromofuscus*, *Klebsiella pneumoniae* and *Acetobacter liquefaciens* (Banat *et al.* 1996, Hao *et al.* 2000). Studies utilising pseudomonads to decolourise azo dyes have been numerous, such as Kanekar and co-workers in 1996 who observed 60% decolourisation of the azo dye Methyl Violet using *P. alcaligenes* and *P. mendocina*, and Hu (1998) who observed 99% decolourisation of three reactive azo dyes within 5 days using *P. luteola*.

A study by Sarnaik and Kanekar in 1995 observed that Methyl Violet could be decolourised by 77% within 24 h using pure cultures of *P. alcaligenes*, *P. mendocina*, *P. putida* biovar B and *P. stutzeri*, and that mineralisation occurred as measured by a 61% reduction in TOC. Mineralisation of aromatic amines by *Pseudomonas* sp. was observed much earlier than this, such as the study by Nörtemann and co-workers in 1986 who found that mineralisation of 6-aminonaphthalene-2-sulphonic acid could be ascribed to a mutualistic interaction of two isolated *Pseudomonas* spp. strains BN6 and BN9. Thurnheer and co-workers (1988) found that a co-culture of *Alcaligenes* sp. strain 0-1, *Pseudomonas* spp. strains T-2 and PSB-4 and two unidentified species strains M-1 and S-1 were able to completely mineralise seven sulphonated aromatic compounds. The last two studies highlight that degradation of sulphonated aromatic compounds is

usually accomplished using mixed cultures, as these compounds tend to require unusual catabolic activities that will not be found in a single organism (Rajaguru *et al.* 2000).

It should also be noted that aerobic dye decolourisation may not proceed via breakdown of the dye molecule, as demonstrated in a study by Pagga and Brown in 1986. Using aerobic biodegradation tests with activated sludge they observed that, of 87 water soluble dyes tested, most did not show any significant biodegradation, and those that did were deemed to be removed by adsorption. Thus it has been shown that an alternation from anaerobic to aerobic conditions may be required to achieve decolourisation and subsequent degradation of dyes in textile wastewater e.g. anaerobic fluidised bed followed by aerobic activated sludge (Zaoyan *et al.* 1992, Banat *et al.* 1996, Hao *et al.* 2000). Most current treatment technologies for decolourisation of textile wastewater involve aerobic processes, and a simple modification to these systems can result in a process with an anaerobic and aerobic configuration (Panswad *et al.* 2001*a*).

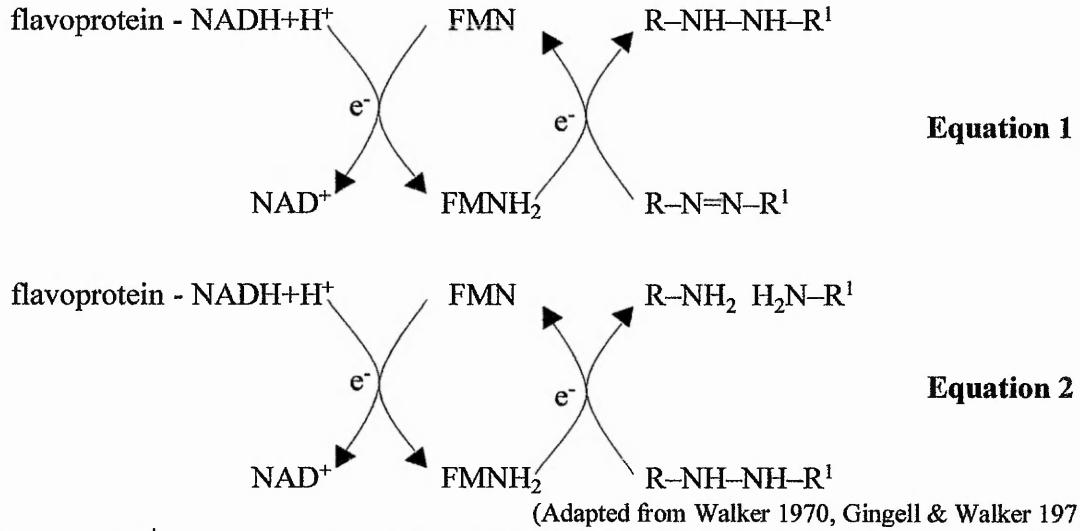
Examples of combined anaerobic-aerobic treatment processes for dyes include that of Brown and Hamburger in 1987 who decolourised 14 azo dyes of the acid, basic, mordant and direct classes using an anaerobic sludge by 90-100% at periods ranging from 7-56 days, with subsequent breakdown of the dyestuff metabolites at levels of <25% for acid dyes and >75% for direct dyes using activated sludge. In 1991, Haug and co-workers used a sulphonated aromatic amine degrading bacterial community, previously identified as consisting of *Pseudomonas* spp. strains BN6 and BN9 (Nörtemann *et al.* 1986), to completely decolourise the sulphonated azo dye C.I. Mordant Yellow 3 within 90 h under anaerobic conditions and with the addition of 10 mM glucose. Subsequently the culture was aerated, and the breakdown products 6-aminonaphthalene-2-sulphonate and 5-aminosalicylate were completely mineralised within 80 and 10 h respectively. Overall, although there are limitations to the processes, microbial decolourisation and degradation of textile dyes are seen as practical and cost-effective methods in terms of manpower and running expenses (Banat *et al.* 1996).

1.3.5 The mechanism of biological dye decolourisation

It has been noted in section 1.3.4 that azo bonds can be degraded biologically under anaerobic conditions. This degradation was first demonstrated as early as 1911 by Sisley and Porcher, who observed sulphanilic acid in the urine of dogs fed with the azo food dye Orange I, which has sulphanilic acid as one of its constituent aromatic amines (Daniel 1962). Today, it is well known that a wide variety of intestinal anaerobic

bacteria (e.g. *Citrobacter*, *Fusobacterium*, *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Peptococcus* and *Peptostreptococcus* spp.) have the ability to cleave the azo linkage (Chung *et al.* 1978, Chung *et al.* 1992, Cao *et al.* 1993, Cerniglia & Somerville 1995, Chung 2000). One of the first *in vitro* studies of decolourisation of azo food dyes was conducted by Brohm and Frohwein in 1937, who used lactic acid bacteria that could commonly be found in the intestine (Walker 1970). However, it was not until 1971 that the mechanism of biological azo reduction was investigated by Gingell and Walker. Using cell-free extracts of *Streptococcus faecalis* (now reclassified as *Enterococcus faecalis*), they established that soluble flavins could act under anaerobic conditions as electron shuttles between reduced nicotinamide adenine dinucleotide (NADH) -linked flavoproteins and azo dyes which acted as artificial electron acceptors. The dye was then decolourised concurrently with re-oxidation of the NADH attached to the flavins due to the dissociation of the azo bond. This mechanism is represented diagrammatically in Figure 1.4.

Figure 1.4: The biological mechanism of azo reduction under anaerobic conditions



(Adapted from Walker 1970, Gingell & Walker 1971)

Where NAD^+ is nicotinamide adenine dinucleotide, FMN is flavin mononucleotide, R represents the chromophore of an azo dye and R^1 the auxochrome. The product of equation 1 is colourless but unstable, and may be reoxidised to the coloured parent compound. The products of equation 2 are colourless and stable. The process requires 4 electrons (Willmott *et al.* 1998, Nam & Renganathan 2000).

Gingell and Walker proposed that the protein responsible was probably a flavoprotein as the reaction was not inhibited by cytochrome-complexing compounds, and the ubiquitous nature of flavoproteins explained the wide species and substrate specificity of azo reductase activity (Gingell & Walker 1971, Walker *et al.* 1971). These findings were subsequently confirmed by the work of Brown and co-workers (1981).

Thus it is commonly thought that azo reduction is due to the action of electron transfer mediated by flavoproteins, and several studies appear to confirm this. A study by Ghosh and co-workers in 1992 purified two azoreductases from *Shigella dysenteriae*, a dimer of 28 kDa and a monomer of 11 kDa, and concluded that these were flavoproteins. In 1993, Rafii and Cerniglia deduced that the azoreductase activity of *Clostridium perfringens* was due to the same protein as the nitroreductase activity, and that it was probably a flavoprotein (Rafii & Cerniglia 1993*a*). Chang and co-workers in 2000 produced a recombinant *Escherichia coli* (strain NO3) with genomic DNA fragments from an azo-reducing wild-type *Pseudomonas luteola* strain. The *E. coli* had the ability to reduce azo dyes, but it was considered that this ability probably did not originate from the plasmid DNA, and thus was probably due to proteins constitutively present in the *E. coli* such as flavoproteins. A study by Russ and co-workers in 2000 also utilised a recombinant *E. coli* to transfer a flavin reductase to *Sphingomonas* sp. strain BN6, and observed that both species reduced sulphonated azo dyes under anaerobic conditions and to a lesser extent aerobic conditions. They inferred that the ability to reduce azo compounds was probably due to the presence of flavoproteins.

The requirement for soluble flavins, such as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), for azo reductase activity has also been observed by several groups (Douch 1975, Brown 1981, Rafii *et al.* 1990, Chung *et al.* 1992). However, some studies have reported that soluble flavins are not required for or have no beneficial effect on azo reduction (Rafii *et al.* 1990, Russ *et al.* 2000). This has bought the role of flavoproteins as proposed by Gingell and Walker into question. Further questions as to whether flavoproteins are responsible for azo reduction have been raised by studies that have isolated azo degrading enzymes.

In 1984, Zimmermann and co-workers compared the azo degrading enzymes of two strains of *Pseudomonas* sp. K24 and KF46 adapted to grow on different azo dyes. They found that the two enzymes, which were 21 kDa and 30 kDa respectively, did not cross-react immunologically with each other. Studies by Rafii and co-workers in 1990 on 10 anaerobic intestinal bacteria capable of reducing azo dyes showed that at least three types of protein were produced by all of the azo reducing organisms, based upon molecular weight and charge, but that each isolate expressed only one type (Rafii *et al.* 1990, Rafii & Cerniglia 1990). More recently, they have shown that an antibody against the *C. perfringens* azoreductase also hybridised to other azo reducing obligate and facultative anaerobes (e.g. *Clostridium* sp., *Eubacterium* sp., *Enterobacter cloacae*,

Citrobacter amalonaticus and *E. coli*), but not to other azo reducing species (Rafii & Coleman 1999).

In 1998, Kakuta and co-workers purified two azo reducing enzymes from a dye decolourising strain of *Candida curvata*, which were different in terms of molecular weight (47 and 56 kDa), co-factor requirement (NADPH and NADH) and temperature optima (50 and 40°C) (Kakuta *et al.* 1998*a*, Kakuta *et al.* 1998*b*). These studies have provided evidence that the enzymes responsible for azo reduction can be very different between genera, species or even strains, and thus it is possible that azo reduction is not mediated by such commonly preserved molecules as flavoproteins. The effect of pre-exposure of the microorganisms to azo dyes has also been studied. Rafii and co-workers in 1990 used 10 anaerobic intestinal bacteria that had no prior exposure to dyes, and found that each could reduce azo dyes and thus that enzymes responsible are produced constitutively. However, studies by Ghosh and co-workers in 1992 and Hu in 1998 showed that the azo reducing enzymes of *S. dysenteriae* and *P. luteola* decolourising azo dyes in a first order reaction were inducible by the dyes. In 1999, Mazumder and co-workers purified an azo reducing enzyme of *Caulobacter subvibrioides*, which was noted to be produced semi-constitutively. It seems unlikely that the ubiquitous flavoproteins were responsible for azo reducing activity at least in these cases.

Whether the reduction of azo bonds occurs intracellularly, by the dye passing through the cell membrane, or extracellularly, by the soluble flavins being excreted through the membrane, is also a point of contention. The permeability of the bacterial cell membrane to azo dyes was highlighted by Walker and co-workers in 1971, who suggested that differences in permeability between different species or even young and old cultures will influence the rate at which reduction of azo compounds occurs. A study by Mechsner and Wuhrmann in 1982 showed that azo reduction was limited by the degree of sulphonation of the dyes, and thus azo reduction must occur intracellularly and be limited by permeation of the dye molecules through the cell membrane. In 1983, Kulla and co-workers observed that sulphonated aromatics formed upon cleavage of sulphonated azo dyes interrupted degradation of the dyes, and concluded that the sulphonated aromatics were reacting with non-sulphonated intermediates and being channelled into a dead-end pathway, and thus the reaction must be occurring intracellularly. Recently, Russ and co-workers in 2000 showed that whole cells of *Escherichia coli* and a *Sphingomonas* sp. reducing sulphonated azo dyes showed less than 2% of the specific activities found with cell free extracts. They suggested that cell

membranes have low permeability to highly polar sulphonated azo compounds, and that membrane permeability is a limiting factor in azo dye degradation.

However, several studies have shown that cell permeability does not appear to influence azo dye degradation. Brown in 1981 observed that a variety of obligate and facultative anaerobes had the ability to reduce high and low molecular weight azo dyes. A study by Yatome and co-workers in 1991 on azo dye degradation by whole cells and cell-free extracts of different species and genera observed that *Pseudomonas stutzeri* cells and extracts could decolourise unsulphonated dyes whilst only extracts could decolourise sulphonated dyes, *P. cepacia* cells and extracts could decolourise unsulphonated and sulphonated dyes, and *Bacillus subtilis* cells and extracts could decolourise unsulphonated dyes but not sulphonated dyes. They concluded that, whilst permeation of the dyes into the cells is a limiting factor for *P. stutzeri*, both *P. cepacia* and *B. subtilis* must contain azo reductase enzymes with different specificity for unsulphonated and sulphonated dyes.

Studies by Rafii and co-workers using gold labelled antibodies to azoreductase with *Clostridium perfringens* have shown that azo reducing enzymes are distributed throughout the cytoplasm, not associated with any organised structures and released extracellularly (Rafii *et al.* 1990, Rafii & Cerniglia 1993*b*). They suggested that extracellular secretion of the enzymes following synthesis without accumulation could explain why high and low molecular weight azo dyes are metabolised at comparable rates despite large differences in cell membrane permeabilities. More recently they have also shown that lytic and lysogenic cultures of *E. coli* infected with a recombinant phage containing DNA from *C. perfringens* showed increased azo reductase activity, suggesting that the enzyme is released only from lysed cells (Rafii & Coleman 1999). This work, in conjuction with the suggestion by Walker and co-workers in 1971 that membrane permeability differences may exist between young and old cultures, may illuminate some of the differences that have been observed in the importance of permeability on azo dye reduction, as the age of the cultures used for decolourisation experiments will determine the quantity of lysed cells and thus the ease with which azo dye reduction will occur.

It has also long been known that azo bonds can be reduced in the mammalian liver (Gingell & Walker 1971, Rinde & Troll 1975, Chung *et al.* 1992). This was first demonstrated by Mueller and Miller in 1948, who observed that an enzyme capable of reducing azo groups was located mainly in the microsomal fraction, active under anaerobic conditions and required nicotinamide adenine dinucleotide phosphate

(NADPH) as an electron donor (Walker 1970). Subsequent studies by Hernandez and co-workers in 1967 inferred that NADPH-cytochrome P-450 was involved in azo fission (Walker 1970). Later studies on azo reducing enzymes purified from mammalian liver have shown them to be identical to quinone reductases, such as that of Huang and co-workers in 1979 (Levine & Raza 1987) and Kim and Shin in 2000. It was also discovered that azo reduction by liver microsomes was oxygen insensitive, proceeding both in anaerobic and aerobic conditions (Levine 1985), whereas most studies of bacterial azo reductase enzymes have found them to be oxygen sensitive (Rafii *et al.* 1990, Chung *et al.* 1992, Zaoyan *et al.* 1992, Banat *et al.* 1996). It has also been stated that water-soluble azo dyes, such as those belonging to the reactive and direct groups, are reduced by intestinal microorganisms in the gastrointestinal tract, whilst water-insoluble azo dyes, such as those belonging to the azoic and disperse groups, are metabolised by enzymes in the liver (Chung 1983). It may therefore be postulated that the mechanism of azo reduction within mammalian livers and by intestinal bacteria is very different, although a definitive mechanism has yet to be elucidated for both systems.

1.4 Toxicity and genotoxicity

1.4.1 Toxicity of textile dyes and aromatic amines

Studies of the toxicity of textile dyes to aquatic microorganisms have shown that a large number are non-toxic at concentrations of up to 100 mgL^{-1} (Brown *et al.* 1981), with only a few types, such as the basic dyes, showing inhibitory effects (Chung 2000). Reactive dyes appear to have the lowest toxicity of all the dye classes (Hao *et al.* 2000). Carliell and co-workers in 1995 showed that C.I. Reactive Red 141 was not inhibitory to an anaerobic sludge at concentrations of up to 100 mgL^{-1}, and only slightly inhibitory at concentrations of up to 500 mgL^{-1}, as measured by total gas production. A study by Beydilli and co-workers in 1998 showed no significant toxic effects for RB, C.I. Reactive Red 2 and 120 or C.I. Reactive Yellow 3, 15 and 17 at 300 mgL^{-1} in a methanogenic culture, as measured by total gas and methane production. Willetts and Ashbolt in 2000 showed that C.I. Reactive Red 235 was non-toxic to the biomass of an up-flow anaerobic sludge blanket (UASB) reactor at concentrations of up to 100 mgL^{-1}.

As has been noted in section 1.3.1, commercial textile dyes exhibit low toxicity to mammals and fish (Little & Lamb 1973 and Clark & Anliker 1980, as quoted by Chung 2000). A review of toxicity data on textile dyes by the Ecological and Toxicological Association of Dyes and Organic Pigment Manufacturers (ETAD)

showed that 88% of the listed dyes had an LD_{50} (dose required to kill 50% of the population) of >10 mgL^{-1} in fathead minnow lethality tests (Easton 1995), which would exceed the normal range of concentration of dyes present in water courses (DiGiano *et al.* 1992, Waters 1995). Bioaccumulation in natural watercourses is also not expected to occur for dyes with a water solubility of >2000 mgL^{-1}, which many including the reactive dyes have (Easton 1995, O'Neill *et al.* 1999). In 1997 Kopponen and co-workers tested 13 textile dyes against mouse hepatoma cells in the Hepa-1 cytotoxicity test, which measures the reduction in protein content of the cells using a dye binding method. It was found that the least toxic dyes were the black and yellow mix reactive dyes, which had IC_{50} values (the concentration at which the protein content of test cells was 50% of that of control cells) of 825 mgL^{-1} and 703 mgL^{-1} respectively. In terms of human health, it has been reported that reactive dyes may be respiratory and/or skin sensitisers (Health and Safety Executive 1997), due to the covalent binding of the dye to protein nucleophilic groups of the epithelial cells (Griem *et al.* 1998). However, skin sensitisation resulting in reactions such as dermatitis seems to be rare (Health and Safety Executive 1997).

Despite the fact that most dyes seem to be fairly non-toxic, especially the reactive dyes, it must be noted that some azo dyes including those belonging to the reactive class have been reported to be toxic in some mammalian and bacterial systems. The azo dye C.I. Mordant Orange 1 was shown to be toxic to a methanogenic sludge in a UASB reactor by Donlon and co-workers in 1997, while Razo-Flores and co-workers have stated that C.I. Mordant Orange 1, C.I. Mordant Yellow 1 and C.I. Mordant Yellow 12 are toxic to methanogenic sludge with IC_{50} (concentration reducing methanogenic activity by 50%) values of 0.014-0.25 mM (Razo-Flores *et al.* 1997*a*). A recent study by Hu and Wu in 2001 showed that a red reactive azo dye was highly inhibitory to the growth of a species of *Anabaena*, with IC_{50} values as low as 5 mgL^{-1} (based on dry weight) and 7 mgL^{-1} (measured by chlorophyll a). Hildenbrand and co-workers in 1999 showed the azo dye C.I. Direct Blue 15 to exhibit cytotoxicity in cultured kidney epithelial cells with an EC_{50} (effective concentration reducing cell proliferation by 50%) value of 40 mgL^{-1}. A study by Kalyuzhnyi and Sklyar in 2000 showed the azo dye C.I. Direct Brown 106 to be toxic to a methanogenic sludge, with an IC_{50} value of 410 mgL^{-1}. Two blue reactive azo dyes were shown to be toxic in the Hepa-1 cytotoxicity test by Kopponen and co-workers in 1997, with IC_{50} values of 40-65 mgL^{-1}.

It has been suggested that the toxicity of azo dyes in both bacterial and mammalian systems is due to the degradation of the azo bond to generate the constituent aromatic amines (Chung *et al.* 1978). Under anaerobic conditions azo bonds are reduced, which results in the fission of the azo bond and subsequently yields aromatic amines as they are the building blocks for production of azo dyes (Rafii *et al.* 1990, Brilon *et al.* 1981*a*, Haug *et al.* 1991, Banat *et al.* 1996). This process is discussed in detail in section 1.3.5. It is well known that many aromatic amines are toxic (Cripps *et al.* 1990, Haug *et al.* 1991, Chung 2000). Often, they are far more toxic than the intact parent dye (Easton 1995, Sosath & Libra 1997, Walsh *et al.* 1994, Hao *et al.* 2000), and thus azo reduction is considered as an activation step in the respect that reduced azo dyes are often more toxic to cells than the unreduced forms (Chung 2000). The toxicity of the aromatic amine breakdown products of azo dyes is also a limiting factor in the biodegradation of these dyes (Goncalves *et al.* 2000, Hao *et al.* 2000). It is therefore interesting to note that recent work has shown that genetic adaptation can occur which allows microorganisms to utilise novel pathways that generate non-toxic as opposed to toxic intermediates, or to produce enzymes with decreased sensitivity to the toxic intermediates (van Hylckama Vlieg *et al.* 2000).

However, it is far too simplistic to state that all reduced azo dyes will exhibit cytotoxic effects in all prokaryotic and eukaryotic systems. The processes involved with the toxic end-point of an assay will vary, thus giving different toxic effects. For example, a study by Hildenbrand and co-workers in 1999 showed that, whilst the azo dye C.I. Direct Blue 15 was toxic to cultured kidney epithelial cells (EC_{50} 40 mgL^{-1}), the dye was non-toxic to hepatocyte cell lines Hep G2 and Chang liver (EC_{50} >250 mgL^{-1} for both). These variables are considered further in section 1.4.3. Also, structurally related dyes may not produce the same toxicity in the same system. For example, the azo dye C.I. Mordant Yellow 12 was found to be ~18-fold less toxic than its group substituted analogue C.I. Mordant Orange 1 in methanogenic cultures (IC_{50} 0.25 and 0.014 mM respectively) (Razo-Flores *et al.* 1997*a*). The relationship between structure and activity is discussed further in section 1.4.8.

1.4.2 Genotoxicity of textile dyes and aromatic amines

Some azo dyes have been known to be genotoxic for some time. In 1940, sufficient evidence was gathered to establish the food azo dye Butter Yellow (C.I. Solvent Yellow 2, dimethylaminoazobenzene [DAB]) as genotoxic, which led to its gradual prohibition by governing bodies throughout the world (Combes & Haveland-

Smith 1982). The azo food dye Amaranth (C.I. Food Red 9, C.I. Acid Red 27) has also been established as genotoxic and carcinogenic in rats, and has been banned from the US since 1976 (Chung *et al.* 1978, Combes & Haveland-Smith 1982). Some azo textile dyes have also been found to be genotoxic. A study by Al-Sabti in 2000 tested low doses of up to 10 mgL^{-1} of the chlorotriazine textile dye C.I. Reactive Red 120 for genotoxicity in fish, as measured by micronucleus induction in erythrocytes. It was found that micronuclei increased not only in a dose-dependent manner but also in a time-dependent manner. A survey of the literature by Moller and Wallin in 2000 on 9 azo textile dyes revealed that C.I. Solvent Yellow 14, C.I. Solvent Yellow 7, C.I. Pigment Orange 5, C.I. Pigment Red 4, and C.I. Pigment Red 23 were genotoxic in short-term tests. Also in 2000, Tsuda and co-workers used the *in vivo* Comet (alkaline single cell gel electrophoresis) assay with eight mouse organs for 24 azo compounds including textile dyes. They observed that 14 and 12 compounds induced DNA damage in the colon and liver respectively, with the genotoxic effect being greatest in the colon.

Concern over the safety of azo dyes is not only ascribed to their genotoxic potential, but also that they may bioaccumulate in the food chain as not all azo dyes are degraded completely in the liver or gastrointestinal tract (Razo-Flores *et al.* 1997*a*). However, the literature concerning genotoxicity of azo dyes has shown that genotoxicity cannot be ascribed to the mere possession of an azo chromophore, as many azo dyes exhibit no activity and there is no correlation between potency and number of azo bonds (Combes & Haveland-Smith 1982). Indeed, it has long been known that most azo dyes require metabolic activation to exhibit a genotoxic effect, such as mutagenicity in the Ames (*Salmonella*/microsome) test (Chung *et al.* 1978, Brown & Dietrich 1983). This initiation of mutagenicity is thought to be due to reductive cleavage of the azo bond under anaerobic conditions, such as by mammalian intestinal microorganisms (Chung 1983, Manning *et al.* 1985), which produces the constituent aromatic amines. Aromatic amines are known to exert genotoxic effects, and thus the observed genotoxicity of most azo dyes is probably due to these components (Chung & Cerniglia 1992).

Many aromatic amines are known to be genotoxic, mutagenic and/or carcinogenic (Cripps *et al.* 1990, Rafii *et al.* 1990, Sweeney *et al.* 1994, Banat *et al.* 1996). Carcinogenicity, mutagenicity and genotoxicity are types of reactions that involve any interaction with cellular DNA. More research into the carcinogenicity and mutagenicity of aromatic amines has been undertaken than their genotoxicity, which are mainly screening assays. Nitro-containing polycyclic aromatic hydrocarbons (nitro-PAHs), which are important intermediates in the manufacture of dyes, have been shown

to be mutagenic to bacteria and carcinogenic in mammalian cells (Cerniglia & Somerville 1995). The azo dye components *p*-phenylenediamine and benzidine have been shown to be mutagenic in the Ames test, whilst benzidine is carcinogenic and has been shown to produce tumours in the bladders of rats, mice, rabbits, dogs and humans (Chung 2000). A study by Forsythe in 1990 showed that the carcinogen *N*-nitroso guanidine, a reduced nitro-PAH, was genotoxic in an *Escherichia coli* differential kill assay. Metabolic activation by enzymes is often required for initiation of the genotoxic, mutagenic or carcinogenic potential of most aromatic compounds. For example, the mutagenicity and carcinogenicity of nitro-PAHs has been shown to be dependent on bioactivation by bacterial and mammalian nitroreductases, which reduces the nitro group (Cerniglia & Somerville 1995). This bioactivation leads to the formation of a highly reactive electrophilic species, such as an ion or a radical, which can covalently interact with DNA thus producing the genotoxic effect (Blunck & Crowther 1975, Chung 2000). However, bioactivation of azo dyes can either increase or decrease their genotoxicity, and so the genotoxicity of not only the constituent aromatic amines but also the by-products of dye reduction should be studied (Chung 2000).

1.4.3 Toxicity bioassays

In terms of pollution potential of a chemical, toxicity is among the most important criteria to be analysed (Leisinger 1983, Baughman & Perenich 1988). As has been discussed, textile dyes themselves appear to present a minimal toxicological threat, but the major point of concern is the toxicity of dye breakdown products in systems designed to decolourise dye containing wastewaters. Various analytical methods can be utilised to determine both the identification and quantification of chemicals in a complex mixture, such as atomic absorption spectroscopy, high-performance liquid chromatography, gas chromatography, and antibody-based assays (Costanzo *et al.* 1998). However, in addition to being expensive, time consuming and labour intensive, the most distinct disadvantage of analytical methods with respect to analysing treated wastewaters is that they are unable to determine toxicity of the effluent to living organisms (Thomulka *et al.* 1993, Billard & DuBow 1998). They are also unable to fully account for synergistic or antagonistic effects of different chemicals in complex mixtures (Costanzo *et al.* 1998).

In 1996, the Environment Agency (EA) proposed the introduction of Direct Toxicity Assessment (DTA), which is the assessment of toxicity of whole effluent or receiving water samples (Environment Agency 1998). DTA is designed to take into account the additive, antagonistic or synergistic effects of all substances present in these

samples, thus providing a more holistic measure of harm. Hitchcock and co-workers showed an example of the additive effect on toxicity that treated dye wastewaters can present in a study in 1998. In the process of dyeing, an electrolyte is required to increase the affinity of the dye for the fibre by increasing its chemical potential. The most common electrolyte used in dyeing with reactive dyes, such as RB, is sodium chloride (Carliell *et al.* 1998). Hitchcock and co-workers (1998) observed that reactive dye wastewaters containing high concentrations of sodium chloride (89-112 gL^{-1}) were significantly more toxic to the nematode *Caenorhabditis elegans* than simulated wastewater samples that did not contain sodium chloride. It is therefore clear that toxicity assessment of treated wastewaters that take into account the total toxicity of the sample is essential to ensure safe discharge to natural watercourses (Hao *et al.* 2000). This would require the use of a toxicity bioassay (Thomulka *et al.* 1993, Salizzato *et al.* 1998), of which there are many different types (Fielden *et al.* 1996).

Toxicity bioassays commonly use whole, aquatic organisms from several trophic levels (Coleman & Qureshi 1985). These include fish, such as the fathead minnow (*Pimephales promelas*), golden orfe (*Leuciscus idus*), and salmon (*Salmo gairdneri*) (de Zwart & Sloof 1983, Blum & Speece 1991, Dannenberg 1996, Sweet *et al.* 1997); invertebrates such as daphnia (e.g. *Daphnia magna*, *Daphnia pulex* and *Ceriodaphnia dubia*) (de Zwart & Sloof 1983, Kaiser & Palabrica 1991, DiGiano *et al.* 1992) and the nematode *Caenorhabditis elegans* (Guven *et al.* 1994, Hitchcock *et al.* 1997); oyster larvae (Fielden *et al.* 1996, Environment Agency 1998); rotifers and hydra (e.g. *Hydra littoralis*) (Dannenberg 1996, Fielden *et al.* 1996); and green algae (e.g. *Chlorella pyrenoidosa*, *Scenedesmus pannonicus*, *Scenedesmus quadricauda* and *Raphidocelis subcapitata* [formerly *Selenastrum capricornutum*]) (de Zwart & Sloof 1983, Kaiser & Palabrica 1991). The EA have identified a battery of test organisms to be used as standard toxicity bioassays in DTA of treated effluents, and have issued appropriate standardised test protocols. These test organisms cover three trophic levels, and consist of algae (e.g. *R. subcapitata*), daphnia (e.g. *D. magna*) and fish (e.g. *P. promelas*) (Heijerick *et al.* 2000, Johnson 2000).

All of the above bioassays have been extensively used for screening environmental samples for toxic substances (Billard & DuBow 1998, Costanzo *et al.* 1998), but each has their own set of disadvantages. Comparative studies on the sensitivity of each of these test organisms to environmental pollutants have shown that sensitivity increases towards the lower trophic levels. Fish tests using *P. promelas* and *Poecilia reticulata* have been shown to be less sensitive than daphnia tests using *C.*

dubia and *D. magna*, which in turn are less sensitive than the green algae *R. subcapitata* (Dannenberg 1996, Rojickova-Padrtova *et al.* 1998). In addition to this, work has been conducted on comparing the sensitivity of other test organisms to the standard organisms, to assess the suitability of other bioassays for ecotoxicological monitoring. For example, a study by Toussaint and co-workers in 1995 showed that the freshwater rotifer *Branchionus calyciflorus* was as sensitive a test organism to single chemicals as the standard organisms daphnia (*D. magna* and *C. dubia*), green algae (*R. subcapitata*) and fathead minnows (*P. promelas*). A recent study by Novak and co-workers in 2000 showed that the microcrustacean *Thamnocephalus platyurus* was as sensitive to toxic storm water samples as daphnia (*D. magna*) and the rainbow trout (*Oncorhynchus mykiss*).

As well as relative insensitivity, and the obvious ethical problems related to the use of vertebrates, one of the major disadvantages of fish bioassays is the requirement of a licence from the Home Office to run these tests (Hayes *et al.* 1999). However, there are also numerous disadvantages that apply to all of the types of bioassays. These include high cost, fragility of the test organisms, the use of sophisticated facilities, difficulty in obtaining reproducible growth rates (and hence reproducible toxicity results) and the requirement for a high level of professional competence for data interpretation (Coleman & Qureshi 1985, Fielden *et al.* 1996, Costanzo *et al.* 1998). The major disadvantage that these bioassays suffer from is one of relatively long assay times, which can run to several hours or even weeks. Therefore, the trend has moved towards the use of short-term tests for ecotoxicological assessment, such as bacterial bioassays.

Microorganisms can be used as ecotoxicological bioindicators, as they detect the biologically active components of a sample as opposed to the total amount of toxic materials (Balicka & Wegrzyn 1994). Bacterial bioassays have several attributes that make them attractive for use in wastewater toxicity testing (Coleman & Qureshi 1985). They are relatively inexpensive to perform, have standardised test procedures, utilise robust test organisms which can be easily stored through freeze drying and reproducibly reconstituted and utilise biochemical pathways common to higher organisms (Fielden *et al.* 1996, Sweet *et al.* 1997). The major advantage that bacterial bioassays have over the other types of bioassays listed above are that they have a rapid response to toxicants present in a sample (Bitton & Dutka 1986, Fielden *et al.* 1996), and hence are known as short-term toxicity assays. Examples of short-term bacterial bioassays include those based on inhibition of metabolic activity, as measured by colony counts, most probable number, turbidimetry or enzymatic activity (e.g. ECHA Biocide Monitor which uses a

tetrazolium salt to indicate metabolic activity) (Trevors 1986, Costanzo *et al.* 1998); inhibition of enzymes such as oxidoreductases (e.g. dehydrogenase detected using tetrazolium salts) and hydrolases (e.g. phosphatase detected using an organic phosphorus substrate) as measured by spectrophotometry, fluorometry or titration (e.g. Toxi-Chromotest which detects β-galactosidase) (Bitton & Koopman 1986, Costanzo *et al.* 1998); inhibition of respiration as measured by reduction of BOD of an aerobic sludge (e.g. PolyTox which measures reduction of respiration in wastewater cultures) or reduction in nitrite conversion by *Nitrobacter* sp. (King & Dutka 1986, Trevors 1986, Costanzo *et al.* 1998); reduction of ATP levels (detected using firefly luciferin) as measured by photometry (Bitton & Koopman 1986); and inhibition of motility (e.g. reversal of 90% motility in *Spirillum volutans*) (Coleman & Qureshi 1985, Trevors 1986). One of the most commonly used short-term bacterial bioassay is that based on the bioluminescence of aquatic microorganisms (e.g. Microtox®, Biotox™, Lumistox™) (Costanzo *et al.* 1998, Environment Agency 1998). The principles behind this bioassay and its use in the ecotoxicological monitoring of wastewaters is discussed further in section 1.4.5.

Several studies have been conducted to assess the sensitivity of bacterial bioassays in comparison to the standard bioassays that utilise test organisms such as fish and daphnia. In 1991, Blum and Speece tested 25 chemicals including chlorinated aliphatic hydrocarbons, substituted benzenes and phenols for toxicity to methanogenic sludge, as measured by inhibition of gas production, and aerobic heterotrophs from activated sludge, as measured by inhibition of oxygen uptake. They found excellent correlation between the sensitivity of fathead minnows with aerobic heterotrophs and methanogens (r^2 = 0.9 and 0.94 respectively). Comparisons of the MetPLATE™ assay, which is based on inhibition of the enzyme β-galactosidase by heavy metals, have shown it to be similar in sensitivity to *Ceriodaphnia dubia* (Bitton *et al.* 1994, Jung *et al.* 1996). A study by Botsford in 1998 showed that the metabolic activity of the bacterium *Rhizobium meliloti*, as measured by the reduction of a tetrazolium dye, showed comparable sensitivity to the fathead minnow *Pimephales promelas*. However, a study by Toussaint and co-workers in 1995 on 11 different chemicals showed that the toxicity values of the Polytox test, which measures inhibition of sludge biomass respiration, were one or more orders of magnitude different from values observed in bioassays using green algae (*Raphidocelis subcapitata*), daphnia (*Daphnia magna* and *C. dubia*) and fathead minnows (*P. promelas*). In 1997, Tisler and Zagorc-Koncan demonstrated that a mixed bacterial culture was less sensitive to formaldehyde and phenol than the algae

Scenedesmus quadricauda and the daphnid *Daphnia pulex* (EC_{50} = 34.1, 14.7 and 5.8 mgL^{-1} respectively for formaldehyde; EC_{50} = 510, 403 and 25 mgL^{-1} respectively for phenol). It has long been known, and these studies illustrate, that one problem encountered by bacterial bioassays is that no single assay can detect all categories of environmental toxicants, such as organics, inorganics and heavy metals, with equal sensitivity (Trevors 1986). Therefore, when the ecotoxicological assessment of wastewater effluents is concerned, a battery of test approaches incorporating several microbial assays is recommended (Costanzo *et al.* 1998).

1.4.4 Short-term genotoxicity bioassays

There are several methods available for the analysis of genotoxicity of a chemical. One type is the *in vitro* use of mammalian cell cultures, to monitor the ability of a suspected genotoxicant to induce chromosomal aberrations, such as aneuploidy (the loss or gain of individual chromosomes), or some form of DNA damage, such as unscheduled DNA synthesis (UDS) (Ashby 1986). Another is the *in vivo* use of whole organisms, such as rats and mice, to study the carcinogenic potential of a suspected genotoxicant in various organs or cell lines. However, there are disadvantages to the use of mammalian assays. Often the results of the assay are difficult to interpret or do not give adequate information as to the mechanism of action. For example, the UDS assay does not supply information as to whether attempts to repair DNA adducts were successful, or whether the cell will survive (Fox 1988). The main disadvantage that mammalian assays suffer from is the length of time required to run an assay, which is often days or weeks. Therefore, short-term genotoxicity assays, which measure mutation, chromosome abnormality or some other genetic endpoint, were developed during the late 1970s and early 1980s (Rosenkranz & Ennever 1988) and are now more commonly used. Bacterial bioassays, such as the use of *Salmonella* to detect gene mutations, or the use of the SOS repair pathway to detect DNA damage, are usually employed (Ashby 1986, de Maagd & Tonkes 2000). The major advantages of short-term assays over mammalian assays are their relative simplicity to conduct and speed of completion, which is usually between a few hours and a few days. Disadvantages include the fact that no single test is equally sensitive to all types of genotoxicants, and they also require highly trained personnel to critically evaluate the results (Poole & Schmähl 1987).

In 1975, Ames and co-workers developed a mutagenicity assay known as the *Salmonella*/microsome assay, or more commonly as the 'Ames' or 'Salmonella test'. This is a short-term bacterial reverse mutation assay specifically designed to detect a

wide range of chemical substances that can produce genetic damage that leads to gene mutations. It involves the incubation of histidine auxotrophs (his^-) of *Salmonella* Typhimurium, using strains carrying different mutations in various genes in the histidine operon that can differentiate DNA damage caused by insertional and frame shift mutations, on minimal media. Induced reversion to prototrophy (his^+) by a mutagen will result in the formation of colonies, which are then compared to the number of colonies formed by spontaneously induced reversion (Forsythe 1990, Mortelmans & Zeiger 2000). The Ames test is now the most commonly used screening assay for mutagens (Costanzo *et al.* 1998), and international guidelines have been developed for use in testing protocols to ensure uniformity of testing procedures (Mortelmans & Zeiger 2000).

Studies have shown that the Ames test provides data that is an acceptable alternative to mammalian bioassays for the prediction of carcinogenicity of a compound (Rosenkranz & Cunningham 2000). In 1998, Zeiger assessed the data provided by Ames test, chromosome aberration, mouse lymphoma cell mutation, rodent bone marrow micronucleus induction and rodent carcinogenicity assays collated by the US National Toxicology Program (NTP). It was found that a positive Ames result was most predictive of carcinogenicity, and a battery of the other tests did not increase the prediction of carcinogenicity of a chemical. A similar survey of data for 82 chemicals held in the NTP database by Kim and Margolin in 1999 assessed the Ames test, chromosome aberration and micronucleus induction assays in mouse bone marrow cells. It was found that only the Ames test affects the predictivity of carcinogenicity, and once the Ames test result is available, the other assays provide little additional information for the prediction of carcinogenicity of a chemical.

However, there are some disadvantages to the Ames test. The test is complicated to perform, and requires a lengthy incubation period of two days (Forsythe 1990, Billard & DuBow 1998). It has also been noted that the traditional tester strains used are largely insensitive to nitrosamines, which are environmentally significant carcinogens associated with azo textile dye production (Cooper & Porter 2000). Another type of short-term bacterial bioassay is the SOS Chromotest. This assay, developed by Quillardet and co-workers in 1982, utilises the induction of the SOS repair mechanism in *Escherichia coli* in the presence of a suspected mutagen. The "stress gene" *sul*A, which is depressed upon exposure to mutagens causing induction of the SOS response, is fused to the *lac*Z reporter gene so that production of β-galactosidase is up-regulated by the SOS response. An increase in the production of β-galactosidase, caused by a

mutagen, is assayed using colorimetric substrates (Billard & DuBow 1998). The SOS Chromotest has been shown to correlate well with tissue contamination in the bivalve molluscs *Mya arenaria* and *Mytilus edulis* with polycyclic aromatic hydrocarbons (r^2 = 0.75) (White *et al.* 1997). Another assay that involves the use of the SOS repair mechanism is the λ Inductest, in which prophage λ is fused to the SOS response genes in lysogenic *E. coli*. DNA damage caused by a mutagen induces excision of prophage λ, which triggers the lytic cycle of the phage thus resulting in clear plaques on a lawn culture (Costanzo *et al.* 1998). Lee and co-workers further developed the λ Inductest in 1992, by utilising a bioluminescent sensing system using a recombinant luc^+ phage (Billard & DuBow 1998). Other bioluminescent systems have been developed for short-term bacterial genotoxicity bioassays, such as the Mutatox® test by Azur Environmental Ltd. (Costanzo *et al.* 1998).

1.4.5 Toxicity bioassays using bacterial bioluminescence

Short-term bacterial toxicity bioassays based on bioluminescence have been developed. The major advantages that bioluminescent assays have over toxicity bioassays from higher trophic levels are that they are extremely rapid, sensitive and their ease of operation means that highly trained personnel are not required (Sim *et al.* 1994, Dannenberg 1996, Billard & DuBow 1998, Salizzato *et al.* 1998). They are also highly reproducible, with coefficient of variation (standard deviation) ranging from 3-20% (de Zwart & Sloof 1983, Mayfield 1997). The major advantages that bioluminescent assays have over other bacterial bioassays are that they do not require cultivation of organisms, and standardised testing protocols are available due to the enormous amount of validation work that has been carried out (Dannenberg 1996, Salizzato *et al.* 1998). However, bioluminescent assays do suffer from some disadvantages. The apparatus required to conduct the assays, including a photomultiplier tube, a fluorimeter and a cooling block, are expensive (Dannenberg 1996). The low temperature and high sodium chloride concentrations that are required by the test organism often reduces sensitivity (Billard & DuBow 1998). Also, coloured and opaque samples present problems for these kinds of tests (Hayes *et al.* 1999). As early as 1942 Johnson, Caruer and Harryman showed that luminous bacteria were sensitive to a wide range of heavy metal chlorides and narcotics. However it was not until after Bulich described a test using freeze-dried *Photobacterium phosphoreum* in 1979 that luminescent bacteria began to have a widespread application in toxicity testing (Mayfield 1997). Examples of commercially available systems that utilise

bioluminescent bacteria are the ToxAlert™ (Merck Speciality Chemicals Ltd.), Biotox™ (BioOrbit) and Lumistox™ (Dr. Lange), but first available and still the most commonly used system is the Microtox® (Azur Environmental Ltd.) (Billard & DuBow 1998, Salizzato *et al.* 1998).

The commercially available luminescent bioassays most commonly use the bioluminescent bacterium *Vibrio fischeri* NRRL-B-11177 (Kaiser & Palabrica 1991, Kahru & Borchardt 1994, Kahru *et al.* 1996, Sweet *et al.* 1997, Azur Enviromental Safety Data Sheets). This organism, formerly known as *Photobacterium fisheri*, belongs to a group of marine microorganisms which occur ubiquitously in the ocean, either free living or in symbiotic association with higher marine organisms (e.g. in the light organ of fish), and include *V. fischeri*, *P. phosphoreum* and *V.* (*Beneckea*) *harveyi* (Bitton & Dutka 1986). These microbes are heterotrophic, facultatively anaerobic, Gram negative rods, with 2-8 sheathed polar flagella (Kahru 1993, Madanecki 2000). A negatively stained light microscope image of *V. fischeri* can be seen in Figure 1.5. These cells constitutively produce light as a function of metabolism, and thus light production reflects the metabolic activity of cells. A decrease in light production after contact with a suspected toxicant is an indication of metabolic inhibition of the test organism, and thus is suggestive of cytotoxicity (Sim *et al.* 1994, Billard & DuBow 1998). The mechanism of luminescence by these organisms is discussed below.

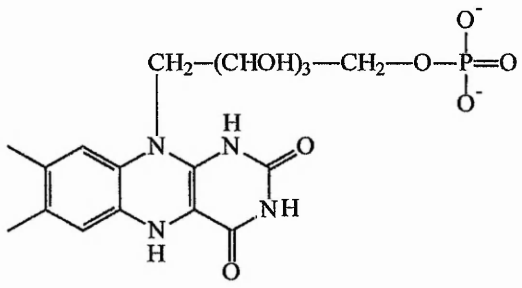
Figure 1.5: Light microscope image of negatively stained *Vibrio fischeri*

(Madanecki 2000)

Bacterial bioluminescence is achieved by an enzyme known as luciferase, which is a mixed-function oxidase (Bitton & Dutka 1986, Kahru 1993). Luciferase is a heterodimeric enzyme, with two non-identical subunits α (40 kDa encoded by *lux*A) and β (37 kDa encoded by *lux*B) that are inactive individually (Nealson & Hastings 1979, Billard & DuBow 1998). Luciferase catalyses the oxidation of a reduced riboflavin phosphate ($FMNH_2$) and the concomitant oxidation of a long-chain aliphatic

aldehyde (RCHO), which is usually 8-16 carbons long (e.g. tetradecanal, dodecanal), with molecular oxygen (Nealson & Hastings 1979, Bitton & Dutka 1986). The structure of the riboflavin phosphate, known as luciferin (Haddock *et al.* 2000), is shown in Figure 1.6. The oxidised luciferin-luciferase complex emits light at 490 nm, which is in the blue-green region of the visible spectrum (Fielden *et al.* 1996). Reduced nicotinamide adenine dinucleotide (NADH) is required as a co-factor to generate the electron transfer between the electron donating substrate (usually glucose) and luciferin, as well as to reduce the aliphatic acid back to the aldehyde (Kahru 1993). This process is represented diagrammatically in Figure 1.7. The *lux* operon in *V. fischeri* contains three genes, *lux*C, D and E, which encode proteins that associate to form a fatty acid reductase protein complex which is required for the synthesis of the aldehyde. Immediately downstream of *lux*E are *lux*G and H, which encode the synthesis of luciferin (Billard & DuBow 1998).

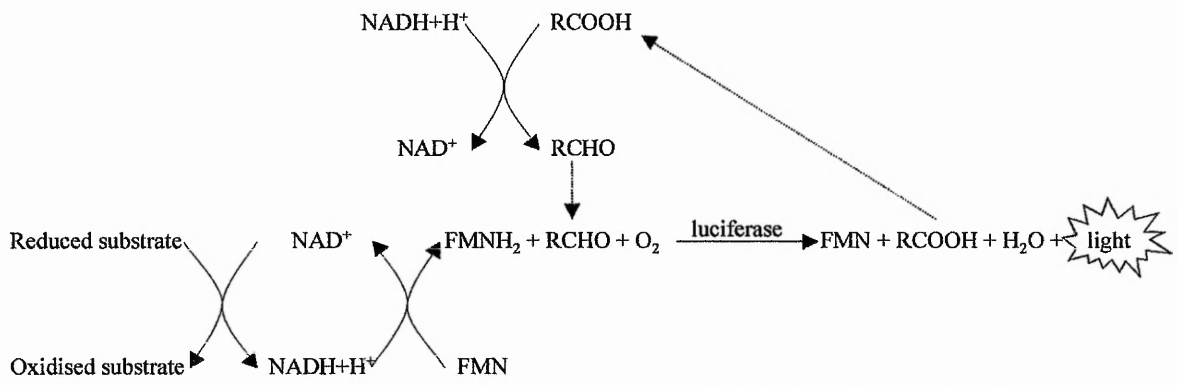
Figure 1.6: The structure of bacterial luciferin



(Haddock *et al.* 2000)

Light production by bioluminescent organisms is regulated mainly by a process known as autoinduction, which was first described by Nealson and co-workers in 1970. Molecules known as homoserine lactones (HSLs) are produced by the cells and excreted extracellularly, at a constitutive rate during early growth stages and then at increasing concentrations during stationary phase. In *V. fischeri*, where the *lux* genes are arranged in two divergently transcribed operons, the right operon (*lux*ICDABEGH) contains *lux*I that directs synthesis of the autoinducer N-(3-oxohexanoyl) homoserine lactone. This interacts with *lux*R in the left operon and forms a complex that stimulates expression of the right operon, thus producing the light reaction (Billard & DuBow 1998). Light production is up-regulated by increasing cell density, as higher concentrations of HSLs are available to enter neighbouring cells and stimulate light production.

Figure 1.7: Mechanism of bacterial bioluminescence



(Adapted from Nealson & Hastings 1979)

Where NAD^+ is nicotinamide adenine dinucleotide, FMN is flavin mononucleotide, RCOOH is a long-chain fatty acid, RCHO is the corresponding long-chain aliphatic aldehyde, and light is emitted at 490 nm.

Light production is also regulated by catabolite repression, in which induced synthesis of luciferase is repressed by glucose. This repression can be overcome by exogenous cyclic adenosine monophosphate (cAMP) (Nealson & Hastings 1979). The affinity of the luminescent mechanism in *V. fischeri* for oxygen is very high, and so light production is up-regulated by increasing oxygen availability (Nealson & Hastings 1979, Billard & DuBow 1998). Bioluminescence is intrinsically linked to metabolic respiration through the electron transport system, and so only viable cells will produce light (Bitton & Dutka 1986, Kahru 1993, Fielden *et al.* 1996). In very bright cells in a rich medium it has been estimated that luciferase accounts for 5% of cellular protein, and *V. fischeri* have a particularly high luciferase content due to the fact that luciferase production is not halted when cellular growth is inhibited (Nealson & Hastings 1979).

In order for any toxicity bioassay to be useful for predicting toxicological effects, not only on the organisms in the environment but ultimately on man, the sensitivity of the assay needs to be assessed with regards to other types of bioassay and correlations about agreement of data need to be drawn. Several studies have been conducted on the correlation between the Microtox® assay, which uses *V. fischeri*, and other bioassays, including bacterial bioassays and standard toxicity bioassays that use test organisms from higher trophic levels. A summary of some of these studies, which correlate the Microtox® assay to other bioassays for compounds with similar structures to the aromatic amines that will be considered in this study, can be seen in Table 1.3.

Table 1.3: Correlation of Microtox® toxicity data with other bioassays for aromatic compounds

Test organism	Endpoint	Toxicant	Correlation (r^2)	Reference
Aerobic heterotrophs	IC_{50} (oxygen uptake)	~60 aromatics inc. naphthalene and phenol	0.70	Blum & Speece 1991
Methanogens	IC_{50} (methane production)	~60 aromatics inc. naphthalene and phenol	0.82	Blum & Speece 1991
V. fischeri (Biotox™)	EC_{50} (light loss)	23 compounds inc. phenols	0.97	Kahru 1993
V. fischeri (Biotox™)	EC_{50} (light loss)	39 compounds inc. phenol	0.83	Kahru & Borchardt 1994
V. fischeri (Biotox™)	EC_{50} (light loss)	13 solvents inc. benzene	0.95	Kahru *et al.* 1996
Fathead minnow	LD_{50} (96 h lethality)	~60 aromatics inc. naphthalene and phenol	0.85	Blum & Speece 1991
Fathead minnow	LD_{50} (96 h lethality)	201 compounds inc. aromatics and phenols	0.81	Kaiser & Palabrica 1991

It can be seen that good correlations exist between Microtox® and two other bacterial bioassays, the Biotox™ (which also utilises *V. fischeri*) and the fathead minnow *Pimephales promelas* for aromatic-based compounds. The least correlation (r^2 = 0.70) was shown with a sludge of aerobic heterotrophs, which is interesting as both *V. fischeri* and the heterotrophs depend upon high concentrations of dissolved oxygen. The Microtox® assay has also been shown to give good correlations to bioassays using other test organisms with other compounds. Comparison of Microtox® with fish, such as the rainbow trout (*Oncorhynchus mykiss*) and fathead minnow, have shown correlations of r^2 = 0.70-0.97 using a wide range of chemicals (Kahru 1993, Mayfield 1997). However, metal compounds showed more variation, and lower correlations were observed with extended incubation of the fish (Mayfield 1997). Comparison with daphnia (e.g. *Ceriodaphnia dubia*, *Daphnia magna* and *D. pulex*), algae (e.g. *Scenedesmus quadricauda*) and ciliates (e.g. *Tetrahymena pyriformis*) have also shown generally positive correlations (Kaiser & Palabrica 1991, Kahru 1993), with typical correlations for daphnia of r^2 = 0.7-0.9 (Mayfield 1997). However, the quality of the fit varies widely dependant upon the types of chemicals and water quality (Kaiser & Palabrica 1991).

In addition, good correlations have been observed between Microtox® and rat and mouse assays for over 600 compounds (Fielden *et al.* 1996). Kahru and co-workers demonstrated high correlation between Biotox™ and rodent oral LD_{50} data for 39 compounds including phenol (r^2 = 0.69-0.71) (Kahru & Borchardt 1994), 14 solvents

including benzene ($r^2 = 0.95$) and 9 pesticides ($r^2 = 0.80$) when pesticide outliers were excluded (Kahru *et al.* 1996). Good correlations between Biotox™ and various human and animal cell lines ($r^2 = 0.90$) and human oral/blood LD_{50} data ($r^2 = 0.76$) have also been found (Kahru & Borchardt 1994). The least collinearity is observed with acute oral LD_{50} for the rat, but rat data is known to correlate little with any other biological endpoint (Kaiser & Palabrica 1991).

The sensitivity of the Microtox® assay to potential toxicants has also been studied. In 1985, Coleman and Qureshi showed that Microtox® was more sensitive than the *Spirillum volutans* mobility inhibition assay, as 10 out of 12 industrial wastewater samples were significantly more toxic to Microtox® than to *S. volutans*. Blum and Speece in 1991 showed that Microtox® sensitivity was significantly greater than that of aerobic heterotrophs and methanogens, to ~60 aromatic compounds including naphthalene and phenol. A study by de Zwart and Sloof in 1983 showed that daphnia *C. dubia*, *D. magna* and *D. pulex* were 2.97 times as sensitive as Microtox® to 15 compounds, including solvents such as benzene, with heavy metals showing least correlation. However, Doherty and co-workers in 1999 assayed 16 effluent samples and showed that all positive samples in the 15 min Microtox® tests were positive with *C. dubia*. It must also be remembered that one of the main advantages that Microtox® has over daphnia tests is the rapidity of the assay, as demonstrated by the fact that *C. dubia* required 48 h of exposure to produce a toxic response in this study.

It has long been known that the correlation between luminescent bioassays and other toxicity bioassays depends strongly on the type of chemicals tested (Kaiser & Palabrica 1991). For example, Kahru and co-workers in 1996 observed that the correlation between Microtox® and Biotox™ assays was very poor when tested against 13 pesticides ($r^2 = 0.37$), and most studies have reported lower collinearity with heavy metals. However, for aromatic compounds such as those under consideration in this study, high correlation between Microtox® and bioassays utilising organisms from all trophic levels has been observed. Therefore, although the complexity of biological processes in humans means that no one bacterial bioassay will give an accurate prediction of human toxicity, it is evident that, if one assay must be chosen, the advantages of bioluminescent bioassays and the good correlation they have demonstrated with higher trophic bioassays means that the Microtox® is an excellent choice for monitoring toxicity of treated wastewaters.

1.4.6 Genotoxicity bioassays using differential kill

One type of short-term bacterial genotoxicity bioassay are the bacterial DNA repair tests, also known as differential killing tests. Examples include the *Escherichia coli pol*A and *rec* assay, which have lethal mutations as their genetic end points (Poole & Schmähl 1987). These assays use bacterial strains defective in one or more DNA repair pathways, such as *pol*A (involved in excision repair via polymerase I) and *rec* (involved in recombination and error-prone repair) (Tweats *et al.* 1984). The principle of the test is that failure to repair DNA damage results in cell inactivation, and thus when repair proficient and repair deficient strains are exposed to a mutagen, a certain concentration will be used that allows repair proficient strains to repair DNA damage and survive, whilst the repair deficient strains are killed. The *E. coli pol*A assay was improved by Tweats and co-workers in 1981 by using more sensitive repair deficient strains (Forsythe 1990). It has been observed that most mutagens and carcinogens covalently bind to nucleophilic sites in DNA, or induce the formation of free radicals (e.g. activated oxygen) which result in DNA damage (Tweats *et al.* 1984), which will allow their detection in an assay based on DNA damage.

Advantages that differential kill assays have over mammalian bioassays are that they are extremely rapid, inexpensive, require limited resources and inexpensive apparatus, have a high degree of accuracy in detecting direct acting mutagens/carcinogens, and detect small differences in the number of lethal events in the repair proficient and deficient strains when metabolic activation is efficient (Tweats *et al.* 1984). Whilst the Ames test is an internationally recognised mutagenicity assay, differential kill assays may be preferable to the Ames test for a number of reasons. Differential kill assays are far more sensitive to chemical mutagens and carcinogens, as *E. coli* encode a gene product (from *umu*C) involved in generating mutations which *Salmonella* lack (MacPhee 1988), and they can detect a wide range of genotoxins as they are sensitive to both specific and non-specific types of DNA damage. However, one disadvantage that differential kill assays suffer from is that they are less sensitive to mutagenic agents that undergo metabolic activation at low efficiency than the Ames test. This is because, of all the mutagenic lesions induced in the genome of cells, at least one must be lethal in the differential kill assays, whereas a single lesion may be sufficient to produce a revertant response in reverse mutational assays such as the Ames test (Tweats *et al.* 1981, Tweats *et al.* 1984). Another problematic area in the use of bacterial bioassays including differential kill tests is that fact that standard culturing methods using microbiological media is required, which involves a one day incubation

period. To overcome this, the rapidity of this assay can be increased by the use of electrical impedance to assess bacterial viability (Forsythe 1990, Silley & Forsythe 1996).

Advantages in the use of impedance over standard culturing techniques include increased rapidity, simplicity and flexibility of the technology and automatic logging of operational parameter data for in-depth analysis via computer (Forsythe 1990, Silley & Forsythe 1996). Detection times with impedance methods usually occur within 12 h, as compared to culturing on currently available defined substrate media (18-24 h) or membrane filtration (minimum 42 h) (Timms *et al.* 1996). Impedance techniques, especially using the indirect measurement method, show good correlation with other viability assessment methods, such as disc diffusion and most probable number (Timms *et al.* 1996, Wu *et al.* 1997). Disadvantages of the use of impedance include the relatively high cost of the initial equipment, although running costs are minimal (Schulenburg & Bergann 1998). In addition, it has been noted that the sample material can affect the metabolic activity of the organisms when utilising impedance to assay foodstuffs, and thus it may be beneficial to determine separate calibration curves for each kind of material assayed when determining total bacterial counts using high nutrient samples (Wawerla *et al.* 1998).

In order to assess the suitability of short-term bacterial genotoxicity assays for the prediction of hazards to human health, correlations must be made to mammalian carcinogenicity testing using whole organisms or cells. However, there is virtually no reference in the literature to correlations between differential kill assays and carcinogenicity tests. The most widely used genotoxicity assay is the mutagenic screening Ames test, and thus it is this assay that is most commonly correlated with mammalian carcinogenicity. Poor correlation between short-term bacterial tests and rodent carcinogenicity bioassays in whole organisms have been observed, but these studies have usually utilised large groups of compounds (Fetterman *et al.* 1997). For example, a survey of the US National Toxicology Program (NTP) database by Bogen in 1995 identified 134 chemicals from various groups that were reported as positive in both the Ames test and rodent bioassays, and found only weak correlation between the two assays (r^2 = 0.39). White and Rasmussen (1996) observed a relatively weak correlation (r^2 = 0.51) when a mixture of only 51 compounds were compared using data from the genotoxic *E. coli* SOS Chromotest and rodent carcinogenicity. However, it has been observed that the correlation observed between the Ames test and mammalian assays is dependent upon the type of compound under investigation.

Certain agents can exhibit genotoxic, mutagenic or carcinogenic effects without any prior alteration in their chemical structure or behaviour, and these are known as direct-acting agents. Other agents require an alteration in their structure or behaviour, which is carried out by metabolic enzymes such as enzymes from the S9 fraction of rat liver homogenates, and are therefore considered to be metabolically activated agents. Aromatic amines, which are substituents of azo dyes, and other aromatics such as naphthols are metabolically activated agents (Knasmuller *et al.* 1999, Chung 2000) that are only able to bind to proteins after insertion or de-masking of functional groups by cytochrome P450 isoenzymes (Griem *et al.* 1998). When only metabolically activated compounds are studied, the correlation between the Ames test and both whole organism and cellular mammalian assays significantly improves. In 1992, a study by Hatch and co-workers showed significant correlation between mutagenicity in the Ames test and carcinogenicity in rodent bioassays using 24 aromatic amines and 10 heterocyclic aromatic amines (HAAs). Knasmuller and co-workers (1999) used a micronucleus induction assay with a human derived hepatoma (HepG2) cell line, and the Ames test using *Salmonella* Typhimurium strain YG1024 to study the correlation between these bacterial and mammalian mutagenicity assays for 5 HAAs. All compounds were positive in both assays. It may therefore be postulated that the weak correlation observed between mutagenicity assays such as the Ames test and carcinogenicity assays such as rodent bioassays when a large number of groups of chemicals are studied may be due to insensitivity of the Ames test to direct-acting agents, as studies of metabolically activated agents such as aromatic amines have shown good correlation between the two.

Good correlations have also been observed between bacterial genotoxicity assays and the Ames test, with both direct-acting and metabolically activated agents. In the study by White and Rasmussen mentioned above (1996) a large number of chemicals were compared in the SOS Chromotest and the Ames test. Good correlations were revealed with 268 direct-acting substances ($r^2 = 0.76$) and 126 metabolically activated substances ($r^2 = 0.65$). A study by De Meo and co-workers in 1992 showed high correlation ($r^2 = 0.85$) between the SOS Chromotest and the Ames test when tested against 84 aromatic compounds. Helma and co-workers (1996) compared the sensitivity of the Ames test with the *Escherichia coli* differential kill assay when tested against 23 wastewater samples with and without metabolic activation, including industrial effluents passed to the wastewater plant. It was observed that, out of a total of 20 genotoxic samples, 19 and 13 tested positive with the differential kill assay and Ames test

respectively, which showed that the differential kill assay was more sensitive than the Ames. The sensitivity of the differential kill assay to azo-based compounds was demonstrated in a study by Sweeney and co-workers (1994). In this study, the azo dyes Amaranth and Sunset Yellow were decolourised by *Enterococcus faecalis* and *Bacteroides thetaiotaomicron* and tested for genotoxicity using the *E. coli* differential kill assay and mutagenicity using the Ames test. It was found that the decolourised dyes were gentoxic in the differential kill assay, and not mutagenic in the Ames assay when using strains TA98 and TA100 but mutagenic when using TA102. Strain TA102 detects a variety of oxidants that are not detected by TA98 and TA100, and the production of the oxygen radical O_2^- was confirmed by reduction of nitroblue tetrazolium and inhibition by superoxide dismutase. This shows that azo-based compounds may exert a genotoxic or mutagenic effect via mechanisms other than those normally detected by standard Ames strains, and that the differential kill assay is a suitable method for detecting a variety of mechanisms that may exhibit gentoxicity.

It has been shown that there is good correlation between the mutagenic Ames assay and mammalian cell and whole organism carcinogenicity bioassays for metabolically activated compounds, including aromatics, and good correlation between the Ames test and bacterial genotoxicity assays such as the SOS Chromotest and those based on differential kill. Therefore, it can be suggested that bacterial genotoxicity tests would correlate well with the results of whole organism carcinogenicity assays for metabolically activated compounds, including the aromatic amines assessed during this study.

The reliability of any short-term genotoxicity bioassay depends upon the sensitivity (the proportion of carcinogens giving positive results) and specificity (the proportion of non-carcinogens giving negative results) of the test (Ennever & Rosenkranz 1988). The sensitivity of an assay is reduced when false negative results arise, which occurs when a carcinogen does not act via a mechanism that involves direct DNA damage. However, a study by Ennever and co-workers in 1987 showed that compounds seen to be non-genotoxic in a variety of short-term tests, such as aniline and 1,4-dioxane, are probable non-carcinogens or possibly weakly carcinogenic in humans, whilst most human carcinogens are genotoxic. This suggests that the possibility of a very hazardous human carcinogen being missed by a short-term genotoxicity assay is relatively low (Rosenkranz & Ennever 1988). The specificity of an assay is reduced when false positive results arise, which occurs when a non-carcinogen is positive in the assay. Data from the NTP database suggests this happens frequently, and may be due to

the use of higher concentrations of test chemical in *in vitro* assays than is possible to achieve *in vivo*, and that detoxifying reactions may occur *in vivo* at these lower concentrations (Dashwood & Combes 1987, Rosenkranz & Ennever 1988). Also, the accuracy of extrapolation from high doses of chemicals administered to rodents to the effects of low doses of that chemical in humans may be questionable.

If interspecies correlation (known as concordance) between carcinogenicity data in mice and rats is high, then this lends credence to interspecies extrapolations between rodents and humans. It has been observed that concordance between rats and mice in the NTP database is approximately 75%, which may be considered to be relatively low considering that these are closely related species tested under the same experimental conditions (Lin *et al.* 1995). It has been proposed that bacterial genotoxicity bioassays can be used in the prediction of potential hazard of a chemical to human DNA, as the primary structure of DNA is the same in bacteria as it is in humans (Tweats *et al.* 1984). However, the use of prokaryotes in genotoxicity assays can lead to inaccuracy in assessing potential mutagenicity or carcinogenicity in eukaryotic cells, due to more complex packaging of eukaryotic DNA into higher order structures such as protein-linked chromatin. These chromatin structures may preferentially protect or increase susceptibility of crucial parts of the genome to damage, which could lead to major differences in the way specific lesions are produced or repaired between bacteria and mammalian cells (Fox 1988, Hanawalt 1989). Therefore, it is apparent that any genotoxicity bioassay, especially bacterial assays, will lead to difficulties in prediction of effects on human health. However, based on the empirically good correlation between differential kill assays and whole organism carcinogenicity bioassays, differential kill assays are likely to give as good an indication of the carcinogenic potential of a compound to humans as many other non-human indicators.

It must be remembered that the monitoring of environmental samples such as wastewaters requires the processing of a large number of samples in a relatively short period of time (White & Rasmussen 1996). Therefore, in terms of cost, simplicity, rapidity and extrapolation of the result to human health, differential kill assays are amongst the most advantageous for use in wastewater monitoring.

1.4.7 Structure-activity evaluation of the toxicity and genotoxicity assays utilised with simple azo dyes

In order to assess the toxicological effects of azo dye decolourisation, the test system utilised must be appropriate for the compounds under consideration. In addition

to the criteria discussed in section 1.4.3 that toxicological monitoring assays must meet, comprising rapidity, reproducibility, sensitivity, standardisation of test protocols, low cost and ease of use, the biological endpoint utilised must also be appropriate for the compounds under consideration. Based upon evidence in the literature, it has been previously discussed that the use of bacterial bioluminescence for toxicity assessment and bacterial differential kill for genotoxicity assessment is eminently suitable for monitoring the toxicological potential of decolourised wastewaters (see sections 1.4.5 and 1.4.6). However, before the test methods were applied to a complex bioreactor system involving many unknown intermediates formed by the biodegradation of an simulated textile effluent (STE), their practical suitability in the laboratory was established. To achieve this, a simple structure-activity evaluation was conducted during this study.

Structure-activity relationships (SARs) are used to predict a biological response, such as toxicological activity, without the use of experimentation (Basketter 1989). As the potential hazard to human health and the environment of any new chemical should be considered, the use of SARs can spare a considerable amount of time, cost and resources (Nakadate 1998). Where an estimate of the extent of the biological response produced is required, quantitative structure-activity relationships (QSARs) are used. A mathematical equation is derived to produce a model, which relates the biological response of a system to one or more parameters representing molecular structure and/or physicochemical properties. The physicochemical properties considered when developing a QSAR can usually be divided into three groups, hydrophobicity, electronic and steric effects, of which hydrophobicity (usually as the log of the octanol-water partition coefficient) is the most common (Basketter 1989). It has long been possible to determine a QSAR for a defined system, such as the acute aquatic toxicity of unreactive chemicals to many different species, with considerable accuracy (Basketter 1989, Cronin & Dearden 1995), and many QSARs have been developed using *V. fischeri* with single compounds (e.g. Schultz & Cronin 1997, Cronin & Schultz 1998).

However, a comprehensive QSAR for a complex biological system is often difficult to establish. Often, it is not possible to establish that a particular toxicological effect is due to the original compound or its predicted metabolites. This is due to the fact that when chemicals undergo metabolism *in vivo* the structure of the resultant metabolites is not always known (Vereczkey 1995), and thus an effect cannot always be related to an exact cause. The mechanism of expression of the response may not be fully understood, a problem which is exacerbated if the effect is realised through different

receptors under the high dose conditions of toxicology studies to the receptors involved in normal metabolism (Nakadate 1998). Most importantly, consideration must be given to the quality, quantity and accuracy of the toxicological data available for QSARs to be modelled from (Cronin & Dearden 1995), meaning that the development of reliable toxicological QSARs will still be dependant on direct testing for a while (Vereczkey 1995). Therefore, the structure-activity evaluation performed in this study used direct testing of simple azo-based dyes, decolourised using pure cultures of known organisms, in a simple background matrix under controlled laboratory conditions. This evaluation was used to confirm expected toxicological activity based upon data reported in the literature, which is not a prediction of toxicity and thus is not a SAR.

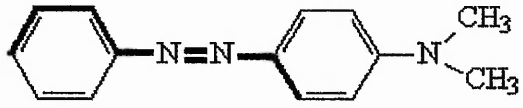
1.4.8 Effects of structural changes on toxicity and genotoxicity of azo dyes

The relationship between structure and toxicological activity for many organic molecules has been studied for many years. The type of relationship that has most often been investigated is the effect of sulphonation on mutagenicity. It has long been observed that sulphonation of a molecule can either decrease or eliminate both its mutagenic and carcinogenic potential (Lin & Solodar 1988, Rosenkranz & Klopman 1989). Originally, the mechanism by which the mutagenic potential is reduced was thought to involve absorption of the molecule in the intestinal tract. As the rate of intestinal absorption is primarily determined by the polarity of a molecule, increasing the polarity via the introduction of sulphonic substituents would decrease intestinal absorption and thus mutagenicity (Combes & Haveland-Smith 1982). The structural effect was also thought to be due to an increase in the electronegativity of the molecule after the addition of the sulphonate group, which would subsequently decrease its electrophilicity and thus its ability to attack DNA bases (Lin & Solodar 1988). Later, a SAR computer program called CASE (computer-assisted structure evaluation) was developed that used molecular structures to predict the mutagenicity and carcinogenicity of a compound (Basketter 1989). In 1989, Rosenkranz and Klopman used CASE to study the mutagenic activity of the carcinogenic azo dye Butter Yellow (*N*,*N*-dimethylaminoazobenzene). It was found that Butter Yellow contains a biophore (R-1) that predicts the observed mutagenicity of this dye, which is shown in Figure 1.8.

A biophore is a single, continuous structural fragment embedded in the complete molecule of a compound that activates its mutagenicity (Rosenkranz & Klopman 1989). Sulphonation of Butter Yellow interfered with this biophore, resulting in a loss of mutagenicity. This work suggested that sulphonation of a compound does not reduce its

genotoxic potential via a change in its absorption or electrochemical characteristics but by a change in the structural composition of the molecule, and also that sulphonation of azo dyes will only reduce or eliminate mutagenicity or carcinogenicity when it occurs at certain sites. A study by Razo-Flores and co-workers in 1997 showed that several azo dyes exhibited far greater inhibitory effects on the activity of acetoclastic methanogens than their respective aromatic amine breakdown products (Razo-Flores *et al.* 1997*a*). This indicates that the intact azo group increases the toxic effect on methanogens normally exhibited by the aromatic amines, which is similar to the findings of Rosenkranz and Klopman in 1989 with Butter Yellow and thus may be due to the presence of biophores in the dye molecules. However, there appears to be far more evidence in the literature that the toxicological potential of many azo dyes increases upon fission of the azo bond to produce the constituent aromatic amines, and thus it may be suggested that most azo dyes either do not contain highly active biophores, or that the constituent aromatic amines have greater toxicological potentials than any biophores present.

Figure 1.8: The structure of Butter Yellow showing the mutagenically activating biophore R-1



Butter Yellow

(Rosenkranz & Klopman 1989)

N.B. Biophore R-1 is indicated by a bold line. Due to the symmetry of the dye molecule, R-1 appears 4 times.

It has been found that most of the mutagenic and carcinogenic azo dyes contain the *p*-phenylenediamine and the now prohibited benzidine moieties (Chung & Cerniglia 1992, Chung 2000). However, it should be noted that less carcinogenic substitutes for many of these dyes, which belong mainly to the Direct and Acid dye groups, have been readily available for use in textile dyehouses for some time (Health & Safety Executive 1996 [Dyeing and Finishing Information Sheets 3&4]). Sulphonation of these *p*-phenylenediamine and benzidine dyes decreases or eliminates their genotoxic potential (Chung & Cerniglia 1992, Puntener *et al.* 1993, Chung 2000). Other structural changes to these types of azo dyes have also been considered with respect to their mutagenic potential. For example, substitution of the hydrogen of the amino group for an ethyl alcohol, carboxylation and deamination of *p*-phenylenediamine moieties decreases mutagenic

activity, whilst methylation of the amino group or substitution with a nitro group does not (Chung & Cerniglia 1992, Chung 2000). For benzidine moieties, alkyl substitution at the position *ortho* to the amine functional group, complexation with metal ions and the use of the hydrochloride salts decreases mutagenic activity, whilst methylation, halogenation or substitution of an ethyl alcohol within the amino group does not (Chung & Cerniglia 1992, Chung 2000).

Changes in genotoxic or mutagenic potentials due to structural alterations of azo dyes not containing *p*-phenylenediamine and benzidine moieties have been studied. A survey of the literature by Moller and Wallin in 2000 assessed nine azo textile dyes for genotoxic activity in short-term assays. Of these only 2 were non-genotoxic (C.I. Pigment red 53:1 and C.I. Pigment red 57:1), both of which would form sulphonated aromatic amines upon hydrolysis of the azo bond. Certain group substitutions, particularly electrophilic groups, have been associated with mutagenicity and carcinogenicity. In 1988, Shelby compared the inclusion of electrophilic substituents (e.g. $^{-}NH_2$, $^{-}NO_2$ and $^{-}CH_2Cl$) in 23 human azo-based carcinogens, including benzene, to their mutagenic potential (as determined by the Ames test or induction of chromosome aberrations or micronuclei in the bone marrow of mice or rats). It was found that, of 20 mutagenic compounds, 2 did not contain any electrophilic groups or behave as mutagens in the Ames test. This suggests that an *in vivo* mutagenicity assay is required to detect mutagens where their potential is not anticipated by structure nor detected by the Ames assay.

The relationship between toxicity and structure of aromatic compounds has been less well studied. In 1997, Razo-Flores and co-workers assessed the toxic effect of *N*-substituted aromatic compounds that constitute azo dyes on the activity of acetoclastic methanogens. It was observed that aromatic amines (-NH_2 substituent) were ~500-fold less toxic than their corresponding nitroaromatic analogues (-NO_2 substituent) (Razo-Flores *et al.* 1997*a*). A survey of the literature in 1989 by Benigni and co-workers showed that there was a good correlation between mutagenicity, as determined by several short-term assays including the Ames test (data from the International Program for the Evaluation of Short-Term Tests for Carcinogens (IPESTTC)), and toxicity, as determined by acute oral mouse data (LD_{50}) for 42 compounds including aromatics. This suggests that chemical interaction with cellular DNA is a common, important determinant for both cell death on one hand and genetic damage on the other. Therefore, whilst it may be anticipated that sulphonation of dyes and aromatic amines will reduce genotoxcity, it may also be suggested that sulphonation will decrease toxicity due to the good

correlation between toxicity and mutagenicity. However, the toxicological response to other structural changes of aromatic compounds, such as group substitution, cannot be easily predicted by structural evaluation, and thus it is extremely important that toxicological assessment of any compound of concern be continued.

1.4.9 Effects of structural changes on biodegradation of azo dyes

As has been discussed previously (see section 1.3.4), microorganisms can be utilised to degrade azo dyes. Complete biodegradation, via decolourisation under anaerobic conditions and mineralisation under aerobic conditions, is the ultimate goal. However, there are many factors that influence the success of these processes, which largely focus on the suitability of the microorganism(s) involved and environmental parameters. One other major factor that influences the biodegradation of azo dyes is their molecular structure, particularly the type and position of substitution groups on the aromatic rings of the dyes (Hao *et al.* 2000). Carboxylated dyes are decolourised more readily than sulphonated dyes. For example, a study by Pasti-Grigsby and co-workers in 1996 decolourising monoazo dyes with *Pseudomonas* sp. showed 16-35% more decolourisation for carboxylated dyes after 7 days than for their sulphonated analogues. This shows that replacement of the sulphonic ($-SO_3H$) group of an aromatic ring with a carboxylic (-COOH) group enhances degradability of the structure. The position of the hydroxy (-OH) group is also important. It has been shown that dyes with the hydroxy in the *para* (*p*)-position, where the groups are opposite each other (positions 1,4), are decolourised more readily than when in the *ortho* (*o*)-position, where the groups are next to each other (positions 1,2) (Hao *et al.* 2000). For example, the study by Pasti-Grigsby and co-workers in 1996 mentioned above showed that dyes with hydroxy groups in the *p*-position were decolourised by 79-95% within 7 days, whilst those with groups in the *o*-position were decolourised by 0-35%.

Mineralisation of aromatic rings is also dependent on group substitution. As with decolourisation, carboxylated aromatic compounds are degraded readily, whereas mineralisation of analogues with a sulphonate group is more difficult (Sangaleti *et al.* 1995). Most of the reports in the literature on mineralisation of sulphonated aromatic amines usually describe mixed cultures with unusual catabolic activities (Rajaguru *et al.* 2000). For example, Thurnheer and co-workers (1988) observed complete degradation of 7 sulphonated aromatic compounds in a chemostat, using a co-culture consisting of *Alcaligenes* sp. (strain O-1), two *Pseudomonas* spp. (T-2 and PSB-4) and two unidentified rod-shaped bacteria (M-1 and S-1). This reduction in the ease of

mineralisation of sulphonated azo dyes causes an obvious paradox for the design of azo dyes, in that sulphonation will decrease the toxicological response (see section 1.4.8) to the dye but increase its resistance to biodegradation. It may be suggested that biodegradation should not be considered for use in azo dye mineralisation, if the dye is highly sulphonated to reduce toxicity. However, biodegradation is the cheapest, cleanest and above all least toxic dye decolourisation method, and so a compromise must be made between the safety of the dye, the ease of its decolourisation and the safety of the resultant compounds.

1.5 Bioreactors

1.5.1 Bioremediation of textile wastewater

Wastewaters from industrial processes commonly contain a wide variety of organic and inorganic compounds. In the natural environment, degradation of organic matter occurs under anaerobic conditions whilst degradation of inorganic matter usually occurs under aerobic conditions. These processes can be exploited as simple and effective biotechnological processes for reducing the pollution caused by industrial wastewaters, especially in terms of organic waste (Godon *et al.* 1997*a*, Delbès *et al.* 2000, Zumstein *et al.* 2000), by the use of bioreactors. There are many types of bioreactor used for treating industrial wastewaters, such as aerated tanks (e.g. activated sludge processes), rotating biofilm reactors in which a biofilm is formed on a series of rotating discs (e.g. rotating biological contactor (RBC)), upflow sludge blanket systems in which wastewater flows through a blanket of flocculated sludge (e.g. upflow anaerobic sludge blanket (UASB)), expanded bed reactors in which biofilm-covered particles are mixed by gaseous treatments (e.g. aerobic-anaerobic fluidised-bed), and single tank reactors in which operational parameters can be altered within a single reactor (e.g. aerobic-anaerobic sequential batch and continuous-flow reactors) (Banat *et al.* 1996, Willmott *et al.* 1998). Bioreactors containing biofilms have been used successfully to degrade complex mixtures of recalcitrant substances, such as phenol, benzene and naphthol compounds. This is because continuous cultivation of the biofilm allows selection of microbes capable of degrading the compounds possible, whilst adhesive growth provides high degradation capacity and process stability (Guieysse & Mattiasson 1999).

Treatment of textile wastewaters, which contain high concentrations of organic compounds and recalcitrant dyes, is commonly carried out using aerobic and anaerobic bioreactors. Anaerobic treatment of textile wastewaters has many advantages over

aerobic treatment systems. Firstly, and most importantly, it is well known that dyes can be decolourised under the reducing conditions present in anaerobic bioreactors, especially those with azo based chromophores (Razo-Flores *et al.* 1997*b*, Setiadi & van Loosdrecht 1997, Rajaguru *et al.* 2000). Also, anaerobic systems are cheaper to operate than aerobic systems as expensive aeration is omitted, effluents with high organic loads which are produced by desizing and scouring can be treated, and problems caused by bulking of sludge such as costly disposal are avoided (Delée *et al.* 1998). Bulking of sludge occurs under aerobic conditions as a large portion of the energy derived from the oxidation of organic wastes is converted to new cells, resulting in unchecked microbial growth (Sacks 1998). Attempts to reduce bulking by uncoupling of aerobic metabolism have been made, but have generally resulted in decreases in treatment efficiency. Low and co-workers (2000) used para-nitrophenol, an uncoupler of oxidative phosphorylation, in a bench-scale activated sludge process, and observed that whilst biomass production was reduced by 49%, total substrate removal rate was also reduced by 25%. However, it is equally well known that the amines produced by azo dye decolourisation are resistant to further degradation under anaerobic conditions, and thus require treatment under aerobic conditions for complete mineralisation (Zaoyan *et al.* 1992, Banat *et al.* 1996, Delée *et al.* 1998, Chung 2000). Therefore, complete treatment of textile effluents can only be achieved by a sequenced anaerobic-aerobic treatment to remove organic load, colour and recalcitrant inorganics such as aromatic amines.

1.5.2 Decolourisation of azo dyes under anaerobic and anaerobic-aerobic conditions in bioreactors

Many studies have utilised anaerobic bioreactors to decolourise azo dyes, usually with high levels of colour removal. An extensive study on decolourisation of reactive dyes, including many with azo chromophores, by anaerobic digestion was reported by Carliell and co-workers in 1994. Of 18 reactive dyes, 14 were decolourised between 70 and 98%. A detailed study on C.I. Reactive Red 141 showed that the 85-90% decolourisation observed occurred via an azo reduction mechanism, which was supported by identification of the dye degradation product 2-aminonaphthalene-1,5-disulphonic acid (Carliell *et al.* 1995). In 1997, Setiadi and van Loosdrecht used an anaerobic digester to decolourise two reactive azo dyes at 100 mgL^{-1} in both parent and hydrolysed forms. For parent dyes, decolourisation of 87 and 66% was achieved in 4 and 10 h for C.I. Reactive Red 198 and C.I. Reactive Red 141 respectively. Similar results were observed with the hydrolysed dyes.

A study by Goncalves and co-workers in 2000 used an UASB reactor to decolourise several azo dyes in the acid, direct and disperse classes. The dyes were readily decolourised even at high concentrations of 700 mgL^{-1}, with an average removal efficiencies of 80-90 and 81% for acid and direct dyes respectively. However, experiments were unsuccessful with disperse dyes even at low concentrations of 35 mgL^{-1}, which was attributed to the toxicity of the dyes to the biomass. In 2000 Bell and co-workers used an anaerobic baffled reactor to decolourise an industrial wastewater from a food dye manufacturer at 10% (v/v), which contained many azo dyes including tartrazine, and observed approximately 90% colour removal. In 1997, Razo-Flores and co-workers used a continuous flow UASB reactor, seeded with anaerobic granular sludge, under anaerobic conditions to decolourise the azo dyes C.I. Mordant Orange 1 and azodisalicylate at 100 and 75 mgL^{-1} respectively. C.I. Mordant Orange 1 and azodisalicylate were reduced by >99 and 99% respectively, with a 30 and 1% recovery of aromatic amines respectively. When glucose (a co-substrate used to provide the reducing equivalents needed for azo reduction when the azo compound is the main substrate) was omitted, azodisalicylate reduction was 89% with a <0.1% recovery of aromatic amines (Razo-Flores *et al.* 1997*b*). This suggests that the azo cleavage products of azodisalicylate (two 5-aminosalicylic acid units) can be fully mineralised under anaerobic conditions, which was confirmed by batch biodegradability assays (Donlon *et al.* 1997).

Further study of the anaerobic mineralisation of aromatic amines was carried out by Razo-Flores and co-workers in 1999. Using a UASB reactor, it was observed that the nitroaromatic compounds 5-nitrosalicylate and 4-nitrobenzoate were transformed to their corresponding aromatic amines, and then subsequently mineralised to methane and carbon dioxide. However, there are very few examples of studies reporting anaerobic mineralisation of aromatic amines in the literature, and even those that do also report some compounds that are not degraded (e.g. 2,4-dinitrotoluene was partially transformed to a non-degradable metabolite in a UASB; Razo-Flores *et al.* 1999). Therefore it seems that, although anaerobic mineralisation of aromatic amines is sometimes achievable, it is highly dependent upon factors such as structure and group substitution, and few compounds are fully degradable.

The use of sequential anaerobic-aerobic treatment to decolourise azo dyes have also been assessed. In 1992, Zaoyan and co-workers used an anaerobic RBC followed by an aerobic RBC, in combination with an activated sludge process, to treat the wastewater of a textile dyeing factory which contained dyes (including reactive azo),

auxiliaries, polyvinyl alcohol, detergents, salts and other organic and inorganic compounds. After anaerobic-aerobic treatment colour removal was 71.6 ± 12.7%, but this was reduced after aerobic treatment under the same conditions (often <60%). A similar study by Jiang and Bishop in 1994 used an aerated biofilm reactor to degrade the azo dyes C.I. Acid Orange 10 and C.I. Acid Red 14 at 5 mgL^{-1}. Decolourisation of up to 60% was observed, but only when the dissolved oxygen (DO) level was below 1 mgO_2L^{-1} and thus part of the biofilm under anaerobic conditions. A study by Fitzgerald and Bishop in 1995 used a two stage reactor system, consisting of anaerobic fixed-film fluidised bed reactor followed by an aerobic suspended growth activated sludge reactor, to decolourise C.I. Acid Orange 10, C.I. Acid Red 14 and C.I. Acid Red 18. Decolourisation levels of >65% were achieved for C.I. Acid Orange 10, and of >90% for the two red dyes, after anaerobic treatment. It was observed that there was very little additional decolourisation achieved after aerobic treatment. In 2000 O'Neill and co-workers reported on the use of a 30 L UASB to decolourise a synthetic textile effluent (STE) containing hydrolysed C.I. Reactive Red 141 at 450 mgL^{-1}, the effluent of which was then treated in a 20 L aerobic vessel. Colour was measured in true colour units (TCU), which is the OD at 436, 525 and 620 nm in a centrifuged or highly filtered sample, and was measured on- (within the effluent flow) and off- (out of the flow) line. Colour was found to be 2.88 ± 0.6, 1.04 ± 0.02 and 0.72 ± 0.12 TCU in feed, anaerobic effluent and aerobic effluent respectively, which shows a 63.9 ± 7.0 and 11.1 ± 7.5% reduction of colour in anaerobic and aerobic stages respectively. It was noted that colour measurements were shifted down by 1 TCU from on- to off-line measurements, and so colour measurements should be made on-line where possible, and that an increase in starch concentration resulted in a decrease in effluent colour (Esteves *et al.* 2000). These studies demonstrate the importance of an anaerobic treatment stage in decolourisation of textile dyes, as reduced efficiency or little improvement is observed after aerobic treatment.

Some studies on anaerobic-aerobic treatment of textile dyes have also commented on the effects of an aerobic system on the aromatic amine breakdown products of azo dyes. In 1992 Harmer and Bishop used rotating drum biofilm reactors seeded with activated sludge to decolourise a synthetic municipal wastewater containing C.I. Acid Orange 7 (O2) at various DO levels. Removal ranged from 18 to 97%, with maximum values at low DO levels. The aromatic amine intermediates 1-amino 2-naphthol and sulphanilic acid were not detected at high DO levels, and only sulphanilic

acid was present at low DO levels, indicating that the dye was completely mineralised under aerobic conditions.

A study by Rajaguru and co-workers in 2000 utilised a two-stage anaerobic-aerobic bioreactor system, consisting of an upflow fixed bed column for the anaerobic stage and an agitated tank for the aerobic stage, to decolourise 4 sulphonated azo dyes at up to 100 or 200 mgL^{-1}. Complete reduction of C.I. Acid Orange 10 was observed under anaerobic conditions up to 50 mgL^{-1} with concomitant release of aromatic amines, but a decrease in reduction was observed at 100 mgL^{-1} and 41% of the dye was recovered as amine. After aerobic treatment, aromatic amines were not detected at dye concentrations up to 50 mgL^{-1}, but a small amount (0.016 mM) was detected at dye concentrations of 100 mgL^{-1}. Similar results were observed with C.I. Naphthol Blue Black. The same trend was revealed with C.I. Direct Red 2 and C.I. Direct Red C, where a decrease in reduction under anaerobic conditions was observed at 200 mgL^{-1} and 37 and 46% of the dye was recovered as amine (C.I. Direct Red 2 and C.I. Direct Red C respectively). Aromatic amines were detected at dye concentrations of 200 mgL^{-1} under aerobic conditions (0.024 and 0.044 mM for C.I. Direct Red 2 and C.I. Direct Red C respectively). It was also observed by Kalyuzhnyi and Sklyar in 2000 that azo dye breakdown products may not be mineralised under aerobic conditions. In this study, the anaerobic and aerobic phases of treatment of a synthetic wastewater containing the azo dye C.I. Direct Yellow 44 were combined into a single unit called the anaerobic-aerobic hybrid reactor. Removal of 56% of the dye was possible at high loads of 0.3 g dye $CODL^{-1}day^{-1}$, however the effluent still had a high COD content. This was suggested to be due to the presence of non-biodegradable autooxidation products of the dye intermediates, which are most probably aromatic amines. These studies demonstrate that although complete mineralisation of azo dyes can be achieved under sequential anaerobic-aerobic conditions, the extent depends on the concentration and type of dye present in addition to parameters such as reactor design, atmospheric conditions, etc.

1.5.3 Decolourisation of C.I. Reactive Black 5 under anaerobic and anaerobic-aerobic conditions in bioreactors

Most of the studies on decolourisation of RB, occasionally in its commercial form as Remazol Black B, have concentrated on anaerobic treatment. High values for colour reduction are usually obtained. In 1994 Carliell and co-workers reported an extensive study of reactive dye decolourisation using anaerobic digestion. At a concentration of 100 mgL^{-1}, RB was decolourised at 80-85% within 4.5 h. A study by

Oxspring and co-workers in 1996 used an upflow anaerobic filter, seeded with a microbial consortium consisting mainly of *Alcaligenes faecalis* and *Commamonas acidovorans*, to decolourise RB at 500 mgL^{-1}. Decolourisation of >95% was observed after 48 h, although most of the reduction had occurred during the initial stages of the experiment. Anaerobic digesters have also been used more recently to decolourise RB. In 1998, Beydilli and co-workers decolourised RB at 300 mgL^{-1}, and observed 79% decolourisation within 10 h. A study by Setiadi and van Loosdrecht in 1997 used an anaerobic digester to decolourise RB at 100 mgL^{-1} in both parent and hydrolysed forms (for an explanation see section 1.2.2), observing 67% decolourisation within 10 h for the parent dye and similar results for the hydrolysed dye. It is interesting to note that, upon analysis of reduction rate of the hydrolysed dye, two slopes were observed. It was hypothesised that either the dye was a mixture of two components that have different decolourisation rates, or that two processes are involved (e.g. adsorption then biodegradation). The hydrolysed and active (vinyl sulphone) forms of RB (see section 1.2.2) were decolourised under aerobic conditions using activated sludge by Ganesh and co-workers in 1994. A rapid decrease in colour was seen when treating the active form, although no evidence of biodegradation in terms of breakdown products was observed. It was therefore concluded that the active form of the dye was being adsorbed to the biomass, thus reducing the colour of the effluent. However, a similar decrease in colour was not observed when treating the hydrolysed form, and so it was hypothesised that this form is neither biodegraded under aerobic conditions nor adsorbed to the biomass. This suggests that the two-stage reduction of hydrolysed RB (RBOH) observed by Setiadi and van Loosdrecht in 1997 was due to the dye being composed of a mixture of two compounds, possibly two different dyes.

Anaerobic-aerobic sequential treatment of RB has been less reported in the literature. In 2000 Luangdilok and Panswad used an anaerobic-aerobic sequencing batch reactor (SBR) to treat a synthetic wastewater containing 100 mgL^{-1} RB, using glucose and acetic acid as carbon sources. A decolourisation level of 58% was obtained, and it was observed that two different rates of colour removal were evident, with the highest rate occurring within the first two hours of the anaerobic stage. Studies by Panswad and co-workers in 2001 have also utilised SBRs seeded with activated sludge, operated under anaerobic-anoxic-aerobic conditions, to decolourise a synthetic wastewater containing low concentrations of RB. Using 10 mgL^{-1} dye with 18 and 5 h anaerobic and aerobic stages respectively, most of the colour removal was noted during the anaerobic stage with a slight attenuation during the aerobic stage (53-63 and 9-13%

respectively; Panswad *et al.* 2001*a*), and similar results were observed using higher concentrations of dye and shorter HRTs (80 mgL^{-1} dye with 8 and 3 h anaerobic and aerobic stages respectively; Panswad *et al.* 2001*b*). In addition to RB, other reactive azo dyes have been studied. In 2000 Lourenco and co-workers used an anaerobic-aerobic sequencing batch reactor (SBR) to treat a cotton STE containing 90 mgL^{-1} of the reactive azo dye Remazol Brilliant Violet 5R. After 40-50 days a stable decolourisation level of 90% was achieved, with almost all of the colour being removed during the anaerobic phase. It was noted that under denitrifying conditions, when the STE contained 45-60 $mgNO_3L^{-1}$, decolourisation was reduced to 70%. A delay in the decolourisation of a reactive azo dye by the presence of nitrate, and thus a reduction in colour removal in a bioreactor system, has also been observed by Carliell and co-workers (Carliell *et al.* 1998).

Studies on anaerobic-aerobic sequential treatment of non-reactive azo dyes have been reported. In 1996, An and co-workers used an UASB reactor followed by an aerobic activated sludge tank to decolourise one acid and two basic azo dyes. Decolourisation of 20 and 72-78% was observed for the acid and basic dyes respectively under anaerobic conditions, with no significant improvement after aerobic treatment. A study by Tan and co-workers in 1999 used an oxygenated methanogenic granular sludge to decolourise the azo dye C.I. Mordant Orange 1. It was found that reduction of the dye occurred in the presence of oxygen only if co-substrates were added, suggesting that the addition of co-substrates stimulated respiration of oxygen by the sludge thereby creating anaerobic microniches in which decolourisation can occur (Tan *et al.* 1999).

These studies demonstrate that decolourisation of RB in a sequential anaerobic-aerobic system is achievable during the anaerobic treatment phase, with common colour removal levels of around 60%, but that subsequent aerobic treatment does little to improve this decolourisation. The same trend can be seen for azo dyes of other classes, although the level of decolourisation that can be achieved appears to be highly variable dependant on dye structure and application class.

1.5.4 Toxicity of azo dyes and decolourised effluents from bioreactors

It has been discussed that dyes generally exhibit low toxicity in bacterial and eukaryotic tests (see section 1.4.1). The reactive class of dyes has been demonstrated to show the lowest toxicity of all the classes (Hao *et al.* 2000). Several studies on the toxicity of reactive azo dyes have demonstrated low levels of toxicity in bacterial assays.

C.I. Reactive Red 141 was shown to be non-inhibitory to total gas production of an anaerobic sludge at concentrations of up to 100 mgL^{-1}, and only slightly inhibitory at concentrations of up to 500 mgL^{-1} (Carliell *et al.* 1995). Hydrolysed C.I. Reactive Black 5 at 300 mgL^{-1} did not show any significant toxic effects to a methanogenic culture, with 10.3, 26.5 and 29.5% inhibition of methane production compared to the control for 83, 64 and 147 h respectively (Beydilli *et al.* 1998). C.I. Reactive Red 235 was non-toxic to the biomass of an up-flow anaerobic sludge blanket (UASB) reactor at concentrations of up to 100 mgL^{-1} (Willetts & Ashbolt 2000). However, some studies have demonstrated toxic effects of reactive azo dyes. Two blue reactive azo dyes were shown to be toxic in the Hepa-1 cytotoxicity test at IC_{50} values of 40-65 mgL^{-1} (Kopponen *et al.* 1997). C.I. Direct Brown 106 was shown to be toxic to a methanogenic sludge with an IC_{50} value of 410 mgL^{-1} (Kalyuzhnyi & Sklyar 2000), although this value is still relatively high. A red reactive azo dye was shown to be highly inhibitory to the growth of a species of *Anabaena*, with IC_{50} values as low as 5 mgL^{-1} (Hu & Wu 2001). Therefore inhibition to biomass in bioreactors decolourising dyes, and thus reduction in decolourising efficiency, may occasionally be caused by the dye itself. However, current thinking suggests that most bioreactor failures in terms of dye decolourisation will be caused by the production of aromatic amines under anaerobic conditions, which are toxic.

There have been occasional reports of anaerobic decolourisation of an azo dye to less toxic compounds, such as that of Donlon and co-workers in 1997 who used a UASB reactor to decolourise C.I. Mordant Orange 1, and observed that the aromatic amines 1,4-phenylenediamine and 5-aminosalicylic acid that were produced were over 25 times less toxic than the dye itself. Conversely, most studies on anaerobic decolourisation of azo dyes show that the aromatic amines produced are more toxic than the unreduced dye. In 1997, Sosath and Libra used an anaerobic RBC to decolourise C.I. Reactive Violet 5, and found that the aromatic amines produced by decolourisation were more toxic than the dye in a bacterial luminescence test. In 2000 O'Neill and co-workers used an anaerobic UASB reactor and a subsequent aerobic vessel to decolourise an STE containing 450 mgL^{-1} hydrolysed C.I. Reactive Red 14. The STE and the effluent produced after anaerobic-aerobic treatment showed no inhibition in a 3 h respiration-inhibition test, whilst the effluent produced after anaerobic treatment showed 17.9% inhibition. HPLC analysis showed that UV-absorbing products were formed during the anaerobic treatment of the STE, and that partial degradation to less aromatic, more polar compounds occurred in the aerobic stage.

It should be noted that other compounds associated with textile dyehouse effluents can cause inhibition in bioreactors. The high sulphate levels produced by the use of sodium sulphate in the reactive dyeing process can lead to toxicity, as sulphate-reducing bacteria will out-compete methanogenic bacteria and toxic hydrogen sulphide (H_2S) rather than methane will be produced (Delée *et al.* 1998). It has been estimated that H_2S levels of >200 mgL^{-1} may inhibit methanogenic bacteria, and a study by Carliell and co-workers in 1996 showed that an anaerobic sewage sludge digester became unstable at H_2S concentrations of 400 mgL^{-1}. High concentrations of salt, mainly sodium, which would be used in the dyeing process are known to be inhibitory to microorganisms. However, studies have reported that bioreactors can be used to treat effluents at sodium chloride concentrations of up to 30 gL^{-1} without inhibition (Delée *et al.* 1998). Textile dyehouse effluents also contain high concentrations of sizes, such as starch, polyvinyl alcohol (PVOH) and carboxymethyl cellulose (CMC). Starch and starch derivatives, like many natural polysaccharides such as dextrin, guar gum and cellulose, are non-toxic compounds (Hoffmann & Beck 1999, Liu *et al.* 2000). A study by Tsuk and co-workers in 1997 showed that PVOH containing gels were not toxic to corneal fibroblasts. CMC and the effluent from an aerobic reactor seeded with activated sludge degrading CMC were shown to be non-toxic to several aquatic organisms. For intact and degraded CMC, no-effect concentrations were observed at >500 mgL^{-1} for the green algae *Raphidocelis subcapitata*, >1000 mgL^{-1} for the water flea *Daphnia magna*, and >1000 mgL^{-1} for the zebra fish *Brachydanio rerio* (van Ginkel & Gayton 1996). In view of the data from these studies, it is expected that any toxicity observed from the bioreactors treating an STE without the inclusion of RB will be due to the presence of metabolic waste products from the sulphate-reducing bacteria, most likely to be H_2S.

1.6 Bioreactor microflora

1.6.1 Microbial interactions within bioreactors

Microbiological processes, anaerobic, aerobic or combined anaerobic-aerobic, which occur naturally in a variety of habitats will degrade inorganic and organic compounds (Godon *et al.* 1997*b*, Stams & Elferink 1997). These processes, especially anaerobic digestion, are commonly exploited on a large scale as simple and effective biotechnological processes for reducing pollution caused by organic wastes (Godon *et al.* 1997*a*, Zumstein *et al.* 2000). The activated sludge process for the purification of wastewater is, in terms of total metabolised matter, today's most important biotechnological process (Amann *et al.* 1995). Wastewater treatment is generally

performed in bioreactors with mixed microbial populations (Stams & Elferink 1997), which are generally robust and can function over a wide range of temperatures (20-42°C) and pHs (5.5-9). Each bioreactor is coupled to a manufacturing process that dictates the organic composition of the waste stream, which contributes to natural selection and thus a unique ecosystem evolves over time to degrade the organic compounds. As the manufacturing processes are modified, the organic composition of the waste stream can change causing microorganisms in the bioreactor to adapt (Bramucci & Nagarajan 2000).

Planktonic life represents one possible survival strategy of microorganisms. However, there is now plenty of evidence that in many different habitats, including wastewater treatment systems such as bioreactors, the microbial biomass is rarely present as suspended cells but rather can be found colonising solid surfaces or other interfaces in organised communities (Stickler 1999). These communities are known as biofilms. Cells in these biofilms are embedded in a polymeric matrix and in this mode are phenotypically different from when they are growing in suspension (Stickler 1999). It is known that biofilms often catalyse important microbial transformations, thus possible advantages of immobilised consortia may include higher availability of nutrients on surfaces and the possibility of long-term positioning in relation to other microorganisms or physicochemical gradients (Amann *et al.* 1995). In wastewater treatment bioreactors, microorganisms are selected on their ability to form loose or dense aggregates by self-aggregation or their ability to adhere to solid material, which immobilises the biomass. Due to this, the liquid retention time can be uncoupled from the biomass retention time which enables high rate conversions of inorganic and organic compounds in relatively small reactors (Stams & Elferink 1997). Settling easily separates any contaminating biomass in the purified wastewater, such as activated sludge flocs that can be regarded as mobilised biofilms (Amann *et al.* 1995).

Insight into the factors that affect ecosystem stability is needed to determine the effects of external parameters on habitats (Fernández *et al.* 1999). Knowledge of the aspects relating to the formation and stability of microbial biofilms, such as the physicochemical characteristics of microorganisms, are required for proper process design and control in biotechnological applications such as bioreactors (Stams & Elferink 1997). To illustrate this, the failure of many anaerobic digestors to operate reliably with consistent performance has underlined this need for more basic information on the biological aspects of the anaerobic digestion ecosystem (Godon *et al.* 1997*b*). Knowledge of the microbial communities involved is a prerequisite to

understanding the activities of microbial ecosystems (Zumstein *et al.* 2000), and ultimately to controlling processes such as those carried out by activated sludge by engineering of the microbial populations (Curtis & Craine 1998). However, correlations between community structure and function in natural or domesticated situations such as the activated sludge process are sill unclear (Amann *et al.* 1995), and the question of whether functional stability implies a persistent community has yet to be fully answered (Fernández *et al.* 1999). This is due largely to the fact that information on community structure in many diverse habitats, including bioreactors which have the advantage of being closed and comparatively homogenous, stable ecosystems, has been limited as they consist of both cultivable and noncultivable organisms (Godon *et al.* 1997*a*). The impact of noncultivable organisms on our understanding of the activities of microbial ecosystems will be discussed below.

1.6.2 Traditional methods of microbial sampling and identification

It has been noted that the bioremediation of organic pollutants is among the most important applications of microbial processes today. There is increasing interest in the investigation of the microbial community structures and population dynamics in these engineered communities, as bioremediation solutions are based upon the coupling of mechanical engineering with biological diversity and functionality (Whiteley & Bailey 2000). Therefore, assessments of diversity, abundance and activity of the microorganisms involved would facilitate understanding of the biotreatment system and improve performance (Yu & Mohn 1999). However, most past measurements of diversity and functionality have been compromised by limitations inherent to the traditional methods of microbiology (Ward *et al.* 1998), principally the requirement for pure-culture isolation and the use of subjective criteria for classification (Amann *et al.* 1990*b*).

The definitive identification of bacterial isolates is among the most difficult practices in microbiology. Direct microscopic analysis has always played an important role in quantification of microorganisms in natural environments (Stams & Elferink 1997). However, identification of individual population members by microscopy is extremely difficult due to the fact that identification is usually based on cell morphology. Most microorganisms have relatively simple and indistinct morphologies (Stams & Elferink 1997, Ward *et al.* 1998), and so only in rare cases does microscopy allow the *in situ* identification of microorganisms (Manz *et al.* 1992). Also, common morphologies can mask considerable genetic diversity among bacteria (Ward *et al.*

1998). Therefore, identification of microorganisms has to proceed via cultivation. The most commonly used method of identifying and enumerating microorganisms from natural environments is the use of liquid or solid media that are selective for certain physiological groups, usually obtaining the microorganisms that are most dominant (Stams & Elferink 1997). Many physiological and biochemical tests exist which allow for identification of microbes, all of which require the prior isolation of pure cultures of these microorganisms (Manz *et al.* 1992).

It has, however, long been noted that comparison of total cell counts obtained by direct microscopy exceed viable cell counts obtained using cultivation techniques by several orders of magnitude (Amann *et al.* 1995). This discrepancy was termed the "great plate count anomaly" by Staley & Konopka in 1985. It has been estimated that ~99% of all microorganisms in nature cannot be isolated in pure culture (Muyzer 1999). The estimated culturability, defined as the percentage of cultivable bacteria in comparison with total cell counts of the highly metabolically active activated sludge, is only 1-15% (Amann *et al.* 1995). This has led to the opinion that many bacteria in natural environments are "uncultivable" (Ward *et al.* 1998). The reason for low culturability is more likely due to ignorance of the real conditions under which most bacteria are growing in their natural environments, and thus required culture conditions in the laboratory (Muyzer & Smalla 1998), and also that many microbes may have specific community associations and interdependencies, such as symbiotic relationships, which prevent the isolation of pure cultures (Whiteley & Bailey 2000). Therefore, classification of microorganisms within natural environments based on physiological and biochemical features is nearly impossible, due to this inability to understand and reproduce the real microenvironmental niches defined by physicochemical and biotic features.

There are other limitations of cultivation techniques for objective analysis of populations within natural communities. Plating as opposed to liquid enrichment may bias against recovery of some populations (Ward *et al.* 1998). Numbers are sometimes underestimated as microbial aggregates that cannot be dispersed completely are counted as originating from a single bacterium. Very long incubation times are needed for correct numbers of slow-growing organisms, and knowledge is only obtained about those microorganisms that are able to grow on artificial media (Stams & Elferink 1997). Those media may be too specific, so that only certain strains of a group may grow, or not specific enough, so that unwanted bacteria affect the result (Harmsen *et al.* 1999). An example of the biased view that can result from cultivation was reported by Wagner

and co-workers in 1993 for an activated sludge system treating municipal wastewater, where three different nutrient-rich media yielded three different population diversities for an identical sample (Amann & Ludwig 2000).

Due to the limitations of traditional culture techniques, it is now well recognised among microbiologists that only a fraction of all bacteria, encompassing a small part of the extant microbial diversity, have been isolated and characterised (Muyzer & Smalla 1998). The lack of knowledge of microbial identification and diversity impacts most heavily on complex, multispecies microbial communities (Muyzer 1999). Populations in these communities are frequently arranged in a very specific way, such as in biofilms, and their activities cannot be achieved by individual microorganisms (Amann & Kühl 1998). Thus it is clear that progress in understanding the role of microbial diversity in the maintenance and functionality of ecosystems will require studies on the activity and distribution of microbes directly in minimally disturbed samples, at high spatial and temporal resolution (Amann & Kühl 1998, Amann & Ludwig 2000). This requires reliable, quantitative identification by objective methods, such as molecular biological techniques, which complement the traditional microbiological procedures (Muyzer & Smalla 1998). An overview of the development of molecular methods for the study of microbial diversity will be discussed below.

1.6.3 Molecular methods of microbial sampling and identification

1.6.3.1 Phylogenetic analysis using rRNA

As plating and most-probable-number techniques are selective, the microbial ecologist could isolate and characterise individual bacterial strains but was not able to tell if these microorganisms were common in the environment (Pernthaler *et al.* 1998). Consequently little knowledge has been gathered about the true community structure and temporal and spatial abundance fluctuations in natural habitats (Snaidr *et al.* 1997, Pernthaler *et al.* 1998). The use of molecular biological techniques has eliminated the dependence on isolation of possibly uncultivable bacteria (Heuer *et al.* 1997, Fernández *et al.* 1999), and thus avoids the bias in assessments of natural environments that are caused by culturing (MacGregor 1999). A major impact of the application of molecular techniques has been to confirm that most of the microflora from natural environments have yet to be cultured (O'Donnell & Görres 1999). Molecular methods can be used to facilitate analysis of diversity, abundance and activity of microbial communities from environmental samples (Godon *et al.* 1997*a*, Rondon *et al.* 1999), and thus the

community structure, population dynamics and succession of natural microbial ecosystems can now be revealed (Pernthaler *et al.* 1998, Whiteley & Bailey 2000).

The most powerful and encompassing tool to analyse microbial diversity in environmental communities is the use of ribosomal ribonucleic acid (rRNA) (Amann *et al.* 1990*a*, Stahl & Amann 1991, Snaidr *et al.* 1997, Felske & Akkermans 1998). The most commonly used molecular marker for nearly three decades has been the small subunit, or 16S rRNA (Muyzer & Smalla 1998, Amann & Ludwig 2000), since its use as a phylogenetic tool was pioneered by Carl Woese in the early 1970s (Madigan *et al.* 2000). 16S rRNA was targeted for a number of reasons. Firstly, it is universally distributed and moderately well conserved in sequence across broad phylogenetic distances (Stahl & Amann 1991, Madigan *et al.* 2000). Secondly, as its ~1500 nucleotide length (Giovannoni *et al.* 1988, Amann *et al.* 1995) contains several regions of quite variable sequence that alter very quickly (Stams & Elferink 1997, Amann & Ludwig 2000), it can be used to differentiate subspecies of bacteria during assessment of phylogenetic relationships (Stahl & Amann 1991). Thirdly, because of the existence of large and expanding databases of complete rRNA sequences (the ARB database contains 22000 aligned 16S rRNAs) (Amann *et al.* 1990*a*, Amann & Ludwig 2000, Ishii *et al.* 2000), identification of unknown microorganisms can easily be made by comparisons to those databases (MacGregor 1999). Fourthly, a single cell will contain between 10^3 and 10^5 ribosomes and consequently as many copies of 16S rRNA (Amann *et al.* 1990*b*, Amann *et al.* 1995), which lends great sensitivity to direct determinative tests (Schleifer *et al.* 1992). Finally, 16S rRNA is more experimentally manageable than 23S rRNA (Madigan *et al.* 2000). However, it must be noted that cells of different species can have up to 100-fold difference in numbers of ribosomes, so relative rRNA abundance cannot be directly translated into cell numbers (Amann *et al.* 1995). Also, it is possible that an organism has multiple operons with different 16S rRNA sequences, so care should be taken when considering whether unique populations of molecules correspond to unique organismal populations (Ferris & Ward 1997).

The most common use of 16S rRNA in the study of natural microbial ecosystems has been as a target for nucleic acid probes (Amann *et al.* 1990*a*, Amann *et al.* 1990*b*). These oligonucleotide probes are fragments of single-stranded DNA that bind with high specificity to complementary sequences of the target rRNA (Schleifer *et al.* 1992, Amann & Kühl 1998). Due to the fact that 16S rRNA contains regions of highly and less conserved sequences, probes derived from 16S rRNAs can have various specificities (Amann *et al.* 1990*a*, Amann *et al.* 1990*b*, Schleifer *et al.* 1992). The

highly conserved regions are useful as binding sites for primers or universal probes, which have been used to measure total rRNA abundance in the environment (Giovannoni *et al.* 1988, Amann *et al.* 1990*b*). The variable regions provide signature sequences that are unique to particular organisms or related groups, such as species, families, genera, orders or the three domains (Archaea, Bacteria and Eucarya) (Amann & Kühl 1998). These signature sequences offer targets for hybridisation probes with various specificities (Giovannoni *et al.* 1988, Amann *et al.* 1990*a*). The use of probes that are increasingly more specific down to the most variable regions makes it possible to identify unknown isolates to the genus, species or subspecies level (Amann *et al.* 1990*b*, Schleifer *et al.* 1992). Probes can also be targeted to DNA, as the reactions are highly specific and binding to complementary sequences will occur even if these sequences account for only a small fraction of the total nucleic acid (Schleifer *et al.* 1992). However, as rRNAs are present in very high copy numbers, 16S rRNA is a naturally amplified target for hybridisation probes which results in considerably increased sensitivity over the use of DNA (Amann *et al.* 1990*b*).

There have been numerous studies of microbial communities from a wide variety of natural habitats utilising 16S rRNA or its operon, 16S rDNA. So far most results have shown an unexpected wealth of microbial diversity (Muyzer & Smalla 1998). Surveys of hot spring microbial communities using 16S rRNA sequences by Stahl and co-workers in 1985 and Ward and co-workers in 1990 were among the first to reveal this impressive diversity of uncultivated microbial populations, and study of the structure and activity of microbial communities in wastewater bioreactors using similar techniques have indicated that microbial populations are diverse (Bramucci & Nagarajan 2000). There seems to be ample evidence that microorganisms in nature are more phylogenetically diverse than has been accounted for by sequence analysis of cultured strains, as many studies that show a lack of a match when comparing environmental sequences with the databases conclude that this indicates that the organism has not been cultured and is different from any in pure culture (MacGregor 1999, Rondon *et al.* 1999). However, it must be remembered that the 16S rRNA genes of fewer than half of the deposited type cultures have been sequenced (Rondon *et al.* 1999). A study by Suzuki *et al.* in 1997 demonstrates this, as it was found that many culturable heterotrophic marine bacterioplankton include strains for which 16S rDNA sequences are not available. 16S rRNA studies have added much to our knowledge of microbial diversity, but it is debatable whether they have advanced our understanding of the relationship between this diversity and important processes, their regulation or how

they might be manipulated (O'Donnell & Görres 1999). Therefore, rRNA-targeted probes must in the future be used to direct new cultivation attempts to those groups that are really important in nature (Amann & Ludwig 2000).

1.6.3.2 Nucleic acid extraction

The first stage of many molecular analyses of natural environments is the extraction of the required nucleic acids from the organisms present. Each physical, chemical and biological step involved in any molecular method, including extraction, is a potential source of bias (Wintzingerode *et al.* 1997), and so it is important to identify and attempt to control the problematic areas. The initial area of concern is sample handling, which can be difficult in terms of preservation of nucleic acids in fragile cells when samples are stored or frozen (Muyzer & Smalla 1998, MacGregor 1999). Analysis of terrestrial ecosystems, such as wastewater treatment plants, is easier in terms of sample handling as sample volumes can be kept small and material can be stored on ice, frozen, or processed immediately (Wintzingerode *et al.* 1997).

The second area of concern involves the lysis of bacterial cells to obtain the nucleic acids. If cells are insufficiently lysed by any method, then their nucleic acids will not contribute to or be under-represented in the final analysis of diversity of the ecosystem, thus resulting in a biased view of the community composition (Wintzingerode *et al.* 1997). Some organisms are resistant to a gentle enzymatic lysis method, such as the use of lysozyme (Muyzer & Smalla 1998), and the presence of inorganic particles or organic matter found commonly in environmental samples affect the efficiency of this method. Therefore, almost all of the currently used methods for nucleic acid extraction from environmental samples are variations on a mechanical lysis treatment, such as bead beating (Krsek & Wellington 1999, O'Donnell & Görres 1999). In bead beating, cells are ruptured by vigorous agitation with glass beads in the presence of phenolic or sodium dodecyl sulfate extractants (O'Donnell & Görres 1999). For analysis of activated sludge samples, bead beating is the most effective lysis method. Activated sludge differs from any other environmental sample as it contains bacterial aggregate flocs which must be disintegrated for efficient lysis of all the cells present, which cannot be achieved by the use of lysozyme, freeze-thaw or chemical methods (Yu & Mohn 1999). One of the major drawbacks to the use of a physical lysis method such as bead beating is shearing of the nucleic acids, especially genomic DNA (Muyzer & Smalla 1998, Yu & Mohn 1999). Fragmentation of nucleic acids is not desirable as, apart from the fact that longer sequences of possible genes of interest cannot be

analysed, they may contribute to the formation of chimeric products during amplification by the Polymerase Chain Reaction (PCR) (Wintzingerode *et al.* 1997), which is discussed further in section 1.6.3.3. It must be noted however that bead beating appears to be a better choice of mechanical lysis treatment, as it has been reported that sonication shears DNA more than bead beating (Krsek & Wellington 1999).

It has been noted that environmental samples contain inorganic and organic materials, such as clays, humic acids and metals, all of which are potentially inhibiting to subsequent molecular analyses (O'Donnell & Görres 1999). Humic acids and other humified substances can cause problems in PCR. They are soluble, difficult to remove and exhibit binding affinities very similar to nucleic acids, therefore they strongly inhibit *Taq* polymerase and can cause other problems such as difficulty in spectrophotometric quantification of nucleic acids (Muyzer & Smalla 1998). Steps can be taken to remove them from the final preparation, such as excision of high molecular mass DNA after agarose gel electrophoresis or the addition of hexadecyltrimethylammonium bromide (CTAB) prior to extraction (Wintzingerode *et al.* 1997). Clay minerals may bind released DNA, thus lowering the extraction yield and inhibiting PCR, but this can be overcome by the use of blocking agents, such as polyvinylpolypyroliddone or bovine serum albumin (Yu & Mohn 1999).

1.6.3.3 PCR as a molecular tool

An approach that has been extensively used to assess the microbial diversity of natural environments in a culture-independent manner is analysis of 16S rRNA. One of the fundamental techniques that allows this analysis is the amplification of 16S rRNA genes (16S rDNA) using the Polymerase Chain Reaction (PCR). By using PCR, 16S rDNA fragments can be selectively amplified from environmental samples containing mixed genomic DNA (Amann *et al.* 1995, García-Martínez *et al.* 1999, MacGregor 1999). It has been discussed that the 16S rRNA gene contains regions of both highly conserved and variable sequence (see section 1.6.3.1). PCR can be used to exploit this sequence variability by the development of primers, which can vary in specificity from all known eubacterial organisms to individual species, facilitated by known 16S sequence information such as that held at the Ribosomal Database project. Microbial community composition is often studied using primers that are homologous to the highly conserved regions of the gene near the 5' and 3' ends (Heuer *et al.* 1997, Amann & Kühl 1998), which then amplifies the interstitial variable regions from mixtures of genes with homologous ends (Suzuki & Giovannoni 1996). Comparison of the

sequences of these variable regions provides information on the microbial populations within the natural habitat (Fernández *et al.* 1999).

Although the study of amplified 16S rDNA sequences will give information on the phylogenetic diversity within a sample, there are several types of bias that can be introduced during PCR that limit inferences about microbial abundance. The first type involves the semi-quantitative nature of 16S PCR-based techniques (Amann & Ludwig 2000). This is due to the fact that the copy number of the rRNA gene varies both within and between species, meaning cells with multiple copies will be over-represented in a comparison of the relative frequencies of genes in the PCR product (MacGregor 1999). The second type concerns the efficiency of amplification of the genes by the PCR reaction, which is dependent upon several factors involving the primary structure of the gene (Amann & Ludwig 2000). One of these factors is the influence of folding of the template gene. Differences in secondary structure, which is dependent upon primary structure, may effect the availability of primer binding sites or the efficiency of PCR (Suzuki & Giovannoni 1996). Another factor is the moles percent guanine-plus-cystosine (GC) content of the gene. Low GC templates dissociate at higher efficiencies than high GC templates, thus genes with low GC content will be over-represented in the PCR product (Suzuki & Giovannoni 1996, Amann & Ludwig 2000). A further factor is inhibition of the reaction due to reannealing of single-stranded template homologs. As the concentration of products increases, the rate of reannealing will also increase, and thus there is less single-stranded template to participate in the reaction (Suzuki & Giovannoni 1996).

It must be noted that amplification of environmental rDNA involves complex template mixtures usually of unknown sequence (Suzuki & Giovannoni 1996), and thus the factors mentioned here will hold especially true for sampling of natural communities. If the rDNA of microbes abundantly present in a sample is inefficiently amplified, they will remain undetected in the sample (Stams & Elferink 1997). Fortunately, high diversity of templates is an advantage in terms of reannealing inhibition, since it is unlikely that the amplification of any particular 16S rDNA will produce products at concentrations high enough to produce inhibition (Suzuki & Giovannoni 1996). The final type of bias that may be introduced by PCR concerns limitations inherent to the method itself. The formation of chimeric molecules, which are hybrids of two or more different sequences produced when templates of high similarity compete for primer binding, has often been observed in analysis of complex communities. The main cause of chimera formation has been suggested to be the use of

sheared DNA, and as rigorous cell lysis conditions are generally used for DNA preparation from environmental samples, this could have a significant impact on 16S rDNA amplification from natural habitats (Wintzingerode *et al.* 1997). A significant limitation of PCR that must be kept in mind is contamination by reaction inhibiting substances or unwanted DNA (Amann *et al.* 1995).

1.6.3.4 Denaturing Gradient Gel Electrophoresis

One of the most powerful and commonly used types of molecular analysis of the phylogenetic diversity in complex natural microbial ecosystems is genetic fingerprinting (Kowalchuk *et al.* 1997, García-Martínez *et al.* 1999), which provides a pattern or profile of the community based on sequence diversity (Muyzer & Smalla 1998, Muyzer 1999). One such genetic fingerprinting technique is Denaturing Gradient Gel Electrophoresis (DGGE). In DGGE, community 16S rRNA (or more commonly its gene) is amplified by PCR which results in products of the same length but with different sequences (Ferris *et al.* 1997, Øvreås *et al.* 1997, O'Donnell & Görres 1999). These products are then separated on the basis of sequence to give an overview of the composition of mixed microbial populations (Suzuki & Giovannoni 1996, Kowalchuk *et al.* 1997, Wintzingerode *et al.* 1997, Zumstein *et al.* 2000). DGGE was first introduced into microbial ecology by Muyzer and co-workers in 1993 in a study of microbial mats and wastewater treatment biofilms (Heuer *et al.* 1997, Muyzer & Smalla 1998), and has since become well established as a technique for the study of natural microbial habitats. The profile produced by DGGE provides high resolution information about the microbial communities as each distinguishable band in the separation pattern is most likely derived from one phylogenetically distinct bacterial species, and the intensity of the band can be used as an estimate of abundance although it must be remembered that PCR is only semi-quantitative (Muyzer *et al.* 1993, Øvreås *et al.* 1997). DGGE can be used to determine the spatial distribution of bacterial populations by sampling from specific locations (Felske & Akkermans 1998, Muyzer & Smalla 1998), and to detect temporal fluctuations in populations or changes after environmental perturbations (Ferris *et al.* 1997, Muyzer 1999, Ishii *et al.* 2000). Many studies have used DGGE to investigate community diversity in natural habitats, such as wastewater treatment plants (Muyzer *et al.* 1993, Curtis & Craine 1998), hydrothermal vents (Muyzer *et al.* 1995), fresh and sea waters (Teske *et al.* 1996) and soil (Felske *et al.* 1996, Heuer *et al.* 1997). Changes in populations within various communities, such as

microbial mats, chemoclines and biofilms, have also been extensively studied, as shown by a comprehensive review by Muyzer and Smalla in 1998.

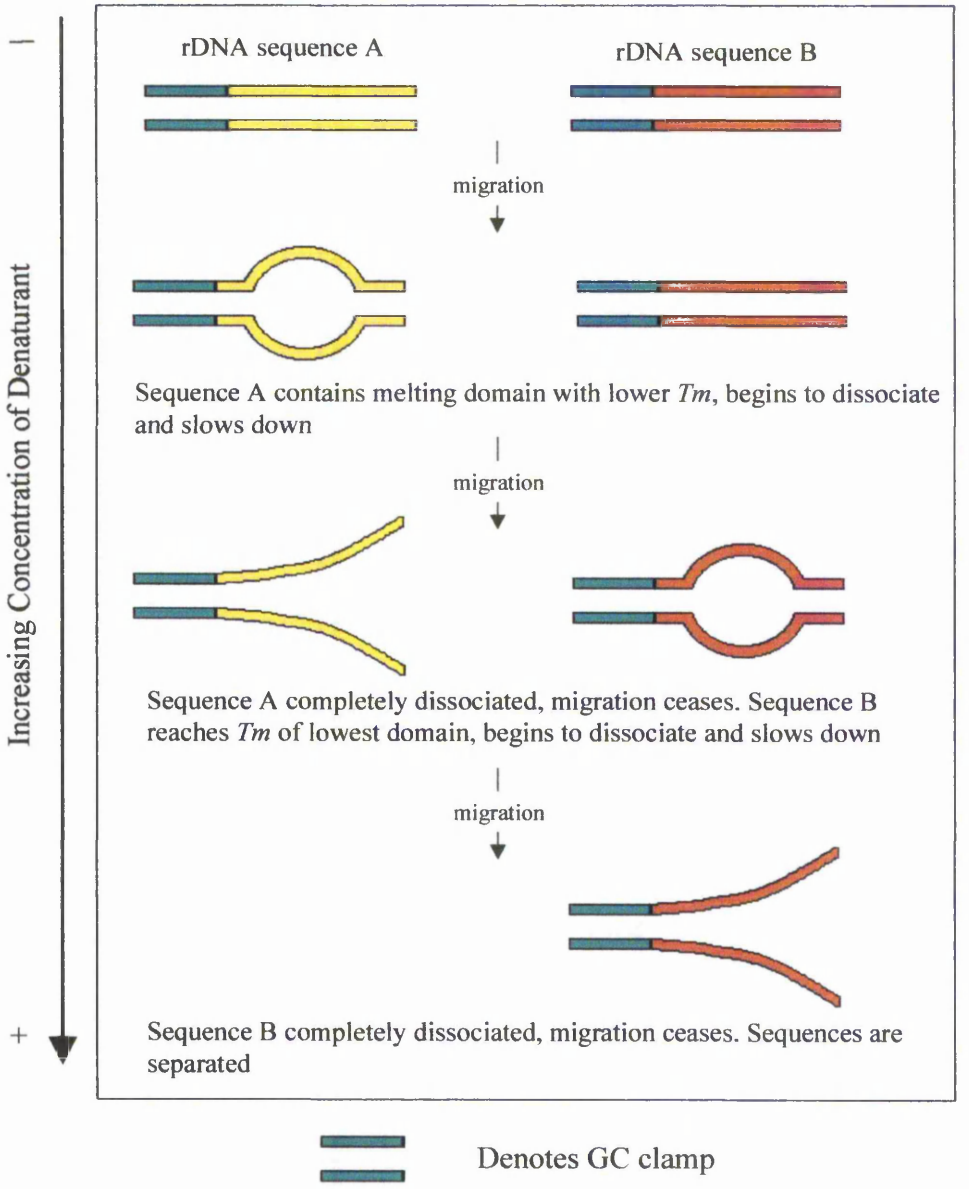
It has been noted that the products of a single PCR of community rRNA or rDNA using primers designed for use in DGGE are all of the same length but have different sequences. The separation of these products in DGGE is based on two basic characteristics of nucleic acids. The first is that denaturation of double-stranded (ds) DNA, or melting, is dependent upon primary sequence (Wintzingerode *et al.* 1997, Ward *et al.* 1998). Melting of ds DNA proceeds in discrete so-called melting domains, which are stretches of base pairs with an identical melting temperature (*Tm*) (Muyzer *et al.* 1993, Muyzer & Smalla 1998). A·T base pairs, being joined by two hydrogen bonds, denature more easily than G·C pairs which are joined by three H-bonds (MacGregor 1999), and thus stretches of DNA containing a higher proportion of A·T pairs will have a lower *Tm* than those with a higher G·C content. The second property is that partially melted DNA fragments have a lower electrophoretic mobility than completely helical fragments (Stams & Elferink 1997, Muyzer 1999). Therefore, fragments of ds DNA being subjected to electrophoresis will essentially stop migrating at the *Tm* of the lowest melting domain (Muyzer *et al.* 1993, MacGregor 1999).

In DGGE, a polyacrylamide gel is used that contains a concentration gradient of DNA denaturants, usually formamide and urea (Heuer *et al.* 1997, Muyzer & Smalla 1998). When a ds fragment reaches the position in the gel that contains the concentration of denaturants equal to the lowest *Tm* of that fragment, it will cease migrating and form a band within the gel (MacGregor 1999). Sequence variation of the rDNA causes different fragments to have different *Tm*, and so different fragments will stop migrating at different positions in the denaturing gradient and hence can be separated effectively (Muyzer *et al.* 1993, Muyzer 1999). If the DNA dissociates completely into its single-stranded (ss) form, it will continue to migrate through the gel. Therefore, one of the PCR primers is synthesised with a GC-rich 'tail' (usually 30-50 nucleotides long) (Wintzingerode *et al.* 1997, Muyzer & Smalla 1998), which results in PCR products with a GC clamp (Muyzer *et al.* 1993, MacGregor 1999). The GC clamp acts as a high melting domain preventing complete dissociation of the fragments into ss DNA (Muyzer & Smalla 1998). The theoretical basis behind this technique is represented diagrammatically in Figure 1.9. The upper size limit of PCR products that can be differentiated by DGGE is 800-1000 bp, and nearly 100% of the possible sequences can be detected in fragments of up to 500 bp (Muyzer & Smalla 1998).

There are many factors which have made DGGE one of the most commonly used techniques for analysis of microbial diversity in natural habitats. The first is that it is extremely rapid (Muyzer & Smalla 1998, Muyzer 1999), giving a profile of the community from a sample within 10 hours. The second is that it is highly reproducible, especially when 16S rDNA is analysed (Felske & Akkermans 1998), with multiple DNA extractions and PCRs showing no difference in banding patterns (Kowalchuk *et al.* 1997, Ishii *et al.* 2000). It is also sensitive, and can detect bacterial populations that make up approximately 1% of the total community (Muyzer *et al.* 1993, Muyzer & Smalla 1998). It also allows for identification of the community members by sequencing or probing the bands (Heuer *et al.* 1997), which is not possible for other fingerprinting techniques such as terminal restriction fragment length polymorphism (T-RFLP) (Muyzer 1999). Finally, it is a convenient technique to monitor spatial or temporal variation in communities, as high numbers of samples can be run next to each other on a gel and easily compared (Felske & Akkermans 1998, Curtis & Craine 1998).

However, there are limitations that are specific to this technique, which must be kept in mind when using DGGE to analyse bacterial communities. The inclusion of a GC-rich sequence in the primers will reduce amplification efficiency during PCR and can cause incomplete strand synthesis, leading to multiple bands for one template (Amann & Ludwig 2000). This is of great concern when analysing highly complex ecosystems, as it may lead to an overestimation of diversity (Wintzingerode *et al.* 1997). Another problem faced by DGGE is the co-migration of fragments with different sequences in the concentration gradient of denaturants used (Kowalchuk *et al.* 1997, Muyzer 1999). This means that a single band on the gel may not consist of fragments of exactly the same sequence, and thus may not be derived from the same species (Muyzer & Smalla 1998). This problem can be addressed by using more narrow gradients to provide high resolution profiles of particular parts of the original profile that are suspected to contain co-migratory fragments (Muyzer *et al.* 1993).

Figure 1.9: The theoretical basis of Denaturing Gradient Gel Electrophoresis



A further limitation of DGGE is that only relatively small DNA fragments can be analysed (upper limit 800-1000 bp), which limits the amount of sequence information that can be gleaned from a community (Muyzer & Smalla 1998). Finally, although species that contribute 1% of the total community can be detected, there will be species present that are less abundant than this. If these species are very important in the ecosystem, they will be missed, and thus the true dynamics of the community will not be elucidated (Heuer *et al.* 1997). Because of these limitations, and also those that are common to all molecular analyses, it cannot be assumed that DGGE provides a complete and totally objective view of community diversity and composition (Ward *et*

al. 1998). There is a clear trend to combine DGGE with other molecular and microbiological techniques such as sequencing and cultivation to obtain a more realistic picture of microbial community structure and function (Muyzer 1999).

1.6.3.5 Sequencing as a tool in microbial diversity analysis

The microbial diversity of complex ecosystems is most often described by phylogenetic characterisation of its members using 16S rRNA or its gene (Alfreider *et al.* 1996, O'Donnell & Görres 1999). Sequencing of 16S rDNA has long been the most powerful method of bacterial identification (Manz *et al.* 1992), and many new phylogenetically distinct species have been discovered in this way (Kowalchuk *et al.* 1997). The most effective method of sequence analysis is cloning and sequencing of the products from PCR amplification (García-Martínez *et al.* 1999, Muyzer 1999). However, the study of spatial or temporal population changes in microbial communities involves analysis of a large number of samples (Alfreider *et al.* 1996). This means that the cloning approach is not well suited to many investigations of microbial diversity, as it is laborious, time consuming and expensive (Kowalchuk *et al.* 1997, Muyzer 1999). One way to circumvent the need for cloning is the reamplification and sequencing of bands from a DGGE gel (Stams & Elferink 1997, Ward *et al.* 1998). Bands appearing on a gel are separated according to sequence variations, and so excision of the bands and subsequent sequencing will provide a phylogenetic identification of the community members which constituted that sample (Øvreås *et al.* 1997). In this way, the presence of bacterial species can be correlated with changes in the community profile, which may be due to changes in key environmental factors (Kowalchuk *et al.* 1997). However, there is a distinct drawback in the use of sequencing to assess diversity in natural habitats. Sequencing depends on databases of sequences already elucidated (such as the NCIB databases), and so the effectiveness of bacterial identification by sequencing is only as good as the completeness and usability of the databases. It has been noted that fewer than half of the deposited type cultures have been sequenced (Rondon *et al.* 1999) and many studies elucidate environmental sequences that do not correspond with the databases (MacGregor 1999). Also, new databases were often created using different formats or software, so they were difficult to search and were stretched to their limits (Baker & Brass 1998). The inclusion of many more complete sequences and re-engineering of the databases using advances in information technology have gone some way to alleviating these problems.

1.6.3.6 Fluorescent *in situ* Hybridisation

One method of analysing the community composition and dynamics in complex microbial ecosystems is the use of *in situ* hybridisation with nucleic acid probes (Amann *et al.* 1995, Muyzer & Smalla 1998). By using this technique, the spatial distributions, abundance and identity and of cells in mixed communities can be resolved (Giovannoni *et al.* 1988, Alfreider *et al.* 1996, Kowalchuk *et al.* 1997, Harmsen *et al.* 1999). Hybridisation is faster, more reliable and provides a more complete understanding of community composition than traditional culture techniques (Amann & Ludwig 2000, Whiteley & Bailey 2000), and the use of whole cell (*in situ*) hybridisation is more rapid and provides more information on the undisturbed ecosystem than other types of hybridisation e.g. slot- and dot-blot (Festl *et al.* 1986). The first applications of nucleic acid hybridisation were described in the early 1960s, and then two decades later in 1988 hybridisation was applied *in situ* to rRNA by Giovannoni and co-workers (Schleifer *et al.* 1992). The use of rRNA conferred a major advantage to nucleic acid hybridisation, as signature sequences in the rRNA could be used to control probe specificity (Madigan *et al.* 2000). This specificity spans the entire phylogenetic spectrum (Amann *et al.* 1990*b*), from species-specific probes targeting the most variable regions of rRNA or general group/genus probes complementary to more conserved regions (Stams & Elferink 1997). Like all previous applications of hybridisation, the study by Giovannoni *et al.* in 1988 used a radioactively labelled probe, but concerns about safety quickly initiated the development of fluorescent dye labelling (Stahl & Amann 1991). Fluorescently labelled probes were first introduced by DeLong *et al.* in 1989, who coined the term "phylogenetic stain" to describe the unique character of these probes (Amann *et al.* 1990*b*), and thus the technique of Fluorescent *in situ* Hybridisation (FISH) was developed. FISH has been used extensively to study spatial relationships in biofilms, such as bioreactor sludge (Stams & Elferink 1997, Stickler 1999). An example of the diversity of phylogenetic groups that have been studied in anaerobic sludge is shown in Table 1.4.

Table 1.4: Selection of oligonucleotide probes useful for the microbial characterisation of anaerobic sludge

Probe	Target Group	Reference
EUB338	Bacteria	Amann *et al.*, 1990*a*
ALF1b	Proteobacteria α-subclass	Manz *et al.*, 1992
BET42a	Proteobacteria β-subclass	Manz *et al.*, 1992
GAM42a	Proteobacteria γ-subclass	Manz *et al.*, 1992
SRB385	Gram-negative mesophilic sulphate reducers	Amann *et al.*, 1990*a*
ARCH915	Archaea	Stahl & Amann, 1991

N.B. ALF1b is not specific only for the α-subclass. Several non-target organisms share the target sequence of SRB385, whereas some target organisms have several mismatches (Stams & Elferink 1997).

As has been noted, most probes used in FISH assays are rRNA-targeted oligonucleotides (Alfreider *et al.* 1996). These oligonucleotides are short, usually 15-25 nucleotides in length (Stahl & Amann 1991), as most microorganisms are permeable to these probes following fixation (Amann *et al.* 1990*a*). The fact that individual cells can be visualised using FISH is based on the high number of ribosomes present in actively growing cells (between 10^3 and 10^5) (Giovannoni *et al.* 1988, Amann *et al.* 1995, Amann & Kühl 1998). There are an equal number of rRNA molecules present, and thus labelling of each molecule enables the technique to be sensitive enough to directly observe single cells by fluorescence microscopy (Amann *et al.* 1990*a*). The fluorescent dye molecule, referred to as the fluorochrome, is attached to the 5' end of the oligonucleotide via an aminoethylphosphate-linker (Stahl & Amann 1991). Commonly used fluorescent dyes include fluorescein (FITC), tetramethylrhodamine and Texas Red (Stahl & Amann 1991, Amann *et al.* 1995). There are recently developed indocarbocyanine dyes available, such as Cy3, Cy5, and Cy7, which are bright, relatively photostable and enable much higher detection rates in environmental samples (Ekong & Wolfe 1998, Amann & Ludwig 2000).

Some of the advantages of the use of FISH in analysis of microbial ecosystems have been noted, such as the speed of the technique and the 'true-to-life' picture of community composition that results from probing whole cells in undisturbed samples. It is also highly significant when studying the temporal changes of environmental populations that samples can be fixed and stored for later analysis (Harmsen *et al.* 1999). However, there are several limitations to this technique that are worth taking into consideration during an investigation of natural microbial habitats. Most importantly, the design of nucleic acid probes relies on sequence data. As the microbial, and thus rRNA, diversity is unknown in many ecosystems, it is possible that many organisms may be

missed (Amann & Ludwig 2000). The probe may be too specific, and miss species that are ecologically similar but phylogenetically unrelated, or too general, and categorise ecologically different but closely related populations as one (Muyzer 1999). Also, studies can only focus on particular microorganisms for which probes have already been developed, unless a molecular inventory of the ecosystem is carried out (Godon *et al.* 1997, Muyzer & Smalla 1998).

One limitation that impacts significantly on enumeration of cells within bioreactor sludge is the presence of humic substances such as humic acids. Humic substances have been shown to interfere with DNA binding to nylon membranes at concentrations of >20 $ng\mu l^{-1}$ (Bachoon *et al.* 2001). The level of humic substances present in sediments such as bioreactor sludges would be higher than this, and are therefore likely to bind the oligonucleotide probes and the generic DNA-staining compound 4',6-diamidine-2-phenylindole (DAPI), resulting in high background levels with oligonucleotide probes and almost complete staining of the sample with DAPI.

Another limitation is that permeability of bacterial cell walls varies (MacGregor 1999, Amann & Ludwig 2000). Different fixatives need to be applied to Gram-negative bacteria, such as paraformaldehyde, and Gram-positive bacteria, such as ethanol (Amann *et al.* 1995), and thus all cells from a single sample will not be equally accessible to probing. Finally, it is difficult to apply FISH quantitatively, especially to environmental samples. Probed cells are counted manually using a fluorescent microscope, which is both labour intensive and subjective (Rondon *et al.* 1999) especially when dealing with large numbers of complex samples. This makes quantitative FISH a laborious method that cannot be considered rapid (Davenport *et al.* 2000). This problem may be alleviated in the future with the use of image processing and automated techniques such as flow cytometry (MacGregor 1999, Amann & Ludwig 2000). Many studies on FISH analysis of bacteria from activated sludge have also reported great variability in counts, to the extent that robust statistical analyses were impossible, and few studies have even attempted to apply statistical methods (Davenport *et al.* 2000). This means that the scientific value of quantitative FISH analyses may be reduced.

1.6.3.7 The use of confocal scanning laser microscopy in Fluorescent *in situ* Hybridisation

One of the greatest obstacles to the use of FISH for analysis of environmental samples is visualisation of the cells. The microorganisms are often densely packed

across many planes of focus, and so light from planes that are out of focus contaminate the signal from the focal plane. A major advance in overcoming this problem is the use of the confocal scanning laser microscope (CSLM) (Fung & Theriot 1998, Palmer & Sternberg 1999). In CSLM, a narrowly focussed spot of laser light illuminates the specimen. Fluorescence from this spot passes through the objective to be focussed on the focal plane. At this point, called the "confocal position", a pinhole aperture only allows the focussed light to pass through, whilst light from outside the focal plane gets blocked. The focussed light is then passed to a photomultiplier tube, and provides the digital image. As the specimen is only illuminated a point at a time, 2D images are obtained by rapidly scanning the illumination over the sample, which is known as raster scanning (Fung & Theriot 1998). By altering the position of the microscope stage in the Z dimension and repeating the process, 3D images are obtained (Palmer & Sternberg 1999). In this way, CSLM has provided an added dimension to the study of natural microbial communities by FISH, as the advantages that are offered by *in situ* analysis in terms of microbial associations and spatial distribution within the sample are not lost upon visualisation (MacGregor 1999). However, one of the drawbacks of using CSLM is that the number of chromophores that can be utilised is limited by the laser frequencies available, which places a restriction on the number of differently labelled probes that can be used in multiple probing experiments (Fung & Theriot 1998).

1.7 Study rationale

This study builds on two previous research projects (Sweeney 1995, Kennerley 2000).

- Sweeney worked on food azo dyes (such as Sunset Yellow) with regard to the generation of mutagenic and genotoxic compounds following anaerobic reduction akin to conditions in the human large intestine. This work showed the generation of oxygen radicals by naphthol compounds and DNA adduct formation.
- Kennerley studied the decolourisation of textile dyes (principally hydrolysed Reactive Black) under anaerobic conditions and used various chemical methods (TLC, HPLC and FTIR) to identify the end products.

Hence this new study builds on previous experience of anaerobic textile dye decolourisation with genotoxicity testing in order to assess the 'safety' of textile dyehouse effluent following anaerobic wastewater treatment. The use of toxicity testing

with the *V. fischeri* bioluminescent bacterium is included since this is a method readily available to industry.

1.8 Aims and objectives

The overall aim of this project is to determine the safety, with regard to toxicity and genotoxicity, of effluents from two laboratory-scale bioreactors decolourising an STE. The anticipated project outcomes are increased knowledge of the requisite physico-chemical and biological operating parameters for biological treatment of dyehouse wastewaters that conform to both current consent limits for colour and future consent limits for toxicity and genotoxicity imposed by government agencies.

The preliminary objective is to establish the applicability of short-term bacterial bioassays for toxicity and genotoxicity assessment of the effluent samples by *in vitro* experiments with textile azo dyes and their products of decolourisation, which are principally thought to be aromatic amines. By reviewing the literature, it has been proposed in this study that the most effective toxicity bioassay for the monitoring of wastewater samples is one based upon bacterial bioluminescence, as discussed in section 1.4.5. Therefore, two commercially available bioluminescent assay systems, the Microtox® (Azur Environmental Ltd.) and the ToxAlert™ 100 (Merck Speciality Chemicals Ltd.) will be used. By reviewing the literature, it has been proposed that the most effective genotoxicity bioassay for the monitoring of wastewater samples is one based upon differential killing of DNA repair proficient and deficient strains, detected using impedance technology, as discussed in section 1.4.6. Therefore, the *Escherichia coli* Differential Kill assay combined with the Rapid Automated Bacterial Impedance Technique (RABIT, Don Whitley Scientific) will be used (Forsythe 1990). A representative set of reactive azo textile dyes will be used, including RB, as these have been identified as the most commonly used and problematic dyes in the textile industry (see sections 1.2.2 and 1.2.3), as will a number of structurally related naphthol compounds similar to the aromatic amines predicted as products of decolourisation. Dyes will be tested in their native (parent), hydrolysed (see section 1.2.2) and decolourised forms. Decolourisation will be achieved by the use of a laboratory strain of *Enterococcus faecalis*, a bacterium known to decolourise azo textile dyes (see section 1.3.4), and an environmental isolate capable of decolourising azo textile dyes identified as *Clostridium butyricum/beijerinckii* (Kennerley 2000).

The subsequent objective is to assess the toxicity and genotoxicity of effluent samples from laboratory-scale bioreactors decolourising an STE. Two types of

bioreactor, a batch-fill and a continuous flow model, will be designed and operated in a joint EPSRC-funded project in conjunction with this study at Loughborough University of Technology, by C. Shaw. Both bioreactors will operate in anaerobic and phased anaerobic-aerobic states, to determine the effect of aeration on the ability of the bioreactors to decolourise dye and the effect on the toxicity and genotoxicity of the effluent. Physico-chemical operational parameters will be monitored by C. Shaw to determine the effect of aeration and increased dye concentration of the performance of the bioreactors. The azo textile dye RB will be used as a model dye for bioremediation of textile wastewaters (see sections 1.2.2 and 1.2.3).

The final objective is to establish the influence of bioflora diversity on the performance of the bioreactors with regard to decolourisation, toxicity and genotoxicity of the effluent. Diversity will be determined by conventional culturing methods and molecular methods. Due to the limitations of conventional culturing methods, as discussed in section 1.6.2, the emphasis of the work will be on the use of molecular methods. Due to its status as the main focus of research on phylogenetic analysis (see section 1.6.3.1), the 16S rDNA gene pool of the microbial community will be analysed. The genetic fingerprinting technique DGGE has many advantages in analysis of complex natural microbial ecosystems (see section 1.6.3.4), and so will be utilised to analyse the bioflora diversity within the bioreactors, with subsequent identification of the predominant or persisting genotypes by gene sequencing. FISH will also be utilised due to the advantages of this technique in determining the spatial arrangement of genotypes within sludge floc and biofilm structures, such as would be present in the bioreactors (see section 1.6.3.6). The FISH technique will be augmented by the use of CSLM, which allows for three dimensional imaging of biofilms and other large cellular structures (see section 1.6.3.7). The effect of aeration, increasing concentration of dye, and the spatial and temporal diversity of the bioflora of the reactors will be assessed with a view to determining the optimal biological conditions for effective treatment of textile dyehouse wastewaters.

Chapter 2: Materials and Methods

2.1 Decolourisation studies of azo dyes

2.1.1 Chemicals and media

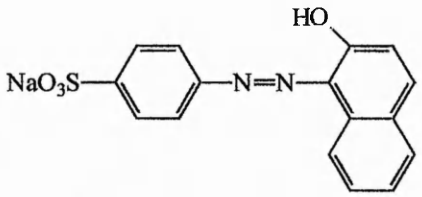
Where standard media are used, such as Tryptone Soya Broth (TSB), Tryptone Soya Agar (TSA) and Schaedler Anaerobe Broth (SAB), they were supplied by Oxoid, Hampshire, England. Schaedler Anaerobe Agar (SAA) was made using 1% Agar No. 1 (Oxoid) in SAB. Whitley Impedance Broth (WIB) was supplied by Don Whitley Scientific, West Yorkshire, England.

The dyes used were C.I. Reactive Black 5 (RB) (55% pure), supplied by Aldrich Chemical Company, Milwaukee, USA, Procion Navy H-EXL (PN) (33% liquid) and Procion Crimson H-EXL (PC) (>75% pure) supplied by Zeneca Colours, Procion Yellow H-EXL (PY) supplied by ICI Organics Division, C.I. Food Yellow 3 (Sunset Yellow FCF, SY) (>80% pure) supplied by BDH Chemicals, Poole, and C.I. Acid Orange 7 (Orange II, O2) supplied by the Department of Textiles, TNTU. The structures of RB, SY and O2, including Colour Index constitution number, are shown in Figure 2.1. The structures of the Procion dyes have yet to be released. It should be noted that none of the dyes utilised were pure compounds, although exact purity of some was unknown, and thus each will contain a mixture of molecules that constitute the final dye including aminonaphthol compounds. The generic name of Procion Yellow H-EXL is C.I. Reactive Yellow 138:1. The generic names of Procion Crimson H-EXL and Procion Navy H-EXL are unknown. All of the Procion dyes are known to be azo dyes with two monochlorotriazinyl reactive side groups (Dystar, pers. comm.), which are different to vinylsulphonyl reactive groups of RB. None of these dyes have a structure or constitution number declared in the latest edition of the Colour Index International (1999).

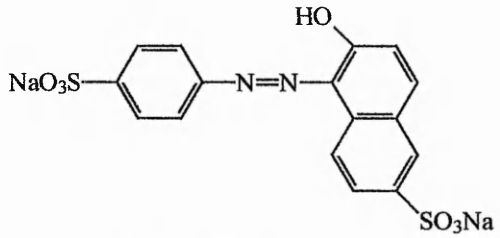
The aminonaphthol compounds related to aromatic amines used in the structure-activity evaluation comprised 1-amino-2-naphthol·HCl, 1-amino-2-naphthol-6-sulphonate, sulphanilic acid, 1-naphthalene sulphonic acid, 2-naphthalene sulphonic acid, 4-amino-1-naphthalene sulphonic acid, 4-amino-1-naphthalene sulphonic acid sodium salt and 1-naphthol-8-amino-3,6-disulphonic acid. All were supplied by Aldrich Chemical Company, Milwaukee, USA, with the exception of 1-naphthol-8-amino-3,6-disulphonic acid (H-acid) which was supplied by Kodak, Rochester, USA and 1-amino-2-naphthol-6-sulphonate which was synthesised by the Department of Chemistry, TNTU. The structures of the aminonaphthol compounds can be seen in Figure 2.2.

Although laboratory grade reagents were used, the purity of each of the compounds is unknown, and thus each is likely to contain a mixture of molecules that constitute the final aminonaphthol compound.

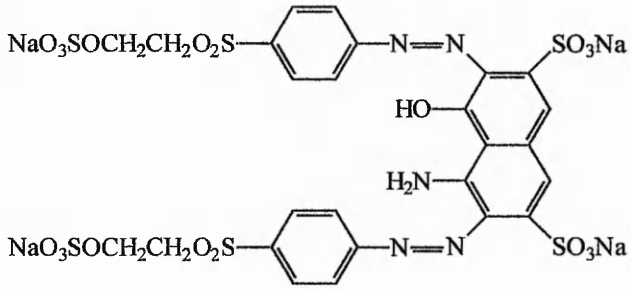
Figure 2.1: The structures of the azo dyes utilised in this study



C.I. Acid Orange 7 (C.I. 15510)



C.I. Food Yellow 3 (C.I. 15985)



C.I. Reactive Black 5 (C.I. 20505)

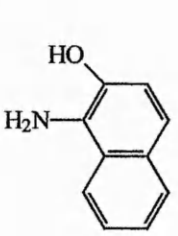
(Colour Index International 1987)

2.1.2 Hydrolysis of dyes

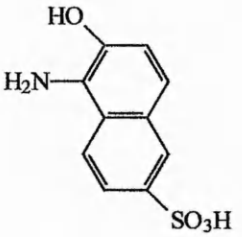
Textile dyes were tested in their parent form and a hydrolysed form, as would be found in a textile waste effluent (Colour Index International 1987, Carliell 1993, Weber & Stickney 1993, Hao *et al.* 2000). The alterations to the molecular structure of a reactive azo dye caused by hydrolysis of the dye, which occurs during the dyeing process (see section 1.2.2), are shown in Figure 1.2. The protocol for hydrolysis of reactive azo dyes shown in Weber and Stickney (1993) and Zubrick (1984) was followed, and is outlined below.

1. The powdered dye was made up to the required concentration (100-500 mgL^{-1}) using distilled water. This is known as the parent form.
2. The pH of the dye solution was adjusted to pH 11 using 1M sodium hydroxide.
3. The dye solution was boiled in a round-bottomed flask containing anti-bumping granules for 3 h. Reflux was achieved by the use of a water-jacketed condenser attached to the top to minimise water loss by evaporation.
4. The pH of the now hydrolysed dye solution was adjusted to pH 7 using 1M hydrochloric acid.
5. Both parent and hydrolysed dye solutions were sterilised by boiling in a water bath for 10 min.

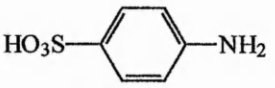
Figure 2.2: The structures of the aminonaphthol compounds utilised in this study



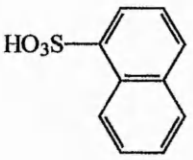
1-amino-2-naphthol



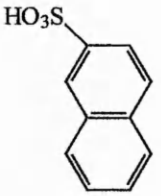
1-amino-2-naphthol-6-sulphonate



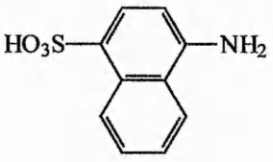
sulphanilic acid



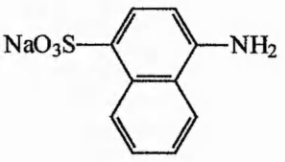
1-naphthalene sulphonic acid



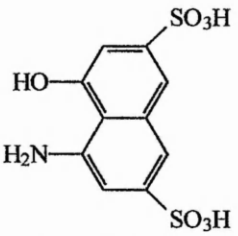
2-naphthalene sulphonic acid



4-amino-1-naphthalene sulphonic acid



4-amino-1-naphthalene sulphonic acid sodium salt



1-naphthol-8-amino-3,6-disulphonic acid (H acid)

(Budvari 1996)

2.1.3 Biological decolourisation of azo dyes

Bacterial cultures used for biological decolourisation of azo dyes were a laboratory strain of *Enterococcus faecalis* (formerly *Streptococcus faecalis*) and an environmental isolate identified as *Clostridium butyricum/beijerinckii* by V. Kennerley at TNTU (Kennerley 2000), referred to as *Clostridium butyricum* in this study. *E. faecalis* was maintained on TSA slopes, grown at 37°C under aerobic conditions, and *C. butyricum* was maintained on SAA slopes, grown at 30°C under anaerobic conditions, which were then stored at 4°C and used a maximum of 4 times.

1. Overnight cultures were set up by inoculating a streak from a stored slope into 10 ml of the appropriate medium (TSB for *E. faecalis* and SAB for *C. butyricum*),

incubated overnight at the appropriate conditions (37°C aerobic for *E. faecalis*, 30°C anaerobic for *C. butyricum*).

2. Precultures were set up by inoculating 100 µl of an overnight culture into 10 ml of the appropriate medium, incubated at the appropriate conditions for 6 and 24 h (*E. faecalis* and *C. butyricum* respectively).
3. Large volume cultures were set up by transferring the entire preculture into 400 ml of the appropriate medium, incubated at the appropriate conditions for 18 h (both species).
4. The large volume cultures were centrifuged at 11,600 g for 20 min at 12°C (*E. faecalis*) or 14,700 g for 20 min at 10°C (*C. butyricum*). Centrifugation with increased *g* and decreased temperature was required for proper adherence of *C. butyricum* to the centrifugation bottle.
5. The supernatant was poured to waste, and pellets were resuspended in 100 mM potassium phosphate buffer at pH7 to ×200 original concentration (final cell density ~5 mg cell wet weight ml^{-1}; Kennerley 2000) and left to stand at room temperature.
6. All assays were conducted using 5 ml final volumes. Assays were prepared in test tubes by adding dye (final concentrations of 100 mgL^{-1} for the structure activity evaluation and 500 mgL^{-1} for textile dyes), glucose (final concentration of 1%, except Procion Crimson with *E. faecalis* which was 0.1%) and 100 mM potassium phosphate buffer at pH7 to a total volume of 4 ml.
7. Tubes were left to equilibrate for 15 min in a water bath at 37°C.
8. A negative control was included by substituting 100 mM phosphate buffer for dye.
9. The reaction was started by the addition of 1 ml of ×200 concentration of cells to give a final cell increase of ×40 original concentration.
10. After complete decolourisation had occurred (see Table 3.5), the assays were centrifuged at 13000 rpm for 5 min in a benchtop microfuge (~18000 *g*).
11. The supernatants were then osmotically adjusted to 2% NaCl using solid NaCl (99.9% pure), and diluted appropriately using 2% NaCl for toxicity testing (see section 2.1.6) or sterile 0.85% saline for genotoxicity testing (see section 2.1.7).

2.1.4 Chemical decolourisation of azo dyes

RB was also decolourised via a chemical oxidation method, using sodium dithionite. The concentrations of sodium dithionite required to give complete decolourisation of the dye solutions were determined in a previous titration experiment conducted by V. Kennerley, TNTU (Kennerley 2000).

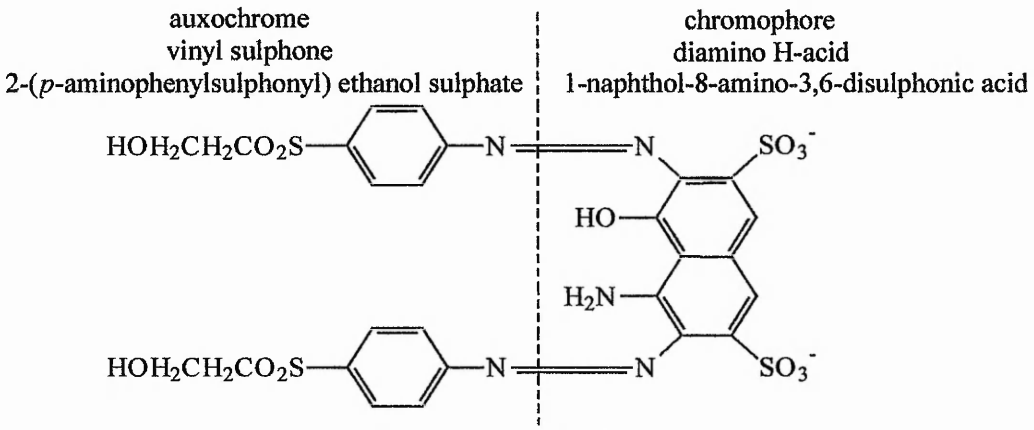
1. A 500 mgL^{-1} solution of RB was made up to a total volume of 10 ml in distilled water.
2. If required, the dye solution was hydrolysed (see section 2.1.2).
3. Sodium dithionite (sodium hydrosulfite) (87% grade, BDH) was added as a dry powder to the dye solution. Final concentrations of sodium dithionite were 0.8 $mgml^{-1}$ and 0.6 $mgml^{-1}$ for parent and hydrolysed dyes respectively.
4. The solutions were swirled gently by hand to mix, and decolourisation was achieved in a few seconds.
5. A negative control was included using 0.8 $mgml^{-1}$ sodium dithionite in distilled water.
6. The supernatants were then osmotically adjusted to 2% NaCl using solid NaCl (99.9% pure), and diluted appropriately using 2% NaCl for toxicity testing (see section 2.1.6) or sterile 0.85% saline for genotoxicity testing (see section 2.1.7).

2.1.5 Fractionation of decolourised hydrolysed C.I. Reactive Black 5

It has been discussed that azo textile dyes are reduced to their constituent aromatic amines during anaerobic decolourisation (see section 1.3.4). Samples of the aromatic amines produced by decolourisation of RB were obtained by fractionation of the decolourised dye using high pressure liquid chromatography (HPLC). Hydrolysed C.I. Reactive Black 5 (RBOH) was assayed due to the environmental significance of this form of the dye (see section 1.2.2). The structure of RBOH, indicating the aromatic amines that would formed upon fission of the azo bonds, is shown in Figure 2.3. The HPLC method used was adapted from Grossenbacher *et al.* 1986 (Kennerley 2000), which is reported as being suitable for the separation of sulphonated azo dyes and their intermediate diamino H-acid.

1. A solution of RBOH at 500 mgL^{-1} was decolourised using *E. faecalis*, using the protocol in section 2.1.3.
2. An Applied Biosystems 785 instrument was used in conjunction with a Perkin-Elmer Series 410 LC pump and a Hibar Lichrocart manu-fix 250-4 C_{18} reverse phase column (Merck), employing a dual solvent system.
3. A programmable absorbance detector (diode array) was used for the analysis, with a detection wavelength of 260 nm.
4. The column was equilibrated with 100 mM phosphate buffer at pH 6.7 (solvent 1) for 10 min prior to injection of 20 µl of decolourised dye.
5. A linear gradient (0-100%) of solvent 1 was performed for a 30 min duration.
6. A reverse linear gradient (100-0%) of 70 % gradient analysis grade methanol in 10 mM phosphate buffer at pH 6.7 (solvent 2), filtered through a 0.2 µm cellulose acetate filter (Sartorius), was then performed for a 20 min duration.
7. The fractions collected were then osmotically adjusted to 2% NaCl using solid NaCl (99.9% pure) and diluted appropriately using 2% NaCl for toxicity testing (see section 2.1.6).
8. Prior to each fractionation, 1 mM RB was assayed for confirmation of reproducibility of the method.

Figure 2.3: The structure of RBOH, indicating its constituent aromatic amines



Hydrolysed C.I. Reactive Black 5

(Colour Index International 1987)

N.B. Vinyl sulphone and diamino H-acid are the generic names of the compounds constituting the auxochrome and chromophore of the dye respectively. The chemical names of each are also shown.

2.1.6 Toxicity assessment using the bioluminescent assay Microtox®

The assay used to determine toxicity of the dyes and aromatic amines was the short-term bioluminescent Microtox® Acute Toxicity test (Azur Environmental Ltd.). This assay uses freeze-dried cultures of the bioluminescent bacterium *Vibrio fischeri* (formerly *Photobacterium fischeri*) to assess cytotoxicity via a reduction in the luminescence of the cells, as luminescence is a function of actively metabolising cells (see section 1.4.5). As *V. fischeri* is a marine organism, the assays must be carried out at 15°C using 2% sodium chloride (NaCl) as the diluent and samples must be osmotically adjusted to 2% NaCl. An initial screen of each dye, reduced and unreduced, and each aromatic amine was carried out to give an indication as to the most appropriate dilution to begin testing. The protocol for the initial screen, given in the Microtox® Acute Toxicity Basic Test Procedures booklet (Azur Environmental 1995) is summarised below.

1. A freeze-dried vial of *V. fischeri*, stored at -20°C, was reconstituted using 0.5 ml Azur Microtox® Reconstitution Solution stored at 4°C, and kept in the reagent well of the instrument at 5°C.
2. Aliquots of 500 µl Microtox® Diluent (2% NaCl in Ultrapure water, Azur Environmental Material Safety Data Sheet) were pipetted into two glass cuvettes (1 cm diameter) per sample, and left to equilibrate in the instrument at 15°C for 15 minutes.
3. Using a small volume repeat pipettor, 10 µl of *V. fischeri* reagent was added to each cuvette and left to equilibrate in the instrument at 15°C for 15 minutes.

4. The initial light level (I_0) of a cuvette was read by placing it in the light tight turret (connected to a photomultiplier tube and luminometer), and automatically recorded by the Microtox® analysis software.
5. Immediately after reading the I_0, 10 µl of sample was added. This was the test cuvette.
6. The I_0 of the second cuvette was read, but no sample was added. This served as the control cuvette for the sample.
7. After exactly 5 minutes of contact time, as indicated by the software, the 5 minute light levels (I_5) of both test and control cuvettes were read and automatically recorded by the Microtox® analysis software.
8. The appropriate dilution for toxicity testing of the sample was indicated by the I_5. Samples were tested undiluted for an I_5 value of >60, at 1:10 dilution for a value of 60-20 and at 1:100 dilution for a value of <20. A test was invalid if the control I_5 fell significantly below the I_0.

Before toxicity testing commenced, the reduced dyes were prepared appropriately (see sections 2.1.3, 2.1.4 and 2.1.5). Unreduced dyes and aromatic amines (only those miscible with water) were also adjusted to 2% NaCl using solid NaCl (99.9% pure). To assess the veracity of the toxicity data produced, the reference toxicant phenol was tested periodically and compared to data from the literature. The average toxicity for phenol was determined to be 19.16 ± 3.18 mgL^{-1} (expressed as EC_{50} after 5 min contact time), which was within the limits set out in the quality control data sheets provided by Azur Environmental Ltd., and compares well to EC_{50} 5 min values of 18-42 mgL^{-1} quoted in the literature (King & Dutka 1986, Blum & Speece 1991, Kaiser & Palabrica 1991, Kahru 1993).

The assay was conducted according to the Azur Environmental Microtox® Acute Toxicity Basic Test Procedures method (Azur Environmental 1995), which conforms to standard protocol EN ISO 11348, and is summarised below.

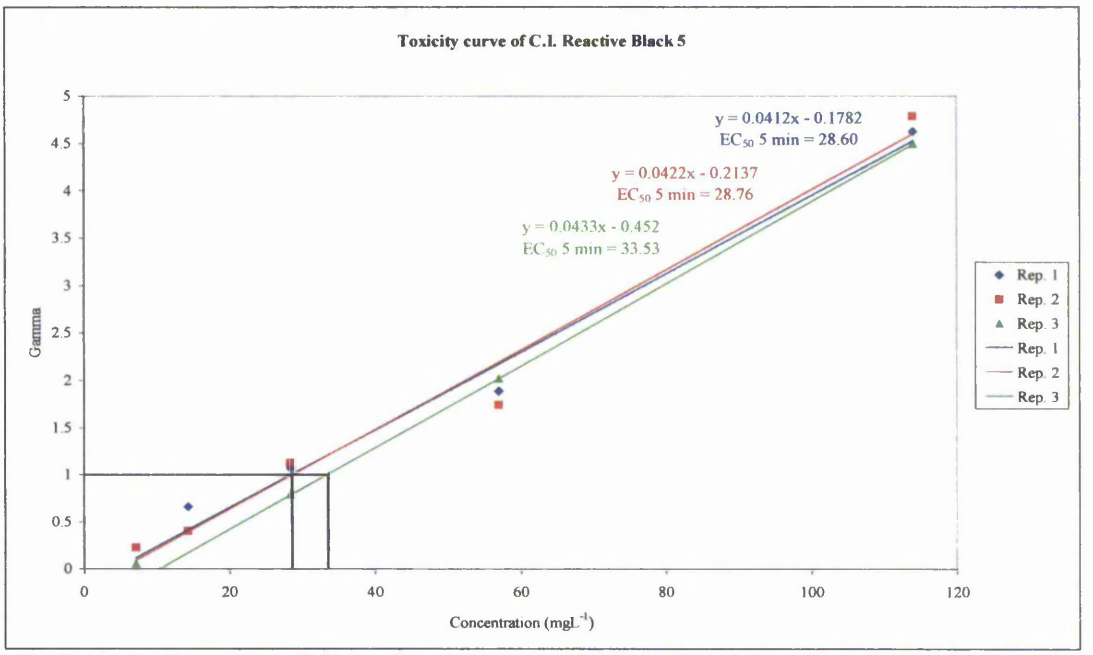
1. A vial of *V. fischeri* reagent was reconstituted as described in the initial screen protocol.
2. In the dilution row, 1 ml of Microtox® diluent (2% NaCl in Ultrapure water) was pipetted into 10 glass cuvettes (1 cm diameter). In two separate assay rows, 500 µl of diluent was pipetted into a total of 20 cuvettes (10 cuvettes in each row).
3. After any requisite initial dilution, 1 ml of sample was added first cuvette in the dilution row and aspirated 3 times. Using the same tip, 1 ml of this dilution was transferred to the next cuvette and aspirated 3 times. This was carried out 9 times, with the final cuvette left as a control.
4. All cuvettes were left to equilibrate in the instrument at 15°C for 15 minutes.
5. Using a small volume repeat pipettor, 10 µl of *V. fischeri* reagent was added to each cuvette and left to equilibrate in the instrument at 15°C for 15 minutes.
6. The initial light level (I_0) of a cuvette in the assay row was read by placing it in the light tight turret (connected to a photomultiplier tube and luminometer), and automatically recorded by the Microtox® analysis software.
7. Immediately, 500 µl of diluent was added. This served as a control cuvette.

8. After a pre-determined interval (ascertained by the instrument), the I_0 of the second cuvette in the assay row was read, and 500 µl of diluent immediately added. This served as a duplicate control cuvette.
9. After a pre-determined interval, the I_0 of the third cuvette in the assay row was read, and 500 µl of the lowest concentration of sample immediately added. This was a test cuvette for that concentration.
10. After a pre-determined interval, the I_0 of the fourth cuvette in the assay row was read, and again 500 µl of the lowest concentration of sample immediately added. This served as a duplicate for that concentration.
11. This process was repeated for all of the dilutions of the sample.
12. After exactly 5 minutes of contact time, as indicated by the software, the 5 minute light levels (I_5) of all cuvettes were read and automatically recorded by the Microtox® analysis software.
13. After exactly 15 minutes of contact time, as indicated by the software, the 15 minute light levels (I_{15}) of all cuvettes were read and automatically recorded by the Microtox® analysis software.
14. The effective concentration at which 50% of the total light is inhibited (EC_{50}) at the 5 and 15 minute contact times were calculated by the software, using the average value of the duplicate cuvettes to plot a toxicity dose-response curve. When highly coloured samples were tested, correction of the EC_{50} value to account for this was carried out as per the manufacturer's instructions (Azur Environmental 1995)

Examples of the dose-response curves produced for the model textile reactive azo dye RB can be seen in Figure 2.3, calculated by conversion of light levels into gamma (Γ) values. Γ is the ratio of light reduction at each sample concentration taking into account the natural light loss observed in the controls in which no toxicant was added.

Each Γ point was calculated from light levels after 5 minutes contact time between the *V. fischeri* cells and RB. Firstly, the ratio R_5 was calculated, which shows natural light reduction over 5 minutes in the control samples that did not have contact with RB. Secondly, Γ values were calculated, which shows light reduction over 5 minutes at each concentration of RB taking into account the R_5 value. The EC_{50} 5 minute value is calculated using the slope of the trendline produced by the dose-response curve, as shown in equations 1-3 below. The EC_{50} values for each of the compounds tested during this study were calculated by automatic production of dose-response curves by the instruments used. For presentation purposes, only EC_{50} values are presented and not the full dose-response curves.

Figure 2.4: Toxicity of the reactive azo dye RB calculated using gamma values



N.B. Rep. 1, Rep. 2 and Rep. 3 refer to replicates of independent experiments. Each sample was assayed in duplicate.

$R_5 = I_5/I_0$ **Equation 1**

(where I_0 and I_5 are light levels of the control at the start of the assay and after 5 minutes respectively)

$\Gamma = ((R_5 \times I_0)/ I_5) - 1$ **Equation 2**

(where I_0 and I_5 are light levels of the sample at the start of the assay and after 5 minutes respectively)

EC_{50} 5 min = (y-c)/m **Equation 3**

(where y = Γ value of 1, c = intercept on y axis and m = slope, derived from y = mx+c)

It must be noted that, whilst the EEC Council Directive 79/831 (1979) specifies unequivocal limits for the classification of 'very toxic', 'toxic' and 'harmful' substances (Anliker *et al.* 1988), these limits apply to LD_{50} data for acute rodent assays. The use of EC_{50} values obtained from short-term microbial bioassays has not yet been standardised, and thus classification of a substance as toxic or non-toxic can only be arbitrary. Various levels have been previously utilised with bioluminescent toxicity assays as the non-toxic cut-off point. Using pure compounds, where the concentration of the suspected toxicant is known, an EC_{50} value of $\geq$100 mgL^{-1} has been used to indicate non-toxicity (Thomulka *et al.* 1993). Using complex solutions or solutions where the concentration of the suspected toxic agent is unknown, a dilution factor (G value) of $\leq$2 has been used to indicate non-toxicity (Dannenberg 1996), which equates to the highest concentration of sample possible to test in the Microtox® assay (i.e. 50% of original concentration) due to the need to dilute the sample 1:2 with bacterial cells in diluent. Therefore in this study, pure compounds with

EC_{50} values of ≥100 mgL^{-1} and bioreactor samples or other complex solutions (where the concentration of suspected toxicant is unknown) with EC_{50} values of ≥50% were considered to be effectively non-toxic.

2.1.7 Genotoxicity assessment using the *E.coli* Differential Kill assay

Assessment of genotoxicity was measured by observing the difference in survival of a wild type and a DNA repair deficient mutant strain of *Escherichia coli* after exposure to a suspected toxicant. Bacterial numbers were estimated using the Don Whitley Rapid Automated Bacterial Impedance Technique (RABIT). This assay is based on a method described by Tweats and co-workers in 1981, and was modified for use on the RABIT by Forsythe in 1990. Whitley Impedance Broth (WIB), specifically designed by Don Whitley Ltd. as a medium that will allow changes in conductance due to bacterial utilisation of the medium components, was used in the RABIT as it has been reported to be the best basal medium for detecting the variations in impedance caused by bacterial growth (Salvat *et al.* 1997). The advantages of using the *E.coli* Differential Kill assay to detect genotoxicity, in combination with bacterial impedance, are discussed in section 1.4.6. The strains of *E. coli* utilised in this assay were *E. coli* WP2, which is a DNA repair proficient wild type (Bridges *et al.* 1972), and *E. coli* CM871, which is a DNA repair deficient *uvrA lexA recA* triple mutant (Tweats *et al.* 1981). The *uvrA* mutation means that the cell is incapable of removing damaged DNA by excision repair, which may be caused by UV exposure (Lehmann 2000). The *recA* mutation means that the cell is deficient in damage inducible repair, where protein-protein repair of double stranded breaks is disabled (Shinohara & Ogawa 1995). Organisms containing the *recA* mutation will detect a different spectrum of genotoxins than *uvrA* mutants, due to the difference in repair mechanisms (Tweats *et al.* 1984). The *recA* mutation does mean that cells are slow growing, but this can be overcome by incorporation of the *lexA* mutation (Tweats *et al.* 1984). It was noted that growth of cells to stationary phase in liquid based tests increases the resistance of repair proficient and *recA* deficient strains to DNA damage (Rosenkranz & Leifer 1980), and thus exponential phase cells were used.

To confirm that CM871 had retained its DNA repair mutations, a UV test was carried out in conjunction with each assay. The protocol is summarised below.

1. Either a broth culture of the *E. coli* strains WP2 and CM871, or a turbid suspension of a colony resuspended in 300 µl sterile 0.85% saline, was used.

2. A drop of each culture/suspension was placed at one edge of a dry tryptone soya agar (TSA) plate using a Pasteur pipette.
3. The plate was tilted slightly so that the drops ran in a straight line across to the other side of the plate, ensuring that they did not mix. A drop of culture/suspension was used as opposed to a streak from a wire loop to ensure that bacterial numbers were not significantly reduced across the plate by the action of streaking.
4. After the lines of culture/suspension had dried, the plate was placed under a UVc lamp with the lid removed but covered with cardboard.
5. The cardboard was repositioned to reveal one third of the plate containing lines of both strains, which was then exposed to UVc for 10 sec.
6. Immediately, the cardboard was repositioned to reveal a further third of the plate, which was then exposed to UVc for a further 10 sec.
7. Immediately, the plate was completely covered and removed, and then incubated at 37°C overnight.
8. The wild type WP2 strain grows at all exposure periods (0, 10 and 20 sec), whilst the mutant CM871 strain will not remain viable at exposure periods of 10 and 20 sec. Growth at 0 sec confirms the viability of the CM871 culture.

Before genotoxicity assessment could be carried out using the impedance detection technique, calibration curves had to be constructed using the RABIT in order that detection times produced by the RABIT could be equated to viable cell numbers (see Appendix 3). The indirect impedance technique was utilised, the protocol for which is summarised below (adapted from Forsythe 1990).

1. Broth cultures of *E. coli* strains WP2 and CM871 were obtained by static incubation of a colony in 10 ml tryptone soya broth (TSB) at 37°C overnight.
2. Exponential phase precultures were then obtained by static incubation of 1 ml of overnight culture in 10 ml TSB at 37°C for 1 and 2 h (WP2 and CM871 respectively).
3. Cell densities were measured by absorbance at 650 nm, and were used if within 0.2 AU of each other.
4. The cultures were serially diluted 10-fold in sterile 0.85% saline to 10^{-8}.
5. Duplicate aliquots of 200 µl of each dilution were pipetted into sterile glass tubes (3 × ½").
6. Sterile 0.85% saline (100 µl) was added to each tube.
7. Tubes were incubated at 37°C for 30 minutes, after which 2 ml Whitley Impedance Broth (WIB) was added.
8. A sufficient quantity of molten 2% Agar No.1 was mixed 1:1 with freshly prepared 1% potassium hydroxide (KOH).
9. Aliquots of 750 µl of the KOH-agar were pipetted into the base of sufficient numbers of conductance cells, one per glass tube, ensuring that none solidified on the sides and left to set.
10. The caps of the glass tubes were removed and the tubes placed inside the conductance cells, which were stoppered with silicon bungs.
11. The conductance cells were immediately connected to the RABIT instrument and the assay commenced. The instrument parameters used in each assay were temperature 37°C, stabilisation period 1 h, test duration 24 h and impedance readings every 6 minutes. The detection criterion used was a -20 µS change in impedance for 3 consecutive readings.

12. Volumes of 100 µl of each dilution were spread onto TSA plates in duplicate, then incubated at 37°C overnight.
13. Viable cell counts (in cfu ml^{-1}) were obtained from the spread plates and compared to the detection times obtained from the RABIT.
14. Calibration curves of cell numbers versus detection time constructed by the RABIT software were used as a reference in subsequent experiments.

Decolourised dyes were prepared appropriately (see sections 2.1.3, 2.1.4 and 2.1.5) and assayed using the protocol summarised below. It should be noted that assays were carried out using supernatants of samples centrifuged in a benchtop microfuge at 13000 rpm (~18000 *g*) for 5 minutes, to reduce the levels of the organisms used to decolourise the dyes. Filtration of the samples was not practical due to the high cell densities involved. In addition, reactive dyes such as RB bind to cellulose filters. The intermediate compounds that form part of the decolourised samples were largely unknown, and the genotoxicity assay employed was designed to test whole sample genotoxicity, and thus it could not be ensured that any intermediate compounds would not have been removed from the samples during filtration using either cellulose or polyvinyl acetate filters. Therefore, in order to determine the effect of any remaining contaminating organisms on the genotoxicity assay, a sample blank (sample supernatant and sterile 0.85% saline) was included to indicate the level of microbial contamination contributed by the supernatants. In all assays, the detection times of the contaminating organisms in the blanks were occurring at least 1 hour after the detection of the test organisms in assays using *Enterococcus faecalis* for decolourisation and >21 hours after the detection of the test organisms in assays using *Clostridium butyricum* for decolourisation. Examples of common detection times provided by the sample blanks with reference to sample tests can be seen in Appendix 4. Therefore, although it is appreciated that centrifugation did not remove all of the contaminating organisms from the decolourised samples, the test organisms in the genotoxicity assay were always present in higher numbers than the contaminating organisms and thus the results produced were a measurement of the growth of the test organism.

1. Exponential phase precultures of *E. coli* strains WP2 and CM871 were obtained as in steps 1-3 of the previous protocol.
2. The cultures were diluted to 10^{-1} in sterile 0.85% saline, which gave a rapid detection time that was within the limits of detection.
3. Aliquots of 200 µl of the diluted culture were pipetted into sterile glass tubes, in sufficient number to test each sample and negative control in triplicate.
4. Triplicate aliquots of 100 µl of each sample were added to the assay tubes, and 100 µl volumes of sterile 0.85% saline were added to the negative control tubes.

5. Sterility control tubes were set up for every assay using 300 µl sterile 0.85% saline, to ensure the sterility of the saline and WIB.
6. Blank tubes were set up for every assay using 100 µl sample and 200 µl sterile 0.85% saline, to determine the microbial contamination contributed by the samples.
7. The assay was performed as in steps 7-11 of the calibration curve protocol. The RABIT instrument parameters used in each assay were temperature 37°C, stabilisation period 1 h, test duration 24 h and impedance readings every 6 minutes. The detection criterion used was a -20 µS change in impedance for 3 consecutive readings.
8. Viable cell numbers (in cfu ml^{-1}) of each strain were determined by the RABIT by reference to the calibration curves previously constructed.

The data obtained from the RABIT needed analysis to assign a value of genotoxic potential to a sample. The detection times produced by the RABIT are dependent upon the number of viable bacterial cells present at the commencement of incubation within the RABIT instrument. The number of viable bacterial cells present at the commencement of incubation within the RABIT is dependant upon the effect of the suspected genotoxicant under examination, as cells are incubated with the suspected genotoxicant for 30 minutes at 37°C prior to incubation within the RABIT. The percentage of surviving cells for each *E. coli* strain can be calculated by comparing the number of viable cells in the assay with the number in the negative control which had no exposure to a suspected genotoxicant. This data can then be converted into a ratio of survival of the repair deficient (CM871) versus the repair proficient (WP2) strains, called the Coefficient of Survival (CS). CS values below 1.0 indicate preferential killing of the DNA repair deficient strain, and thus the sample can be considered a genotoxicant. Within the limits of the assay, differing CS values can be used to categorise the genotoxic potential of a compound: ≤0.30 = genotoxic, 0.31-0.95 = weakly genotoxic, ≥0.96 = non-genotoxic (Rosenkranz *et al.* 1981).

$$\% \text{ survivors} = \frac{\text{No. cells in test (cfu ml}^{-1}\text{)}}{\text{No. cells in control (cfu ml}^{-1}\text{)}} \times 100$$

$$CS = \frac{\% \text{ survivors repair deficient strain}}{\% \text{ survivors repair proficient strain}}$$

(Rosenkranz & Leifer 1980)

2.2 Bioreactors

2.2.1 Simulated textile effluent

It has been discussed that the wastewaters of textile dyehouses are extremely variable, in terms of colour concentration and type, auxiliary chemical concentration and type and pollution load (see section 1.3.2). The purpose of this project was to

determine the optimal conditions (in terms of bioreactor design and operation and bioflora composition) for the safe decolourisation of textile azo dyes (in terms of toxicity, genotoxicity and reduction of in/organic matter). In order the effects on any one of these parameters may be directly linked to a possible cause or causes, the composition of the bioreactor influent must be the same throughout the entire run of the experiment. In order to achieve this, the typical composition of a textile dyehouse wastewater was studied by C. Shaw (Loughborough University of Technology). It was found that the main components of a waste effluent would be dye, sizes and alkalis from a dyehouse using batch exhaustion dyeing (a commonly used process) with reactive dyes (a commonly used group) (Shaw 2000). Sizes such as starch, polyvinyl alcohol (PVOH) and carboxymethyl cellulose (CMC) are released during the desizing process in high concentrations, and are the highest contributor to COD in the effluent (~30 $gCODkg^{-1}$ fabric) (Shaw 2000). Reactive dyes are most often found in dyehouse wastewater in their hydrolysed form due to their low fixation rates and high levels of usage, especially RB (see sections 1.2.1 and 1.2.2). Reactive dyes also require high levels of alkalis, especially NaCl and NaOH, to improve fixation of the dye (see section 1.3.3). Therefore, a simulated textile effluent (STE), which served as a feed for the bioreactors, was devised to represent a typical dyehouse wastewater, the composition of which could be kept constant throughout the experimental period. The STE included nutrients and trace elements required for bacterial growth, the commercially available RB dye known as Remazol Black B (hydrolysed by boiling under reflux with sodium hydroxide for 3 h), potato starch, PVOH (M_w 28000) and CMC (sheared in a blender and boiled for 20 min to simulate conditions under which sizes are applied to yarn), and alkalis such as NaCl, NaOH and Na_2CO_3 (Shaw 2000). The main chemical properties and constituents of the STE can be seen in Table 2.1, whilst the full chemical composition can be seen in Appendix 1.

Table 2.1: Chemical properties and constituents of the STE

Property/Constituent	STE with Dye	STE without Dye
hydrolysed Remazol Black B (mgL^{-1})	533.3	0
COD ($mg\ O_2L^{-1}$)	3916	3547
Starch (mgL^{-1})	2670	2670
PVOH (mgL^{-1})	400	400
CMC (mgL^{-1})	270	270
pH	9.38 ± 0.05	9.44 ± 0.05
NaCl (%)	2.67	2.67

(Adapted from Shaw 2000)

2.2.2 Design and operation of the Sequencing Batch Reactor

Two types of bioreactor (referred to as rig) were designed and operated by C. Shaw of Loughborough University of Technology, Department of Civil Engineering, as part of the joint project by which this project was funded. The aim of this investigation was to compare the performance of two types of single vessel bioreactors, one batch flow and the other continuous flow, to ultimately mineralise a reactive azo dye and other compounds associated with textile dyeing. The first type of reactor operated was a Sequencing Batch Reactor (SBR), which is a single tank reactor operating on a fill and draw system (Irvine *et al.* 1997). A cross sectional diagram of the SBR can be seen in Figure 2.5. The volume of the unit was 10.5 L, the STE and effluent flow rates were 4 L day^{-1} and the HRT was 2.6 days.

Two types of operating cycle were utilised; an anaerobic cycle (consisting of fill, anaerobic reaction, settle, decant and idle phases of 0.25, 19, 4, 0.25 and 0.5 h respectively), and an anaerobic-aerobic cycle (consisting of fill, anaerobic reaction, aerobic reaction, settle, decant and idle phases of 0.25, 18.5, 0.5, 4, 0.25 and 0.5 h respectively). The SBR was mixed by stainless steel paddles at 40 rpm during both reaction phases, and aerated at 5 $Lmin^{-1}$ during aerobic reaction phases using a coarse bubble diffuser to prevent blocking during settling phases. There are three stages of operation in the SBR:

1) after the fill phase, when neither dissolved oxygen (dO_2) nor nitrate (NO_3) are available as electron acceptors, called anaerobic.

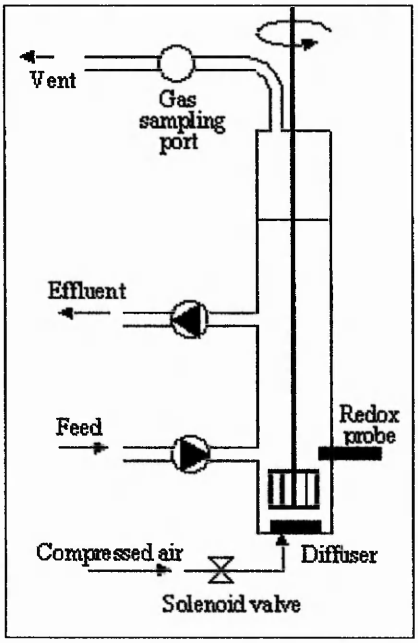
2) during aeration, when dO_2 increases and ammonia is oxidised to NO_3 by nitrifying bacteria, called aerobic.

3) after aeration, when dO_2 is depleted and NO_3 is reduced to nitrogen gas by denitrifying bacteria, called anoxic.

Stage 3 only occurs after stage 2 has been completed, and so the anaerobic cycle will consist of only stage 1 whereas the anaerobic-aerobic cycle will consist of all stages. Two SBRs were run in parallel, the experimental rig (Rig 1) which had the hydrolysed reactive azo dye Remazol Black B (commercial name of RB) included in the STE, and the control rig (Rig 2) which contained all STE components except dye (for full composition see Appendix 1). The organic loading rate (OLR) was 1.49 and 1.35 $kgCODm^{-3}day^{-1}$ for Rigs 1 and 2 respectively. The SBRs were operated at 38 ± 1°C for a total of 244 days, and the STE was also stored at this temperature for a maximum of 72 h to prevent cold shock to the biomass. The main stages of operation for both Rigs 1

and 2 are summarised in Table 2.2, whilst a detailed description of operational changes or events is given in Appendix 2.

Figure 2.5: Design of the SBR



(Shaw 2000)

Table 2.2: Operational stages for SBRs

Day	Operation Stage Symbol	Rig 1 Description	Rig 2 Description
0 - 124	I	533 mgL^{-1} dye, anaerobic	0 mgL^{-1} dye, anaerobic
125 - 137	II	533 mgL^{-1} dye, introduction of air	0 mgL^{-1} dye, introduction of air
138 - 199	III	533 mgL^{-1} dye, anaerobic	0 mgL^{-1} dye, anaerobic-aerobic
200 - 207	IV	533 mgL^{-1} dye, anaerobic	introduction of dye, anaerobic-aerobic
208 - 222	V	533 mgL^{-1} dye, introduction of air	introduction of dye, anaerobic-aerobic
223 - 244	VI	533 mgL^{-1} dye, anaerobic-aerobic	533 mgL^{-1} dye, anaerobic-aerobic

(Shaw 2000)

It can be seen from Table 2.2 that stage III corresponds to conversion of Rig 1 back to anaerobic operation. This is because Rig 1 began to fail with the increasing introduction of air, as shown by a reduction in total organic carbon (TOC) removal efficiency (Shaw *et al.* 2002). Once Rig 1 had again stabilised under anaerobic operation, aeration was reintroduced at a slower rate (stage V), but TOC removal again decreased during anaerobic-aerobic operation (stage VI). To verify that the dye was

causing reactor failure under anaerobic-aerobic conditions, dye was introduced to Rig 2 during stages IV and V until the same dye concentration was included in the STE as that used for Rig 1. A decrease in TOC removal during stages IV and V and then failure during stage VI was mainly attributed to the dye (Shaw *et al.* 2002). This is discussed further in section 4.2.2.

Both Rigs 1 and 2 were seeded with anaerobic granules taken from a UASB (Upflow Anaerobic Sludge Bed) reactor treating high starch wastewater, with a volatile solids concentration of 14.32 gL^{-1} each. The use of anaerobic granules, which are formed by the aggregation of anaerobic sludge within the upflow conditions of a UASB reactor (Razo-Flores *et al.* 1997*a*), allows the bioreactor to be operated under anaerobic-aerobic phase with the obligate anaerobes surviving the aerobic and anoxic phases. This is because anaerobic granules have a multi-layered structure, with syntrophic associations between an external layer consisting of facultative fermenters (hydrogens and acidogens), a layer of obligate anaerobic acetogens and methanogens, and an aceticlastic core consisting mainly of the slow growing obligate anaerobic rods *Methanosaeta* spp. (Guiot *et al.* 1992, Sacks 1998, Shaw 2000). This is a stable structure which enables different types of bacteria to thrive in oxygen concentrations that are not conducive to their survival, as the anaerobic bacteria are protected against inhibitory concentrations of oxygen within the granules (Razo-Flores *et al.* 1997*a*). A study by Bishop and Yu in 1999 showed that gradients of dO_2, redox potential and dissolved sulphide occur in biofilms, with aerobic conditions at the surface and anaerobic conditions at a depth of $\geq$ 0.75 mm. Therefore, if large enough granules were used to seed the SBRs, the constituent bacteria could survive all phases and thus the reactors could function effectively under anaerobic-aerobic conditions.

2.2.3 Design and operation of the Hybrid Anaerobic Baffled Reactor

The second type of reactor operated was a Hybrid Anaerobic Baffled Reactor (HABR), which was a single unit operating on a continuous flow system. The HABR was divided into compartments (referred to as cells) by baffles, and so the different oxygen conditions can be separated spatially as opposed to temporally which is the system used in the SBR. A cross sectional diagram of the HABR can be seen in Figure 2.6. Each compartment (cell) is divided into a downflow section (10 mm wide) by a hanging baffle, and an upflow section (35 mm) wide by a standing baffle. The tips of the hanging baffles are angled at 45° to direct the flow to the centre of the upflow

section, which can be seen in Figure 2.6. Figure 2.6 also shows the HABR with 8 cells, but the reactor was designed so that extra cells could be added by the use of threaded rods that allow the final cell with the outlet pipe to be removed and further cells added before the final cell is replaced. Cells were numbered sequentially, with cell 1 at the beginning of the HABR and cell 8 at the end. Not shown in Figure 2.6 is that fact that the inlet was attached to the reactor via a drip, so that the air space would prevent biological contamination of the STE, and the outlet pipe contained a U-tube to keep the reactor air tight. The volume of the unit with 8 cells was 9 L, and with 10 cells was 11.25 L.

Three types of operating conditions were used:

1) anaerobic only, where STE was allowed to flow through the cells of the HABR.

2) anaerobic-aerobic, where a number of anaerobic cells were included at the beginning of the HABR and then a number of aerated cells included at the end.

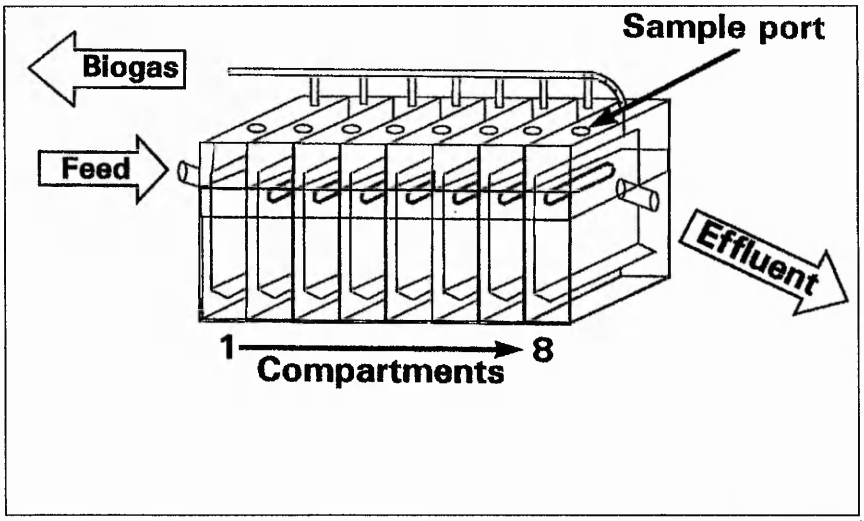
3) anaerobic-aerobic-anoxic, where a number of anaerobic cells were included at the beginning of the HABR, a number of aerated cells at the end, and then a recycle loop which took effluent from the end of the HABR back to an anaerobic cell (as ammonia is oxidised to nitrate by nitrifying bacteria during aerobic operation, this environment is now anoxic).

As oxygen conditions can be separated spatially, each of these stages can be produced sequentially. Aeration was achieved by pumping air into the aerobic cells at 0.4 $Lmin^{-1}$ for each cell through a fine bubble diffuser, and a reticulated foam block was included in each cell to immobilise the biomass and thus improve retention. Biogas from each of the anaerobic cells was channelled away for analysis, whilst off gas from each of the aerobic cells was channelled away separately for venting to the atmosphere. Sampling ports were included in each cell so that individual measurement of parameters could be made. The recycle loop ran from cell 10 (aerobic) back to cell 5 (anaerobic) at a rate of 4.5 Lh^{-1}, with a recycled aerobic effluent to anaerobic effluent ratio of 1:1.

Two HABRs were run in parallel, the experimental rig (Rig A) which had the hydrolysed reactive azo dye Remazol Black B (commercial name of RB) included in the STE, and the control rig (Rig B) which contained all STE components except dye (for full composition see Appendix 1). The HABRs were operated at 38 ± 1°C for a total of 229 days, but the STE was stored at 4°C to increase storage life as no cold shock to the biomass would occur due to the lag stage created before the STE entered the HABRs by the drip. The main stages of operation for both Rigs A and B are summarised in Table

2.3, whilst a detailed description of operational changes or events is given in Appendix 2.

Figure 2.6: Design of the HABR



(Shaw 2000)

Table 2.3: Operational stages for HABRs

Day	Operation Stage Symbol	Rigs A & B Description	Rigs A & B O_2 conditions & n° cells (HRT h)	OLR (kgCOD $m^{-3}day^{-1}$)	
				Rig A	Rig B
0 – 43	a	Cells 1-7 seeded	8 anaerobic (72)	1.31	1.18
44 – 76	b		8 anaerobic (48)	1.96	1.77
77 – 111	c		7 anaerobic (42) 1 aerobic (6)	1.96	1.77
112 – 154	d		7 anaerobic (42) 3 aerobic (18)	1.57	1.42
155 – 203	e	Cells 8-10 seeded	7 anaerobic (42) 3 aerobic (18)	1.57	1.42
204 - 229	f		4 anaerobic (24) 3 anoxic (18) 3 aerobic (18)	1.57	1.42

(Shaw 2000)

It can be seen from Table 2.3 that the OLR was consistently higher for Rig A than B, which was due to the chemical influence of the dye. As the HRT decreased the OLR increased, although these are still fairly low OLRs for an HABR as they have been known to be sustainable at an OLR of up to 36 $kgCODm^{-3}day^{-1}$ in the laboratory (Shaw 2000).

Both Rigs A and B were seeded with anaerobic sludge taken from Loughborough wastewater treatment works, with a volatile solids concentration of 17.4 gL^{-1} each. However, only cells 1-7 were seeded leaving cell 8 empty, as it was

considered that there would be high washout of the biomass before the HABRs settled to steady state operation, and thus cell 8 would provide a 'settling tank' to prevent a high concentration of suspended solids in the effluent (Shaw 2000). During stages c and d, the aerobic cells were not seeded with any biomass. This was to allow the development of biofilms of facultative bacteria washed over from the anaerobic cells upon the reticulated foam. Whilst a small biofilm did gradually develop it was decided to seed the aerobic cells (8-10) with activated sludge taken from Loughborough wastewater treatment works, with a volatile solids concentration of 3.05 gL^{-1} in each rig, which constitutes stage e.

2.2.4 Preparation of bioreactor samples for assessment

Samples of STE and effluent from the reactors were collected by C. Shaw and prepared appropriately for testing using analytical techniques to measure TOC, suspended solids (SS), pH, biogas composition, alkalinity, polyvinyl alcohol (PVOH), oxidation reduction potential (ORP) and absorbance. To measure absorbance of the samples, the standard method recommended by the Environment Agency (formerly National Rivers Authority) was used (see section 1.3.2). Samples were filtered through a 0.45 μm polyvinylidene fluoride filter (textile dyes, especially reactive, can bind to cellulose acetate filters) and then measured in a spectrophotometer fitted with a diode array detector across the range 190-1100 nm at 1 nm intervals, using 1 cm silica cells. Initially samples were not measured immediately, but it was observed that the absorbance spectrum of a sample was unstable when left to oxidise in air for 3 h (~50% increase in the peak at 597 nm and almost total disappearance of the peaks at 754 and 972 nm). This is discussed further in sections 3.2.2 and 3.2.5. Therefore, measurement of absorbance was carried out immediately from day 62 onwards during SBR operation, and at all times during HABR operation. A calibration curve of absorbance at 597 nm (A_{597}), which is the λ_{max} of RB, showed A_{597} to be directly proportional to concentration of the dye. Therefore, as well as assessing absorbance over the 190-1100 nm range of wavelengths, the dye removal efficiency was calculated and expressed as an absorbance reduction at 597 nm. For full details of bioreactor sample analysis see Shaw 2000.

Samples were simultaneously obtained by the author for analysis in this project, and immediately prepared for toxicity and genotoxicity assessment. Sample pH values were adjusted to pH 7.0 ± 0.4 with 0.2 M HCl or NaOH. Toxicity testing was carried out on whole samples, as the use of bioluminescent bacteria requires no growth of the test organisms, and thus is a short-term assay (usually 15 min contact time). Toxicity

assessment with bioluminescent bacteria usually requires osmotic adjustment to 2% NaCl, as the test organism is a marine bacterium (see section 1.4.5). However, the STE used in the bioreactor experiments had a high NaCl content (2.67%, see Table 2.1) as would be common in wastewater effluents from textile dyeing with reactive dyes, therefore osmotic adjustment was unnecessary. Genotoxicity testing was carried out using supernatants of the samples after centrifugation at 4000 rpm in a bench centrifuge (~2700 *g*) for 20 min, transferred to a sterile tube under aseptic conditions (see section 2.1.7). Centrifugation was used as a means of reducing the levels of contaminating microorganisms within the samples, as the differential kill assay utilised requires growth of the test organisms. Filtration of these samples was not practical as extremely high pressures would need to be used, possibly due to the high levels of suspended solids or insoluble metal complexes. Therefore, in order to determine the effect of any remaining contaminating organisms on the genotoxicity assay, a sample blank (sample supernatant and sterile 0.85% saline) was included to indicate the level of microbial contamination contributed by the supernatants. In all assays, the detection times of the contaminating organisms in the blanks were occurring at least 3 hours after the detection of the test organisms. Examples of common detection times provided by the sample blanks with reference to sample tests can be seen in Appendix 4. Therefore, although it is appreciated that centrifugation did not remove all of the contaminating organisms from the bioreactor samples, the test organisms in the genotoxicity assay were always present in higher numbers than the contaminating organisms and thus the results produced were a measurement of the growth of the test organism.

2.2.5 Toxicity assessment

2.2.5.1 Microtox®

The assay used to determine toxicity of the SBR samples was the short-term bioluminescent Microtox® Acute Toxicity test (Azur Environmental Ltd.). After appropriate preparation (see section 2.2.4), samples were assessed exactly as in the protocols in section 2.1.6.

2.2.5.2 ToxAlert™ 100

The assay used to determine toxicity of the HABR samples was the short-term bioluminescent ToxAlert™ 100 Acute Toxicity test (Merck Speciality Chemicals Ltd.). This assay uses the same principle as the Microtox® test, in that cytotoxicity is assessed via a reduction in the luminescence of *Vibrio fischeri* cells (see section 1.4.5). As with

the Microtox® assay, the use of the marine organism *V. fischeri* requires assays to be carried out at 15°C using 2% NaCl as the diluent with samples osmotically adjusted to 2% NaCl, although osmotic adjustment was not necessary for the bioreactor samples (see Table 2.1). Liquid-dried cultures of *V. fischeri* were utilised instead of freeze-dried cultures, as were used in the Microtox® assays, and so a comparison of the reagent performance with reference toxicants was conducted in order that the SBR and HABR data could be directly compared (see Table 3.14).

After appropriate preparation (see section 2.2.4), HABR samples were tested according to the acute toxicity assay method specified by Merck Speciality Chemicals Ltd., which conforms to standards protocol EN ISO 11348, and is summarised below.

1. A vial of 12.5 ml of reconstitution solution, stored at -20°C, was defrosted at room temperature and kept in the reconstitution well of the instrument at 15°C for 10 minutes.
2. A liquid-dried vial of *V. fischeri* reagent, stored at -20°C, was defrosted for 2 minutes at room temperature.
3. The reagent was reconstituted by adding 500 µl reconstitution solution, using a wide bore pipette tip, and kept in the reagent well of the instrument at 15°C for 15 minutes.
4. After this time, approximately half of the reconstitution solution was poured into the reagent vial, swirled, and then replaced into the reconstitution solution bottle at 15°C.
5. The assay was then started immediately on the instrument, by which instruction on manipulation of reagents and samples at specific times was given.
6. In a dilution row, 1.5 ml of diluent (2% NaCl in Ultrapure water) was pipetted into 6 glass cuvettes (1 cm diameter).
7. After any requisite initial dilution, 1.5 ml of sample was added to the first cuvette in the dilution row and aspirated 3 times. Using the same tip, 1.5 ml of this dilution was transferred to the next cuvette and aspirated 3 times. This was carried out 5 times, with the final cuvette left as a control.
8. In two separate assay rows, 500 µl of reagent was pipetted into 12 glass cuvettes (6 cuvettes in each row).
9. All cuvettes were left to equilibrate in the instrument at 15°C for 15 minutes.
10. After exactly 15 minutes equilibration time, as indicated by the instrument, the initial light level (I_0) of the first cuvette in the top assay row was read by placing it in the light tight turret (connected to a photomultiplier tube and luminometer), and automatically recorded by the ToxAlert™ 100 instrument.
11. Immediately, 500 µl of diluent was added. This served as a control cuvette.
12. After a pre-determined interval (ascertained by the instrument), the I_0 of the first cuvette in the bottom assay row was read, and 500 µl of diluent immediately added. This served as a duplicate control cuvette.
13. After a pre-determined interval, the I_0 of the second cuvette in the top assay row was read, and 500 µl of the lowest concentration of sample immediately added. This was a test cuvette for that concentration.
14. After a pre-determined interval, the I_0 of the second cuvette in the bottom assay row was read, and again 500 µl of the lowest concentration of sample immediately added. This served as a duplicate for that concentration.
15. This process was repeated for all of the dilutions of the sample.

16. After exactly 5 minutes of contact time, as indicated by the software, the 5 minute light levels (I_5) of all cuvettes were read and automatically recorded by the ToxAlert™ 100 instrument.
17. After exactly 15 minutes of contact time, as indicated by the software, the 15 minute light levels (I_{15}) of all cuvettes were read and automatically recorded by the ToxAlert™ 100 instrument.
18. The effective concentration at which 50% of the total light is inhibited (EC_{50}) at the 5 and 15 minute contact times were calculated by the software, using the average value of the duplicate cuvettes to plot a concentration dependant toxicity curve. Information on the standard deviation of the duplicate cuvettes and the ISO compliance of the data was also given. When highly coloured samples were tested, correction of the EC_{50} value to account for this was carried out as per the manufacturer's instructions.

2.2.6 Genotoxicity assessment using the *E.coli* Differential Kill assay

The assay used to determine genotoxicity of the SBR samples was the short-term DNA repair test the *E. coli* Differential Kill assay. After appropriate preparation (see section 2.2.4), samples were assessed exactly as in the protocols in section 2.1.7.

2.3 Bioreactor microflora

2.3.1 Sampling and storage of bioreactor microflora

The sludge used to seed the SBRs consisted of anaerobic granules taken from a UASB reactor treating high starch wastewater (volatile solids concentration 14.32 gL^{-1}) (see section 2.2.2). The sludge used to seed the first 7 compartments (cells) of the HABRs consisted of anaerobic sludge taken from Loughborough wastewater treatment works (volatile solids concentration 17.4 gL^{-1}), whilst that used to seed cells 8-10 (under aerobic operation) consisted of activated sludge taken from Loughborough wastewater treatment works (volatile solids concentration 3.05 gL^{-1}) (see section 2.2.3). Approximately 10 ml samples of seed sludges were used to examine the initial populations in each of the bioreactors, and then similar samples from each of the experimental and control bioreactors (referred to as rigs) were taken at ~1 month intervals or as dictated by changes in operational parameters. Sludges were collected separately from the beginning, middle and end of the HABRs (cells 1, 4 and 7), and also from aerobic cells 8 and 10 as added (for an explanation of cell numbering and operational parameters, see section 2.2.3). Only a single sample for each time point was analysed. However, a study by Curtis & Craine 1998 showed no evidence of spatial, diurnal or intrasample variation in a number of activated sludge plants as assessed by DGGE, and concluded that a single sample was sufficient for a plant to plant comparison. Thus a single sludge sample should be sufficient to describe the microflora

at each time point. All sludge samples were stored in 20% sterile glycerol (5 ml final volume) at –20°C. Tables 2.5 and 2.6 list all of the sludge samples used for population analysis throughout this study, listing the dates and days of operation, and the main operational conditions they were subject to which are detailed in Appendix 2.

Table 2.4: Sludge samples from the SBR utilised for population analysis, showing main operational parameters

Date sludge taken	Day of operation (stage symbol)[a]	Atmospheric conditions (amount of air added)[b]		Conc. hydrolysed Remazol Black B in STE (mgL^{-1})[c]	
		Rig 1 (experimental)	Rig 2 (control)	Rig 1 (experimental)	Rig 2 (control)
23.04.98 (seed)	0 (I)	-	-	-	-
19.05.98	26 (I)	anaerobic	anaerobic	533	0
30.06.98	68 (I)	anaerobic	anaerobic	533	0
04.08.98	103 (I)	anaerobic-aerobic (5 min)	anaerobic	533	0
18.08.98	117 (I)	anaerobic	anaerobic	533	0
10.09.98	140 (III)	anaerobic	anaerobic-aerobic (30 min)	533	0
12.10.98	172 (III)	anaerobic	anaerobic-aerobic	533	0
03.11.98	194 (III)	anaerobic	anaerobic-aerobic	533	0
17.11.98	208 (V)	anaerobic-aerobic (5 min)	anaerobic-aerobic	533	53
17.12.98	238 (V)	anaerobic-aerobic (30 min)	anaerobic-aerobic	533	533
22.12.98 (rig failing)	243 (V)	anaerobic-aerobic	anaerobic-aerobic	533	533
23.12.98 (rig failing)	244 (V)	anaerobic-aerobic	anaerobic-aerobic	533	533

N.B. [a]Stage symbol refers to operational stage symbol (see Table 2.2). [b]Air added at 5 $Lmin^{-1}$, each condition refers to the subsequent condition unless otherwise stated. [c]STE added at 4 $Lday^{-1}$.

Table 2.5: Sludge samples from the HABR utilised for population analysis, showing main operational parameters

Date sludge taken	Day of operation (stage symbol)[a]	Cell number	O_2 conditions[b]
21.04.99 (seed cells 1-7)	0 (a)	-	-
25.05.99	34 (a)	1	anaerobic
		4	anaerobic
		7	anaerobic
15.06.99	55 (b)	1	anaerobic
		4	anaerobic
		7	anaerobic
26.08.99	127 (d)	1	anaerobic
		4	anaerobic
		7	anaerobic
23.09.99 (seed cells 8-10)	-	-	-
28.10.99	190 (e)	1	anaerobic
		4	anaerobic
		7	anaerobic
		8	aerobic
24.11.99	217 (f)	1	anaerobic
		4	anaerobic
		5	anoxic
		7	anoxic
		8	aerobic
07.12.99 (sludge)[c]	230 (f)	1	anaerobic
		4	anaerobic
		5	anoxic
		7	anoxic
07.12.99 (biofilm)[c]	230 (f)	1	anaerobic
		4	anaerobic
		5	anoxic
		7	anoxic
		8	aerobic
		10	aerobic

N.B. [a]Stage symbol refers to operational stage symbol (see Table 2.3). [b]Aerobic conditions created by continuous aeration at 0.4 $Lmin^{-1}$, anoxic conditions by recycling aerobic effluent back to anaerobic stage at 1:1 ratio. [c]Sludge samples collected from settled sludge in cells, biofilm samples collected from biofilm lining cell above liquor level. Concentration of hydrolysed Remazol Black B in STE for all samples was 533 mgL^{-1} for Rig A (experimental) and 0 mgL^{-1} for Rig B (control), STE added dropwise at ~3 $Lday^{-1}$.

2.3.2 Chemicals and media used in traditional culturing techniques

The microflora present in the bioreactor sludge samples were assessed for cellular morphology and Gram staining reaction, using standard microscopy and staining techniques. Total cell counts under aerobic and anaerobic conditions were also obtained by using duplicate spread plates incubated at 37°C for 72 h in a standard CO_2 incubator using Tryptone Soya Agar (TSA) (it should be noted that this medium was not expected to give 100% recovery of the microflora), and in a Don Whitley Scientific

anaerobic cabinet using Schaedler Anaerobic Agar (SAA) (see section 2.1.1). The number of different colonial types was also observed. Samples were diluted 1:5 in sterile 0.85% saline for Gram staining, and 10-fold dilutions were used for culturing down to 10^{-9} dilution.

In addition to total cell counts and colonial types, HABR samples taken on day 55 were assessed for the ability of isolated colonies to decolourise RBOH. TSA and SAA plates were made with the addition of 0.1 mM final concentration of RBOH that had been sterilised by boiling for 10 minutes. Isolated colonies from TSA and SAA were inoculated onto a section of a 5×6 grid on their respective dye-containing plates using a sterile needle, and then reincubated under the same conditions (37°C, aerobic and anaerobic respectively) for 24 h. Plates were then assessed visually for decolourisation of RBOH, as seen by an alteration of the medium from dark blue/black to pale purple/yellow. Selected colonies, including those with decolourising ability, were also identified using biochemical techniques (see section 2.3.3).

2.3.3 Biochemical identification of bioreactor microflora

Selected colonies from samples of HABR sludge taken on day 55 were identified using primary and secondary identification tests. The primary tests used were Gram staining reaction, cellular shape, arrangement and motility, oxygen requirement and possession of oxidase and catalase enzymes. Based upon the results of these primary identification tests, organisms could be assigned the group or genus name *Staphylococcus*, *Micrococcus*, *Pseudomonas*, Enterobacteriacae, *Bacillus* or *Clostridium* spp. (according to Bergey's Manual of Systematic Bacteriology, 1989). Secondary tests took the form of API strips (Biomeriůx) to identify the organisms to genus or species level. The choice of API strip used was based on the identification made by the primary tests, as shown in Table 2.6. All anaerobic isolates required secondary testing by API rapidID 32A, and were subcultured onto blood agar before inoculation into the API strip. Blood agar was made using 2.3% bacteriological peptone, 0.1% starch, 0.5% sodium chloride and 2% Oxoid Agar No. 1 (pH 7.3±0.2), autoclaved at 121°C for 15 min, and 5% defibrinated horse blood was added after the agar had cooled before pouring the plates.

Table 2.6: Primary tests to identify organisms and determine type of API strip used for secondary tests

Test	*Staphylococcus* sp. (API STAPH)	*Micrococcus* sp. (API STAPH)	*Pseudomonas* sp. (API 20NE)	Enterobacteriacae (API 20E)	*Bacillus* sp. (API rapidID 32A)	*Clostridium* sp. (API rapidID 32A)
Gram	+ve	+ve	-ve	-ve	+ve	+ve
Shape	cocci	cocci	rod*	rod	rod	rod
Arrangement	clusters, pairs	clusters, tetrads	-	-	single, chains	single, chains, pairs
Motility	-ve	-ve	+ve*	-ve*	+ve	+ve
Oxygen requirement	facultative anaerobe	aerobe	aerobe	facultative anaerobe	facultative anaerobe, aerobe	anaerobe
Oxidase	-ve	+ve	-ve*	-ve	-ve*	-ve
Catalase	+ve	+ve	+ve	+ve	+ve	-ve

N.B. * = not all species give this result.

2.3.4 Fluorescent *in situ* Hybridisation analysis of bioreactor microflora

Before commencing the Fluorescent *in situ* Hybridisation (FISH) assays, sludge samples taken from the bioreactors required fixing and appropriate storage in order that the cells would be amenable to hybridisation. The methods of sample fixation given below are modified from Amann *et al.* 1995, with grateful acknowledgement to Dr. J. Plumb of the Dept. of Chemical Engineering and Chemical Technology, Imperial College of Science, Technology and Medicine.

2.3.4.1 Fixation of Gram negative organisms

Reagents used: 1× phosphate buffered saline (PBS) solution is 2.58 gL^{-1} disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$) + 0.44 gL^{-1} sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$) + 7.54 gL^{-1} NaCl, pH 7.2. Paraformaldehyde solution is 4% paraformaldehyde in 1×PBS, pH 7.2.

1. Paraformaldehyde solution was prepared by heating 65 ml Ultrapure water to 60°C.
2. 4 g of paraformaldehyde was added to the heated water, stirring continuously.
3. The solution was clarified by adding 1 drop of 2 M NaOH.
4. The clarified solution was removed from the heat, and 33 ml 3×PBS was added.
5. pH was adjusted to 7.2 using 1 M HCl.

6. The solution was filtered through a 0.2 μm cellulose filter to remove any paraformaldehyde crystals, and then stored at 4°C.
7. Bioreactor sludge/pure culture broth samples (500 μl) were placed into screw-capped tubes, and 1.5 ml of paraformaldehyde solution was added and mixed gently by inversion.
8. The sludge samples were stored at 4°C for 1 h.
9. Tubes were then centrifuged at 18000 *g* in a microfuge for 5 min.
10. The supernatant was discarded and the pellet washed by adding 1 ml 1×PBS and shaking vigorously.
11. Tubes were then centrifuged at 18000 *g* in a microfuge for 5 min.
12. The supernatant was discarded and 500 μl 1×PBS and 500 μl ice cold ethanol (98%) was added to reconstitute the cells to a concentration of 10^8-10^9 cells ml^{-1}.
13. Fixed samples were stored at -20°C until required.

2.3.4.2 Fixation of Gram positive organisms

Reagents used: 1× phosphate buffered saline (PBS) solution is 2.58 gL^{-1} disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$) + 0.44 gL^{-1} sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$) + 7.54 gL^{-1} NaCl, pH 7.2.

1. Bioreactor sludge/pure culture broth samples (500 μl) were placed into screw-capped tubes, and 500 μl ice cold ethanol (98%) was added
2. The sludge samples were stored at 4°C overnight.
3. Tubes were then centrifuged at 18000 *g* in a microfuge for 5 min.
4. The supernatant was discarded and 500 μl 1×PBS and 500 μl ice cold ethanol (98%) was added to reconstitute the cells to a concentration of 10^8-10^9 cells ml^{-1}.
5. Fixed samples were stored at –20°C until required.

2.3.4.3 Preparation of Fluorescent *in situ* Hybridisation slides and application of samples

Samples consisted of ~5 ml of either sludge taken from the settled sludge layer in the bioreactors or biofilm scraped from the perspex side walls of the HABRs above the liquor level. Samples were fixed using the protocols for both Gram negative (see section 2.3.4.1) and Gram positive (see section 2.3.4.2) organisms. In addition, pure cultures of organisms were analysed to provide positive controls for the probes used. The Gram negative rod *Escherichia coli*, a member of the γ subgroup of the proteobacteria, was grown in Tryptone Soya Broth to ~10^6 cell ml^{-1}, fixed using the protocol in section 2.3.4.1, and used as representative of the domain Bacteria. The Gram negative irregular rod *Haloferax denitrificans* was grown in Payne, Seghal and Gibbons Medium to ~10^6 cell ml^{-1}, fixed using the protocol in section 2.3.4.1, and used as representative of the domain Archaea.

1. Teflon coated microscope slides with 10×7 mm diameter wells imprinted on the surface were thoroughly cleaned in a warm detergent solution in a sonic bath for 1 h.

2. Slides were then rinsed thoroughly in distilled water.
3. A solution of 0.1% gelatin and 0.01% chromium potassium sulphate ($CrKSO_4$) was heated to 70°C.
4. Rinsed slides were then dipped into the gelatin-$CrKSO_4$ solution and left to air dry standing vertically in slide staining jars.
5. 3 µl of fixed sample was applied to a slide well and evenly spread across the well with the pipette tip. Further samples were applied as required.
6. The slide was then left for the samples to air dry.
7. Dried slides were then dehydrated in an ethanol series by placing each slide into solutions of 50, 80 and 98% ethanol for 3 min each.
8. Dehydrated slides were then left to air dry, and stored at 4°C in the dark (in a microscope slide box) until required (no longer than 1 month).

2.3.4.4 Probes and hybridisation conditions for Fluorescent *in situ* Hybridisation

The probes used for the FISH assays were all targeted to conserved regions within the 16S rRNA subunit of different domains of prokaryotes (Stahl & Amann 1991, Manz *et al.* 1992, Amann *et al.* 1995). The probes, their target domains, and sequences are given in Table 2.7. It can be seen that the probe NONEUB is the reverse complement of EUB338, which causes this probe to be a negative control for EUB338. Whilst EUB338 is complementary to the rRNA, NONEUB is complementary to the antisense strand of the rRNA gene, and so will hybridise to the rDNA and not the rRNA itself (Giovannoni *et al.* 1988, Glöckner *et al.* 1999). The bacterial probe EUB927 was labelled with the fluorescent dye fluorescein, which emits at 520 nm and so appears green. The archaeal probe ARCH915 was labelled with the fluorescent dye Texas Red, which emits at 600 nm and so appears red. EUB927 does not hybridise to as many species of the domain Bacteria as EUB338, and so was only used in conjunction with ARCH915 to assess the spatial arrangements of the bacteria and archaea within the bioreactor sludge flocs. Probes EUB338 and NONEUB were labelled with the indocarbocyanine fluorescent dye CY3, which emits at 565 nm and so appears red. Each probe was made up to a stock concentration of ~1 $\mu g \mu l^{-1}$, and diluted to a working concentration of 25 $ng \mu l^{-1}$ in TE buffer.

The hybridisation protocol used was adapted from a method supplied by Dr. J. Plumb, Dept. of Chemical Engineering and Chemical Technology, Imperial College of Science, Technology and Medicine, with reference to methods published in Amann *et al.* 1990*b*, Stahl & Amann 1991, Manz *et al.* 1992, Alfreider *et al.* 1996, Snaidr *et al.* 1997 and Glöckner *et al.* 1999.

Table 2.7: Sequences, target sites and specificities of the probes used in FISH analysis of the bioreactor microflora

Probe	Specificity	Target	Sequence 5′ → 3′	Label	Reference
EUB338	Bacteria	16S, 338-355	GCTGCCTCCCG TAGGAGT	CY3	Amann *et al.* 1995
EUB927	Bacteria	16S, 927-942	ACCGCTTGTGC GGGCCC	fluorescein	Amann *et al.* 1995
ARCH915	Archaea	16S, 915-934	GTGCTCCCCCG CCAATTCCT	Texas Red	Amann *et al.* 1995
NON338	Negative control	16S, 338-355	ACTCCTACGG GAGGCAGC	CY3	Glöckner *et al.* 1999

N.B. Target numbers refer to *E. coli* 16S rRNA numbering.

Reagents used: Stock concentrations of reagents were 5 M NaCl (autoclaved), 1 M Tris-HCl, pH 7.4 (autoclaved), 10% sodium dodecyl sulphate (SDS) and 100% formamide. Hybridisation buffer was 0.9 M NaCl, 0.02 M Tris-HCl, pH 7.4, 0.01% SDS and a certain concentration of formamide dependent upon the specificity of the probe used. Wash buffer was 0.02 M Tris-HCl, pH 7.4, 0.01% SDS, 0.005 M EDTA and a certain concentration of NaCl dependent upon the specificity of the probe used.

1. Hybridisation buffer was made up to 2 ml final volumes in microcentifuge tubes for each slide to be hybridised using RNase free Ultrapure water. The final concentration of formamide was varied for each probe used according to Table 2.8. SDS was added last into the cap of the tubes, as it can interact with NaCl and precipitate.
2. A microfuge tube was mixed gently by inversion, and 8 µl of hybridisation buffer was added to each well of a slide previously seeded with fixed, dehydrated bioreactor sludge (see sections 2.3.4.1, 2.3.4.2 and 2.3.4.3).
3. The remainder of the hybridisation buffer was added to a 50 ml screw-capped polypropylene tube containing absorbent tissue paper. The slide was then placed horizontally on top of the moistened tissue.
4. Steps 2-3 were repeated for all slides to be hybridised.
5. Ensuring that the slides remained horizontal, the polypropylene tubes were placed in a hybridisation oven at 46°C and left to equilibrate for 20 min.
6. Slides were removed from the tubes, and 2 µl of 25 ngμl^{-1} probe were rapidly pipetted into each of the appropriate wells. In the case of a negative control where no probe was used, 2 µl of RNase free Ultrapure water was used. In the case of dual probing, as with EUB338 and NONEUB or EUB927 and ARCH915, 1 µl of each 25 ngμl^{-1} probe was used.
7. Slides were returned to the polypropylene tubes, which were then left in the hybridisation oven at 46°C for 90 min.
8. Wash buffer was made up to 50 ml final volumes in 50 ml polypropylene tubes for each slide to be washed using distilled water. The final concentration of NaCl was varied for each probe used according to Table 2.8. SDS was added last into the cap of the tubes, as it can interact with NaCl and precipitate.
9. The tubes were mixed gently by inversion, placed in a water bath at 48°C and left to equilibrate for at least 45 min.

10. After hybridisation, a slide was removed from a polypropylene tube and rinsed with a small amount of wash buffer to waste. The slide was then placed into the remainder of the wash buffer in the tube, and replaced into the water bath.
11. Step 9 was repeated for all slides hybridised.
12. Slides were left in the water bath at 48°C for 15 min.
13. Slides were removed from the wash buffer, and air dried quickly using a fume hood or a high pressure gas outlet (as water can quench the fluorescent signal by causing dissociation of the probe from the cell due to osmotic pressure).
14. Drops of VECTASHIELD® mounting medium (Vector Laboratories) containing 1.5 $\mu g ml^{-1}$ 4',6-diamidine-2-phenylindole (DAPI), which binds to DNA and RNA and emits at 460 nm and so appears blue, were placed on the surface of the slide.
15. Slides were covered with large (22×64 mm) coverslips, which were sealed with nail varnish.
16. Slides were stored at 4°C in the dark (in a microscope slide box) until required, for up to 1 year.

Table 2.8: Stringencies of hybridisation and wash buffers used

Probe	Formamide in hybridisation buffer (%)	Sodium chloride in wash buffer (M)	Reference
EUB338	20	0.9	Amann *et al.* 1990*b*, Manz *et al.* 1992
EUB927	0	0.9	Giovannoni *et al.* 1988
ARCH915	0	0.9	Amann *et al.* 1990*a*, Snaidr *et al.* 1997
NONEUB	0	0.07	Glöckner *et al.* 1999
no probe	0	0.9	Amann *et al.* 1990*b*

2.3.4.5 Analysis of Fluorescent *in situ* Hybridisation data

Cellular numbers in the sludge samples were counted using an Axioplan epifluorescent microscope (Zeiss). The total number of fluorescent cells in 5 random fields of view for each well (sample) were counted at ×1000 magnification using the correct filter sets for viewing green (fluorescein) or red (Texas Red and CY3) emission, or UV excitation for viewing blue (DAPI) emission. Unprobed samples were viewed with the correct filter set for red emission, as autofluorescent agents such as dust and chlorophyll emit red light and thus would interfere with the CY3 labelled probes (Alfreider *et al.* 1996). The cellular concentration of the sample was calculated using equations 1-5 below.

At ×1000 magnification, field of view diameter = 200 μm

Area of field of view = $\pi \times r^2 = 0.031$ mm^2 **Equation 1**

(where r = 0.1 mm)

Area of slide well = $\pi \times r^2 = 38.5$ mm^2 **Equation 2**

(where r = 3.5 mm)

Microscope factor = 38.5 ÷ 0.031 = 1242 **Equation 3**

∴ number cells on slide well = n × 1242 **Equation 4**

(where n = number of cells in 1 field of view)

∴ number of cells ml^{-1} = (n × 1242) × 333.33 **Equation 5**

(if inoculum volume = 3 μl)

(Adapted from Amann *et al.* 1995)

The numbers of bacteria and archaea in each of the bioreactor types (SBR and HABR) were determined for both spatial and temporal parameters (as applicable). In addition, numbers of cells visualised using the negative controls of the NONEUB probe (DNA binding) and no probe (to assess autofluorescence) were determined, and used to adjust the bacterial and archaeal counts. Sludge samples probed with EUB927 and ARCH915 (to determine spatial arrangements of bacteria and archaea in sludge flocs) were visualised using a confocal scanning laser microscope (CSLM), which can scan images in 3 dimensions. Specific laser wavelengths and filter sets that allow visualisation of the fluorescent dyes were chosen in accordance with the excitation and emission wavelengths of the dyes (490 and 520 nm for fluorescein, 578 and 600 nm for Texas Red and 550 and 565 nm for CY3 respectively).

2.3.5 Extraction and amplification 16S rRNA genes from bioreactor sludge samples

2.3.5.1 Extraction of total nucleic acid

The method of extraction used was a mechanical type known as bead beating. This type of extraction was chosen since enzymatic methods are very intensive, involving many pipetting steps, and time consuming, requiring 1-3 days (Smith 1999). As it was necessary to extract the total nucleic acid (TNA) from the samples, in order that analysis of RNA could be conducted if time and resources permitted, a method that allowed simultaneous extraction of DNA and RNA was required. It is known that mechanical extraction, such as bead beating, is the only method recommended for RNA extraction directly from soil and sediment samples (Smith 1999). Thus a bead beating technique developed by R. Griffiths at the Institute of Virology and Environmental Microbiology, CEH-Oxford, was used (Griffiths *et al.* 2000), and is essentially described in the protocol below.

Reagents used: Initially, all glassware was rendered RNase free by treating with 0.1% DEPC (diethyl pyrocarbonate) for >3 h at 37°C and subsequent sterilisation by autoclaving. Only RNase free H_2O was used and reagents were not touched by hand or equipment. CTAB buffer is 10% hexadecyltrimethylammonium bromide in 0.7 M NaCl, pH 8, mixed 1:1 with 240 mM potassium phosphate buffer, pH 8. TE buffer is 10 mM Tris-HCl + 1 mM EDTA (disodium ethylene diamine tetraacetate), pH 7.4.

1. Approximately 0.5g of sludge was transferred to Multimix FastRNA tubes (Bio 101, US), containing a lysing matrix (including 0.1 mm glass beads) to lyse the cells and chaotropic salts to stabilise RNA.
2. 0.5 ml of CTAB buffer and 0.5 ml of phenol:chloroform:isoamyl alcohol, pH 8 (25:24:1), was added to the Multimix tubes.
3. Cells were lysed by running the tubes in the FastPrep120 (Bio 101, US) at speed setting 5.5 ms^{-1} for 30 s.
4. Tubes were quickly cooled on ice, and then centrifuged at 16000 *g* for 5 min.
5. The top aqueous layer was transferred to a 1.5 ml tube, and an equal volume of chloroform:isoamyl alcohol (24:1) was added.
6. Tubes were shaken vigorously to emulsify the contents, and then centrifuged at 18000 *g* for 5 min.
7. The top layer was transferred to a 1.5 ml tube, and two volumes of ice cold 100% ethanol were added. No sodium acetate was used, as the salt concentration in the sludges was extremely high.
8. Tubes were left to precipitate at –20°C for ~1.5 h, and then centrifuged at 18000 *g* for 10 min.
9. As much of the supernatant as possible was removed, taking care not to dislodge the pellet.
10. The pellet was washed by gentle addition of 100 µl 70% (v/v) ethanol, tubes were stood on the bench for ~30 s, and then as much of the ethanol as possible was removed, taking care not to dislodge the pellet.
11. Pellets were air dried on the bench for 20-30 min.
12. Pellets were resuspended in 50 µl TE buffer (to give TNA).
13. To obtain a separate extract of RNA, a 20 µl aliquot of TNA from each sludge was transferred into a 0.2 ml PCR tube.
14. To this aliquot, 0.1µl of 127 $U\mu l^{-1}$ RQ1 RNase-free DNase (Promega) was added (final concentration 0.64 $U\mu l^{-1}$), incubated at 37°C for 1 h, and then heat inactivated at 80°C for 10 min (to give RNA).

In addition to the use of ethanol, as mentioned in step 7, 50% polyethylene glycol (PEG) and 100% ice cold isopropanol were also utilised for precipitation (two volumes of PEG with precipitation at room temperature for 2 h, one volume of isopropanol with precipitation at –20°C for 1.5 h). Again, no sodium acetate or sodium chloride was used due to the high salt concentration within the samples. However, PEG did not precipitate a good quantity of TNA whilst isopropanol precipitated too much salt and protein (data not shown). Thus it was decided to continue precipitation with ethanol as stipulated in the protocol above.

2.3.5.2 Amplification of 16S rRNA genes using Polymerase Chain Reaction

Extracts of TNA from sludge samples were amplified using primers matching conserved sequences within the V3 region of the 16S rRNA gene of 80% of all bacterial species. The forward primer contained a 39 nucleotide GC sequence (GC clamp), as the DGGE technique requires a length of very high T_m sequence to ensure that the double stranded PCR product does not completely dissociate within the denaturing gradient (see section 1.6.3.4). Primers used were GC338F (forward) (bases 1-38 are GC clamp; target position 338-355 based on *E. coli* numbering) GCCCGCCGCGCCCCCGCCCC GGCCCGCCGCCCCCGCCCACTCCTACGGGAGGCAGC (Griffiths *et al.* 2000; 338F adapted from Amann *et al.* 1995) and 530R (reverse) (target position 516-533 based on *E. coli* numbering) GTATTACCGCGGCTGCTG (adapted from Muyzer *et al.* 1993). This would produce an amplicon of ~230 bp (including the GC clamp).

1. TNA extracts were diluted 1:10 with RNase-free water.
2. Sufficient PCR mastermix for each reaction was made up using 45 µl 1.1×ReddyMix™ PCR Master Mix (2.0 mM $MgCl_2$) (ABgene, UK) and 2 µl of both primers at 25 pmolμl^{-1} (final concentrations of 1.25 U *Taq* DNA polymerase, 2 mM $MgCl_2$, 0.2 mM dNTPs and 50 pmol both primers). The ReddyMix™ contains a red dye and precipitant, which is used for loading samples directly onto a gel for electrophoresis subsequent to PCR.
3. Aliquots of 49 µl of the PCR mastermix were added to 1 µl of diluted TNA in 0.2 ml tubes to make a final volume of 50 µl per reaction.
4. Tubes were then placed in a Peltier Thermal Cycler PTC-225 (MJ Research, UK) using an initial denaturation of 95°C for 2 min followed by 33 cycles of 95°C for 60 s, 59°C for 45 s and 72°C for 90 s, with a final extension of 72°C for 30 min (adapted from Griffiths *et al.* 2000). Initial studies utilised an annealing temperature of 60°C, but it was noted that more bands were appearing in the DGGE profile with an annealing temperature of 59°C, and so this was used for all subsequent samples.
5. Amplicons were then electrophoresed in a 1.5% agarose gel in TAE buffer, and visualised using ethidium bromide.

In addition to amplification of bacterial 16S rRNA genes, samples were also amplified using primers matching conserved sequences within the V3 region of the 16S rRNA gene of archaea. Initial studies using primers chosen from Øvreås *et al.* (1997) did not yield any amplification of archaeal sequences from samples known to contain archaea (from the results of FISH and culture studies). Therefore, it was decided to increase amplification sensitivity by using a nested PCR. Primers used in the first round of amplification were 0025eF (forward) CTGGTTGATCCTGCCAG (target position 9-25 based on *E. coli* numbering) (Achenbach & Woese 1995) and ARCH958R (reverse) (target position 939-958 based on *E. coli* numbering) YCCGGCGTTGAVTCCAATT (where Y = C or T and V = A, C or G) (DeLong 1992). As with the bacterial

amplification, the final amplicon contained a 40 nucleotide GC sequence (GC clamp) so that double stranded PCR product did not completely dissociate within the DGGE denaturing gradient. Primers used in the second round of amplification were PARCH340F (forward) (bases 1-40 are GC clamp; target position 340-357 based on *E. coli* numbering) CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG CCCTACGGGGYGCASCAG (where Y = C or T and S = G or C) (GC clamp from Muyzer *et al.* 1993) and PARCH519R (reverse) (target position 519-533 based on *E. coli* numbering) TTACCGCGGCKGCTG (where K = G or T) (Øvreås *et al.* 1997). This would produce an amplicon of ~230 bp (including the GC clamp).

1. TNA extracts were diluted 1:100 with RNase-free water.
2. Sufficient PCR mastermix for each reaction was made up using 44 µl 1.1×ReddyMix™ PCR Master Mix (2.0 mM $MgCl_2$) (ABgene, UK) and 0.5 µl of primers 0025eF and ARCH958R at 100 pmolμl^{-1} (final concentrations of 1.25 U *Taq* DNA polymerase, 2 mM $MgCl_2$, 0.2 mM dNTPs and 50 pmol both primers). The ReddyMix™ contains a red dye and precipitant, which is used for loading samples directly onto a gel for electrophoresis subsequent to PCR.
3. Aliquots of 45 µl of the PCR mastermix were added to 5 µl of diluted TNA in 0.2 ml tubes to make a final volume of 50 µl per reaction.
4. Tubes were then placed in a Peltier Thermal Cycler PTC-225 (MJ Research, UK) using an initial denaturation of 97°C for 3 min followed by 30 cycles of 94°C for 60 s, 55°C for 60 s and 72°C for 90 s, with a final extension of 72°C for 10 min (adapted from DeLong 1992).
5. Sufficient PCR mastermix for each reaction was made up using 48 µl 1.1×ReddyMix™ PCR Master Mix (2.0 mM $MgCl_2$) (ABgene, UK) and 0.5 µl of primers PARCH340F and PARCH519R at 100 pmolμl^{-1} (final concentrations of 1.25 U *Taq* DNA polymerase, 2 mM $MgCl_2$, 0.2 mM dNTPs and 50 pmol both primers).
6. Aliquots of 49 µl of the PCR mastermix were added to 1 µl of amplicon from the first round of amplification in 0.2 ml tubes to make a final volume of 50 µl per reaction.
7. Tubes were then placed in a Peltier Thermal Cycler PTC-225 (MJ Research, UK) using the same cycling conditions as in step 4.
8. Amplicons were then electrophoresed in a 1.5% agarose gel in TAE buffer, and visualised using ethidium bromide.

Initial studies were also conducted using a different forward primer in the first round of amplification. The primer used was ARCH21F (TTCCGGTTGATCCYGCCG GA where Y = C or T; DeLong 1992). It was found that this primer did not amplify all of the bands seen in the DGGE profile when 0025eF was used, and so this was used for all subsequent samples.

2.3.5.3 Amplification of RNA using Reverse Trancriptase-Polymerase Chain Reaction

Extracts of RNA from sludge samples were obtained by treating TNA extracts with DNase (see section 2.3.5.1, step 14). The RNA was converted to cDNA via the use of the enzyme reverse transcriptase (RT) using only the reverse primer for the region of interest, as the RNA sequence is homologous to the antisense (3' → 5' strand) of the DNA sequence. In this way, double stranded copies of the RNA (cDNA) can then be amplified using a standard PCR reaction (see section 2.3.5.2). The primer used was 530R (target position 516-533 based on *E. coli* numbering) GTATTACCGCGGCTGCTG (adapted from Muyzer *et al.* 1993).

1. RNA extracts were diluted 1:20 with RNase-free water.
2. In a 0.2 ml tube, 1 µl diluted RNA was added. Next, 2 µl of 25 pmolμl^{-1} primer (final concentration 50 pmol) was added and then 12 µl RNase-free water.
3. The tube was incubated at 70°C for 5 min to anneal the primer, then rapidly cooled to 4°C and placed on ice.
4. To the tube, 5 µl 5×RT buffer (ABgene, UK), 1.25 µl of a 10 mM dNTP mix, 0.4 µl of 25 Uμl^{-1} MMuLV RT (ABgene, UK) and 3.35 µl RNase-free water was added to make a final volume of 25 µl (final concentrations of 1×RT buffer, 0.5 mM dNTPs and 10 U MMuLV RT).
5. The tube was incubated at 42°C for 60 min to create cDNA, then at 99°C for 5 min to denature the RT enzyme.

2.3.5.4 Clean-up of unamplified Total Nucleic Acid extracts

In certain cases, especially with samples from the HABR, bacterial amplification could not be achieved (possibly due to the presence of inhibitors). In these cases, TNA extracts were purified using the GeneClean II kit (Bio 101, US) prior to amplification. The method was adapted from the given protocol, and is essentially described in the protocol below.

1. The amount of TNA was estimated by visual inspection of an agarose gel. For more concentrated samples, 5 µl of TNA was added to a 1.5 ml tube. For less concentrated samples, 20 µl of TNA was added to a 1.5 ml tube.
2. Samples were made up to 50 µl using RNase-free TE buffer.
3. Three volumes (150 µl) of sodium iodide were added to each tube.
4. The Glassmilk reagent (Bio 101, US) was resuspended by vortexing for approximately 1 min, and 5 µl was added to each sample.
5. Tubes were left on ice for 5 min, shaking every minute to keep the Glassmilk in suspension.
6. Tubes were then centrifuged at 18000 *g* for 30 s.
7. The supernatant was discarded and 500 µl of New Wash reagent (Bio 101, US) was added. The pellet was resuspended by mechanical distribution with a pipette tip.
8. Steps 6 and 7 were repeated twice.

9. Tubes were then centrifuged at 18000 *g* for 30 s, the supernatant discarded and the pellet left to air dry on the bench for 5-10 min.
10. Pellets were resuspended in 20 µl of TE buffer for 5 min at room temperature.
11. Tubes were then centrifuged at 18000 *g* for 1 min to precipitate the Glassmilk particles.
12. The supernatant (clean TNA) was removed to clean 0.2 ml tubes.
13. The 0.2 ml tubes were centrifuged at 18000 *g* for 1 min to ascertain that all of the Glassmilk particles had been removed (Glassmilk particles interfere with the PCR reaction).
14. If a pellet was observed, step 13 was repeated. Otherwise, the clean TNA can be used for subsequent amplification.

2.3.6 Denaturing Gradient Gel Electrophoresis analysis of bioreactor microflora

For Denaturing Gradient Gel Electrophoresis (DGGE), the DCode™ Universal Mutation Detection System (BioRad, UK) was utilised. In this system, 10% polyacrylamide gels containing a 30-60% gradient of denaturant (where 100% denaturant is 7 M urea + 40% formamide) were made using a manual mixing wheel, and electrophoresed vertically at a set temperature. After electrophoresis, the bands were visualised using SYBR® Gold nucleic acid gel stain (Molecular Probes, US), which is a UV fluorescent molecule that intercalates with nucleic acid. The advantage of using SYBR® Gold over ethidium bromide is that it lacks background staining, and thus the less dominant bands within the profile can also be observed (Muyzer & Smalla 1998). In addition, the SYBR® family of stains are much less mutagenic than ethidium bromide, as demonstrated by Singer and co-workers in 1999 with SYBR® Green I in the Ames test. For every DGGE gel run, a marker ladder was used in order that the position of bands within a profile could be compared across gels. This marker ladder consisted of an amplicon of TNA from a mixture of 8 different organisms produced using the same primers as the bacterial amplification (see section 2.3.5.2). The organisms were all isolates held at Institute of Virology and Environmental Microbiology, CEH-Oxford (strain reference in brackets): *Agrobacterium radiobacter* (293), *Bacillus macerans* (306), *Pseudomonas vesicularis* (309), *Acidovorax delafieldii* (315), *Psychrobacter immobilis* (331), *Moraxella catarrhalis* (366), *Pseudomonas putida lux*cdabe (B5 566) and *Escherichia coli lux*cdabe (XL1). The following protocol describes the construction, loading and electrophoresis conditions for the DGGE gels used.

1. Glass plates (1 large back plate and 1 smaller front plate per gel) were cleaned with 70% ethanol.
2. Spacers (two per gel) were greased well using silicon vacuum grease and placed long edge inwards at the edges of the back plate.
3. The front plate was placed on top of the back plate, flush with the edges, and the plates were secured with plate clamps.

4. Plates were placed vertically on top of a plate cushion in a base and secured in position.
5. A disposable needle was attached to a length of polypropylene tubing using the supplied joining piece, and inserted between the front and back plates in the middle of the plates.
6. Approximately 20 ml of 60% denaturant solution (60% denaturant + 10% acrylamide/bis-acrylamide, Design-a-Gel stock solution, Severn Biotech, UK) was added to a 25 ml disposable Universal, and 2-3 drops of dye solution was added. The Universal was labelled "high".
7. Approximately 20 ml of 30% denaturant solution (30% denaturant + 10% acrylamide/bis-acrylamide, Design-a-Gel stock solution, Severn Biotech, UK) was added to another Universal, and labelled "low".
8. A 0.1 gml^{-1} solution of ammonium persulphate solution was made (to scavenge gases such as oxygen from the denaturant solutions), and 160 µl was added to each Universal which was then mixed gently by inversion to avoid air bubbles.
9. 16 µl of TEMED was added to each Universal and mixed carefully by inversion.
10. Two lengths of polypropylene tubing were attached to two 30 ml disposable syringes using the supplied joining pieces. The supplied metal barrels were fitted over the barrels of the syringes, and the supplied metal end clamps were attached to the plungers of the syringes.
11. A syringe was filled with the high denaturant solution via the polypropylene tube. When all the high denaturant was in the syringe, it was inverted to expel any air from the syringe and tubing.
12. Step 11 was repeated for the low denaturant.
13. The syringes were attached to the mixing wheel, high at the back and low at the front, using the metal barrel and end clamps attached to the syringes, ensuring that the screw on the end clamp is correctly inserted into the groove on the mixing wheel.
14. The polypropylene tubing from each syringe was attached to the supplied Y-shaped joining piece, which was then attached to the polypropylene tubing from the needle.
15. The mixing wheel was then turned slowly with a constant pressure to ensure a steady flow of denaturant solutions between the plates. The high denaturant will be injected first, followed by an increasing volume of low denaturant, until only low denaturant is injected. As the high denaturant contains a dye, a good gradient can be visually assessed by a continuous reduction in colour towards the top of the gel.
16. When the denaturant solutions had reached the top of the front plate, pouring was ceased and a 16-well comb was pushed between the front and back plates. A small amount of TEMED was pipetted along the line of the comb to assist in the formation of wells.
17. A vertical gel running tank (DCode™ System, BioRad, UK) was filled with 0.5×TAE and set to 65°C (5°C above the running temperature to allow for cooling during loading).
18. When the tank was at temperature and the gel set (~1.5 h), the combs were removed from the gels and the plates were attached to the supplied running apparatus which was stood on the bench.
19. Approximately 250 ml of heated 0.5×TAE was transferred to the top of the running apparatus to cover the top of the gels.
20. Unpolymerised acrylamide was removed from the wells by injecting the TAE into each well at high pressure using a needle and syringe.
21. The lid was removed from the tank and the running apparatus gently lowered into the tank with the cathode on the right of the tank, until completely submerged in TAE.

22. Variable volumes of amplicons were added to 5 µl of 5×loading dye. If the amplicon was weak, average or strong (as assessed visually on an agarose gel), 30, 20 or 10 µl volumes were used (to attempt to standardise the intensity of the bands produced on a single gel).
23. Using a long tip, the whole of the amplicon + loading dye samples were pipetted into the wells in the DGGE gel.
24. When all wells had been loaded, the lid of the tank was replaced and the temperature reset to 60°C.
25. Gels were run at 20 V for ~20 min to allow the tank to return to running temperature, and then run at 200 V for 5 h or 85 V overnight (~16 h).
26. After running was completed, the plates were removed from the tank and placed flat on a tray. The front plate was removed to expose the gel.
27. Gels were stained with a 1:10000 dilution of SYBR® Gold in 0.5×TAE (×10000, Molecular Probes, US) and stained in the dark for 20-30 min.
28. Gels were analysed using a UV transilluminator with GeneSnap software (SynGene, UK).

DGGE profiles were then analysed using Phoretix 1D Advanced v.4.01 software (Phoretix International, UK, ©NonLinear Dynamics Ltd.) to determine divergence of profiles, and dendograms were constructed using the UPGMA algorithm.

2.3.7 Identification of bioreactor microflora by sequencing

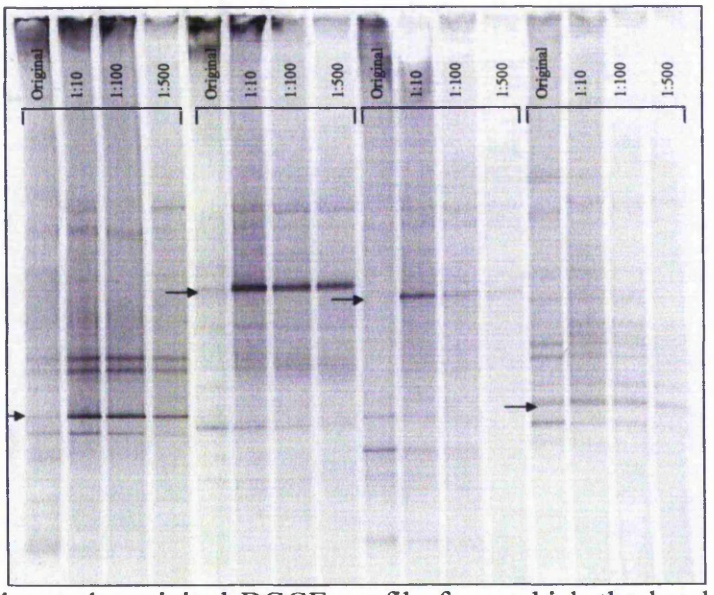
Bands of interest within the DGGE profiles were excised for sequencing using the CEQ (Beckman) capillary sequencer with BigDye™ fluorescent dye termination. Bands should be excised from the DGGE gel as soon as possible as the DNA will diffuse through the acrylamide, as the SYBR® Gold stain used does not fix the DNA to the acrylamide in the same way as Coomassie Blue or Silver staining. It was necessary to re-PCR and re-DGGE the excised bands to determine that only the band of interest had been excised, as there will be a small amount of amplicon from the original amplification that will not have become GC clamped. When the original amplicon was run in a DGGE gel, double stranded products without a GC clamp will become fully dissociated within the denaturing gradient and will therefore run completely through the gel. This means that, when a band of interest is excised, a small amount of single stranded non-target product will also be taken, which may then interfere with the sequencing result. By repeating the PCR and DGGE, the non-target product may become GC clamped, and thus will show as discreet non-target bands within the profile. Bands were diluted before re-PCR and re-DGGE in an attempt to dilute out any non-target product. When it was ascertained that the band of interest was purified, a PCR reaction must be performed without the use of the GC clamp on the primers used, as this will interfere with the sequencing result. The following protocol describes the excision, purification, amplification and sequencing reaction of the bands of interest.

1. DGGE profiles were visualised on a Dark Reader™ DR-45M (Chemical Research, UK) UV transilluminator.
2. Bands of interest were excised by inserting a cut-off pipette tip in the centre of the band and placing the tip into 20µl RNase-free water.
3. Bands were left to elute overnight at 4°C.
4. Bands were diluted 1:100 in RNase-free water and used as a template in a second PCR (see section 2.3.5.2).
5. Reamplified bands were run in a second DGGE gel (see section 2.3.6), and the band of interest was re-excised as in steps 2 and 3.
6. Bands were diluted 1:100 in RNase-free water and used as a template in a third PCR. In this amplification, the forward primer was used without the GC clamp (see section 2.3.5.2).
7. The PCR product was purified using the Ultra PCR Product Clean Up Kit (ABgene, UK). The supplied protocol was followed with the exception that the product was eluted once in 50 µl of elution buffer and then again in 20 µl (total volume 70 µl) to increase the yield. The product was then concentrated using standard ethanol/salt precipitation, and resuspended in 30 µl of TE buffer.
8. The purified PCR products were electrophoresed in 1.5% agarose gels and visualised with ethidium bromide in order to visually quantitate the amount of DNA present. The amount of template required for the sequencing reaction is 10-50 fmol, which corresponds to 1.2-6 ng of DNA for a 200 bp PCR product.
9. Sequencing reactions were prepared in 0.2 ml tubes using the appropriate amount of template, 2 µl of one primer (either forward or reverse) at 1.6 $pmol\mu l^{-1}$, 6 µl of supplied reaction premix (containing 10×sequencing reaction buffer, dNTP mix, ddUTP, ddGTP, ddCTP and ddATP dye terminators and a DNA polymerase enzyme, concentrations unknown), 1 µl of amplification buffer and made up to a final volume of 20 µl with RNase-free water.
10. Tubes were then placed in a Peltier Thermal Cycler PTC-225 (MJ Research, UK) using 30 cycles of 96°C for 20 s, 50°C for 20 s and 60°C for 240 s.
11. To the tubes, 4 µl of stop solution (1.5 M sodium acetate + 50 mM EDTA) and 1 µl of 20 $mgml^{-1}$ glycogen was added and mixed by aspirating with the pipette tip.
12. Reactions were transferred to 1.5 ml tubes, and 60 µl of ice cold 95% ethanol was added.
13. Tubes were immediately centrifuged at 18000 *g* for 15 min at 4°C.
14. The supernatant was discarded and the pellet washed in 200 µl of ice cold 70% ethanol.
15. Tubes were immediately centrifuged at 18000 *g* for 5 min at 4°C.
16. Steps 14 and 15 were repeated.
17. The supernatant was discarded, and the pellets vacuum dried for 20 min.
18. Reactions were resuspended in 35 µl of deionised formamide for 15 min.
19. Reactions were then loaded onto the CEQ sequencer and the appropriate program was run.

Sequences were edited using Chromas v.1.6 (Technelysium) software, and pairwise global and local alignments of forward and reverse sequences to create consensus sequences were completed using BioEdit biological sequence alignment editor v.5.0.9 (T. Hall, North Carolina State University) software. Local alignments of the sequences were made to the non-redundant nucleotides database (incorporating GenBank, EMBL, DDBJ and PDB) held at the National Centre of Biotechnology

Information (NCBI) using the BLAST algorithm. Initially when excising bands of interest to re-PCR, dilutions of 1:10, 1:100 and 1:500 were tested. It was found that non-target bands could still be observed at the lowest dilution used (see Figure 2.7). It was decided to use 1:100 dilutions for all subsequent samples, as this dilution reduced the background but still allowed for a reasonable concentration of target band to be amplified. The band re-excised was chosen on the basis of position in comparison to the original DGGE gel, and was usually the strongest band within the profile.

Figure 2.7: DGGE gel of reamplified bands of interest at various dilutions



N.B. Original shows the original DGGE profile from which the band of interest was excised. 1:10, 1:100 and 1:500 indicate the dilutions of template used in the second PCR. The band of interest is indicated by an arrow in the original profile.

Chapter 3: Results

3.1 Decolourisation studies of azo dyes

3.1.1 Structure-activity evaluation with simple azo dyes and related naphthol compounds

In order to assess the suitability of the toxicity and genotoxicity assays used in this study, a structure-activity evaluation was conducted using the simple, structurally related azo dyes Sunset Yellow (SY) and Orange II (O2) in both their unreduced and reduced forms. The time taken for complete decolourisation to occur can be seen in Table 3.5 (see section 3.1.2). The negative control (i.e. the assay mixture without the dye) was collected at the same time as the dye decolourisation assays. In addition, commercially available naphthol compounds analogous to the substituents of the dyes were tested. The resultant toxicology data on dye reduction and structural changes to the naphthol compounds were compared to the trends expected from the literature.

Table 3.1: Toxicity and genotoxicity of SY, both unreduced and reduced by *Enterococcus faecalis*, and its substituent aromatic amines

Sample	Toxicity (EC_{50} 5 min)	Genotoxicity (CS)
Sunset Yellow unreduced	22.13 ± 2.47 (2)	0.90 ± 0.07 (2)
Sunset Yellow reduced	32.45 ± 1.53 (2)	0.40 ± 0.18 (3)
Sulphanilic acid	21.54 ± 6.72 (3)	0.81 ± 0.06 (2)
1-amino-2-naphthol-6-sulphonate	391.00 ± 21.92 (3)	0.95 ± 0.03 (3)
Negative control	>50.00 (2)*	0.79 ± 0.51 (3)

N.B. All toxicity values are expressed as mgL^{-1}, and values of >100 mgL^{-1} should be considered non-toxic. All genotoxicity values are shown for sample concentrations of 100 mgL^{-1}, and each sample was assayed in triplicate. Numbers in parenthesis are number of replicates of independent experiments. *Units are % of original concentration.

It was observed that unreduced SY demonstrated a toxic potential but was non-genotoxic (see Table 3.1). Reduction of SY slightly decreased the toxicity but increased genotoxicity, conferring a weak genotoxic potential. Of the naphthol compounds, analogous to the substituent aromatic amines liberated by azo fission, the toxicity of sulphanilic acid was equivalent to that of unreduced SY whilst 1-amino-2-naphthol-6-sulphonate was 17.7 times less toxic. No genotoxicity was observed with either of the naphthol compounds tested. The negative control, which comprised *E. faecalis* cells incubated with glucose without the presence of SY for 10 minutes, showed neither toxicity nor genotoxicity.

Table 3.2: Toxicity and genotoxicity of O2, both unreduced and reduced by *Enterococcus faecalis*, and its substituent aromatic amines

Sample	Toxicity (EC_{50} 5 min)	Genotoxicity (CS)
Orange II unreduced	15.70 ± 2.68 (2)	1.14 ± 0.41 (2)
Orange II reduced	0.19 ± 0 (2)	0.04 ± 0.05 (3)
Sulphanilic acid	21.54 ± 6.72 (3)	0.81 ± 0.06 (2)
1-amino-2-naphthol	0.07 ± 0.03 (3)	0 (2)
Negative control	>50.00 (2)*	0.79 ± 0.51 (3)

N.B. All toxicity values are expressed as mgL^{-1}, and values of >100 mgL^{-1} should be considered non-toxic. All genotoxicity values are shown for sample concentrations of 100 mgL^{-1}, and each sample was assayed in triplicate. Numbers in parenthesis are number of replicates of independent experiments. *Units are % of original concentration.

Unreduced O2 exhibited a toxic potential but was non-genotoxic (Table 3.2). Reduction of O2 increased the toxicity by 84.4 times, and conferred an extremely acute genotoxic potential. Of the naphthol compounds analogous to the substituent aromatic amines, sulphanilic acid, also a substituent of SY, was less toxic than unreduced O2 and non-genotoxic. However, the naphthol compound analogous to the second substituent aromatic amine, 1-amino-2-naphthol, was extremely toxic and genotoxic, to the extent that concentrations of >5 mgL^{-1} rendered all test organisms non-viable in the genotoxicity assay. The negative control, which comprised *E. faecalis* cells incubated with glucose without the presence of O2 for 10 minutes, showed neither toxicity nor genotoxicity.

Table 3.3: Toxicity and genotoxicity of structurally related aminonaphthols

Sample	Toxicity (EC_{50} 5 min)	Genotoxicity (CS)
1-amino-2-naphthol	0.07 ± 0.03 (3)	0 (2)
1-amino-2-naphthol-6-sulphonate	391.00 ± 21.92 (3)	0.95 ± 0.03 (3)
1-naphthalene sulphonic acid	29.10 ± 2.46 (2)	0.69 ± 0.04 (2)
2-naphthalene sulphonic acid	8.29 ± 0.23 (2)	1.26 ± 0.06 (2)
4-amino-1-naphthalene sulphonic acid	28.28 ± 3.82 (2)	0.83 ± 0.26 (2)
4-amino-1-naphthalene sulphonic acid sodium salt	456.00 ± 5.66 (2)	0.63 ± 0.07 (2)

N.B. All toxicity values are expressed as mgL^{-1}, and values of >100 mgL^{-1} should be considered non-toxic. All genotoxicity values are shown for sample concentrations of 100 mgL^{-1}, and each sample was assayed in triplicate. Numbers in parenthesis are number of replicates of independent experiments.

As has been previously shown, 1-amino-2-naphthol was extremely toxic and genotoxic. The genotoxic effect of this compound, as opposed to a highly toxic effect resulting in false-positivity in the genotoxicity assay employed, is demonstrated by Table 3.4, which shows that the threshold at which a compound is considered weakly genotoxic (CS = 0.31, Rosenkranz *et al.* 1981) occurs at a concentration between 0.61 and 1.23 mgL^{-1}. Sulphonation of this compound completely eliminated both the toxic and genotoxic potentials (see Table 3.3). It is interesting to note the extent at which the toxicity of 1-amino-2-naphthol is reduced (over 5000 times), by the addition of only one sulphonic acid group. 1-naphthalene sulphonic acid, with the $-SO_3$ group on the 1' carbon of the aromatic ring, demonstrated a toxic potential and a weak genotoxic potential. When the $-SO_3$ group was altered to the 2' carbon, as in 2-naphthalene sulphonic acid, the toxicity increased 3.5 times whilst the genotoxicity was completely eliminated. It can also be seen that 4-amino-1-naphthalene sulphonic acid demonstrated a toxic potential and a weak genotoxic potential. When the sodium salt was tested, toxicity decreased 16.1 times and genotoxicity increased marginally (1.3 times), although this genotoxicity was still weak.

Table 3.4: Genotoxicity of 1-amino-2-naphthol

Conc. (mgL^{-1})	% kill WP2	% kill CM871	Genotoxicity (CS)
2.54	85.69 ± 5.36	99.96 ± 0	0 ± 0
1.23	71.89 ± 0.24	93.15 ± 4.06	0.24 ± 0.15
0.61	34.24 ± 19.49	69.12 ± 14.57	0.44 ± 0.09
0.31	16.12 ± 6.28	41.20 ± 23.02	0.68 ± 0.22

N.B. Each sample was assayed in triplicate. Numbers are average of two independent experiments.

3.1.2 Toxicity and genotoxicity of reactive azo textile dyes

The toxicological responses of the assay systems utilised in this study to four reactive azo textile dyes, C.I. Reactive Black 5 (RB), Procion Navy (PN), Procion Yellow (PY) and Procion Crimson (PC) were assessed, both as whole molecules (unreduced) as after fission of the azo bond to produce the substituent aromatic amines (reduced), at a concentration of 500 mgL^{-1}. Complete decolourisation (as ascertained by visual inspection) required various incubation times for different dyes and organisms. These times are shown in Table 3.5. It should be noted that the negative control (i.e. assay mixture without the dye) was collected at the time analogous to the slowest dye decolourisation (i.e. 5 h with *E. faecalis* and 24 h with *C. butyricum*).

Table 3.5: Times taken for complete decolourisation of dyes in both parent and hydrolysed forms during biological decolourisation assays

Dye	Decolourisation time (h)	
	Enterococcus faecalis	*Clostridium butyricum*
C.I. Food Yellow 3	0.17	ND
C.I. Acid Orange 7	0.17	ND
C.I. Reactive Black 5	2	4
Procion Yellow H-EXL	2	2
Procion Crimson H-EXL	5	4
Procion Navy H-EXL	3	24

N.B. ND = not done

Table 3.6: Toxicity of reactive azo textile dyes, both unreduced and reduced by *Enterococcus faecalis*

Sample	Parent	Hydrolysed
C.I. Reactive Black 5 unreduced	27.48 ± 14.01 (2)	11.40 ± 3.68 (2)
C.I. Reactive Black 5 reduced	0.65 ± 0.09 (4)	0.22 ± 0.03 (3)
Procion Navy unreduced	18.94 ± 5.65 (2)	27.89 ± 3.28 (2)
Procion Navy reduced	0.50 ± 0.17 (3)	0.45 ± 0.07 (3)
Procion Yellow unreduced	70.98 ± 6.00 (2)	66.35 ± 0.93 (2)
Procion Yellow reduced	0.75 ± 0.17 (3)	0.50 ± 0.28 (3)
Procion Crimson unreduced	34.72 ± 0.27 (3)	37.68 ± 1.72 (3)
Procion Crimson reduced	19.90 ± 0.10 (2)	18.22 ± 2.28 (2)
Negative control	0.68 ± 0.19 (3)*	

N.B. All data are EC_{50} values for 5 minute contact time, expressed as mgL^{-1}. Values of >100 mgL^{-1} should be considered non-toxic. Numbers in parenthesis are number of replicates of independent experiments. *Units are % of original concentration.

The toxic potentials of the unreduced parent dyes varied, with PY being the least toxic to PN being the most toxic (see Table 3.6). Hydrolysis of each of the dyes resulted in an increase in toxicity for some (RB and PY) but a decrease for others (PN and PC). However, these decreases or increases were relatively small (1.1 times) with the exception of RB, in which the toxicity was increased 2.4 times. Reduction of the dyes by *E. faecalis* resulted in increases in toxicity, in the ranges 1.7-94.6 and 2.1-132.7 times for parent and hydrolysed forms respectively, presumably from the release of the substituent aromatic amines. The greatest increases were observed with PY and the least with PC, in both parent and hydrolysed forms. In general, reduction of the hydrolysed dyes resulted in slightly more toxic products than reduction of the parent dyes, which is likely to be due to release of more toxic, less sulphonated aromatic amines. Once again, these differences were relatively small (1.1 times) with the exception of RB, where the hydrolysed reduced dye was 3 times more toxic than the parent reduced dye.

The negative control, which comprised *E. faecalis* cells incubated with glucose without the presence of the dyes, showed a toxic response. This is in contrast to the non-toxic response of the *E. faecalis* negative control observed during studies of the simple azo dyes SY and O2 (see Tables 3.1 and 3.2).

Table 3.7: Toxicity of reactive azo textile dyes, both unreduced and reduced by *Clostridium butyricum*

Sample	Parent	Hydrolysed
C.I. Reactive Black 5 unreduced	27.48 ± 14.01 (2)	11.40 ± 3.68 (2)
C.I. Reactive Black 5 reduced	2.15 ± 0.71 (3)	0.28 ± 0.01 (3)
Procion Navy unreduced	18.94 ± 5.65 (2)	27.89 ± 3.28 (2)
Procion Navy reduced	9.12 ± 0.53 (2)	8.55 ± 1.19 (3)
Procion Yellow unreduced	70.98 ± 6.00 (2)	66.35 ± 0.93 (2)
Procion Yellow reduced	7.84 ± 1.67 (3)	5.55 ± 0.89 (3)
Procion Crimson unreduced	34.72 ± 0.27 (3)	37.68 ± 1.72 (3)
Procion Crimson reduced	8.05 ± 0.66 (3)	9.11 ± 1.04 (3)
Negative control	7.85 ± 1.58 (3)*	

N.B. All data are EC_{50} values for 5 minute contact time, expressed as mgL^{-1}. Values of >100 mgL^{-1} should be considered non-toxic. Numbers in parenthesis are number of replicates of independent experiments. *Units are % of original concentration.

The data presented in Table 3.7 for the unreduced dyes in both parent and hydrolysed form are repeated from Table 3.6. Reduction of the dyes by *C. butyricum* resulted in increases in toxicity, in the ranges 2.1-12.8 and 4.1-40.7 times for parent and hydrolysed forms respectively, presumably from the release of the substituent aromatic amines. The greatest increases were observed with RB and the least with PC, in both parent and hydrolysed forms. In general, reduction of the hydrolysed dyes resulted in slightly more toxic products than reduction of the parent dyes. However, as with hydrolysis of the unreduced dye, reduction of PCOH resulted in a lower toxic potential than reduction of the parent form. These differences are relatively small (1.1 times) with the exception of RB, where the hydrolysed reduced dye was 7.7 times more toxic than the parent reduced dye. It was again observed that the negative control, which comprised *C. butyricum* cells incubated with glucose without the presence of the dyes, showed a toxic response.

3.1.3 Decolourisation of C.I. Reactive Black 5 by various methods and HPLC fractionation

Due to the extensive use of RB in both the textile industry and research, this dye was designated as the model dye to be used in the bioreactor degradation studies. In addition, it was noted that RB produced the most significant toxic effects after

hydrolysis and decolourisation. Therefore, RB was studied further in pure culture assays to gain further insight into the parameters affecting the toxic and genotoxic potentials of this dye. As the hydrolysed form is the type present in the wastewater from a textile dyehouse, and thus was used in the bioreactor degradation studies, this is the form that was utilised extensively in subsequent assays. It is also of note that this form had also consistently shown the highest toxic potentials of all the reactive azo dyes assayed, when both unreduced and reduced. The products formed by reduction of RBOH with *E. faecalis* were separated by HPLC, in order that the resultant toxic potentials may be attributed to a certain fraction of the reduced compound.

Table 3.8: Toxicity and genotoxicity of RB reduced by various methods

Sample	Toxicity (EC_{50} 5 min)	Genotoxicity (CS)
Unreduced	27.48 ± 14.01 (2)	1.51 ± 0.37 (2)
Reduced by *E. faecalis*	0.65 ± 0.09 (4)	1.39 ± 0.16 (2)
Reduced by *C. butyricum*	2.15 ± 0.71 (3)	0.63 ± 0.26 (3)
Reduced by sodium dithionite	4.86 ± 1.45 (3)	0.57 ± 0.37 (2)
***E. faecalis* negative control**	0.68 ± 0.19 (3)*	1.32 ± 0.67 (2)
***C. butyricum* negative control**	7.85 ± 1.58 (3)*	0.88 ± 0.14 (3)
sodium dithionite negative control	24.37 ± 17.36 (3)	0.45 ± 0.28 (2)

N.B. All toxicity values are expressed as mgL^{-1}, and values of >100 mgL^{-1} should be considered non-toxic. All genotoxicity values are shown for sample concentrations of 100 mgL^{-1}, and each sample was assayed in triplicate. Numbers in parenthesis are number of replicates of independent experiments. *Units are % of original concentration.

Reduction of RB by the three methods used increased its toxic potential by 5.7-42.3 times (see Table 3.8). Each of the decolourisation methods used, either biological reduction with *E. faecalis* and *C. butyricum* or chemical reduction with sodium dithionite, resulted in different toxic potentials. By comparing each of these toxic potentials to the corresponding negative control, that is the toxic potentials of either the organisms incubated with glucose without the presence of RB or an aqueous solution of sodium dithionite, it can be seen that RB was 3.7 and 5 times more toxic than the negative control after reduction with *C. butyricum* and sodium dithionite respectively, but had an equivalent toxic potential after reduction with *E. faecalis*. It can also be seen that RB was non-genotoxic, and that reduction by *C. butyricum* and sodium dithionite resulted in a weak genotoxic potential, whilst reduction by *E. faecalis* did not. However, comparison to the corresponding negative controls showed that only reduction by *C. butyricum* resulted in a genotoxic potential lower than that produced by the background matrix (1.4 times), and that even this potential was not significantly lower.

Table 3.9: Toxicity and genotoxicity of RBOH reduced by various methods, including analysis of fractions

Sample	Toxicity (EC_{50} 5 min)	Genotoxicity (CS)
Unreduced	11.40 ± 3.68 (2)	0.68 ± 0.05 (4)
Reduced by *E. faecalis*	0.22 ± 0.03 (3)	1.27 ± 0.27 (2)
Reduced by *C. butyricum*	0.28 ± 0.01 (3)	0.80 ± 0.34 (3)
Reduced by sodium dithionite	0.23 ± 0.09 (3)	0.60 ± 0.23 (2)
vinyl sulphone HPLC fraction	0.32 ± 0.19 (4)	-
diamino H-acid HPLC fraction	>2.18 (2)	-
H-acid	48.63 ± 9.03 (3)	1.22 ± 0.15 (4)
***E. faecalis* negative control**	0.68 ± 0.19 (3)*	1.32 ± 0.67 (2)
***C. butyricum* negative control**	7.85 ± 1.58 (3)*	0.88 ± 0.14 (3)
sodium dithionite negative control	24.37 ± 17.36 (3)	0.45 ± 0.28 (2)

N.B. All toxicity values are expressed as mgL^{-1}, and values of >100 mgL^{-1} should be considered non-toxic. All genotoxicity values are shown for sample concentrations of 100 mgL^{-1}, and each sample was assayed in triplicate. Numbers in parenthesis are number of replicates of independent experiments. *Units are % of original concentration.

Reduction of RBOH by the each of the three methods used increased its toxic potential by 40.7-51.8 times (see Table 3.9). It should be noted that each of the decolourisation methods used, which were biological reduction with *E. faecalis* and *C. butyricum* and chemical reduction with sodium dithionite, resulted in extremely similar toxic potentials. Comparison to the toxic potentials produced by the corresponding negative controls, comprising either the organisms incubated with glucose without the presence of RBOH or an aqueous solution of sodium dithionite, shows that each of these reductions produced products that were significantly more toxic than the background matrix (3.1-106 times). Unreduced RBOH exhibited a weak genotoxic potential, which was eliminated after reduction by *E. faecalis* but remained at approximately the same level after reduction by *C. butyricum* and sodium dithionite. However, comparison to the corresponding negative controls showed that there was no significant difference between reduced RBOH and the corresponding negative controls.

The two major fractions formed from reduction of RBOH by *E. faecalis*, termed 'vinyl sulphone' and 'diamino H-acid', were separated by HPLC and assessed for toxicological effects. It can be seen that the vinyl sulphone fraction exhibited a toxic potential similar to that of RBOH reduced by *E. faecalis*, which was 35.6 times more toxic than unreduced RBOH. Due to the limitations of the HPLC technique, an exact toxic potential of the diamino H-acid fraction could not be ascertained. These limitations also precluded genotoxicity analysis of both fractions. The commercially

available compound similar to diamino H-acid, 1-amino-8-naphthol-3,6-disulphonic acid (H-acid), exhibited a low toxic potential (221 times less than RBOH reduced by *E. faecalis*) and was non-genotoxic.

3.2 Bioreactors

3.2.1 Toxicity and genotoxicity of the Sequencing Batch Reactor

Samples of a synthetic textile effluent (STE) used to feed the sequencing batch reactors (SBRs) (known as feed) and their effluents were obtained and assayed throughout the entire 244 day period of operation. After appropriate preparation, samples were assayed within 2 h post-sampling using the Microtox® toxicity assay and the *E. coli* differential kill genotoxicity assay.

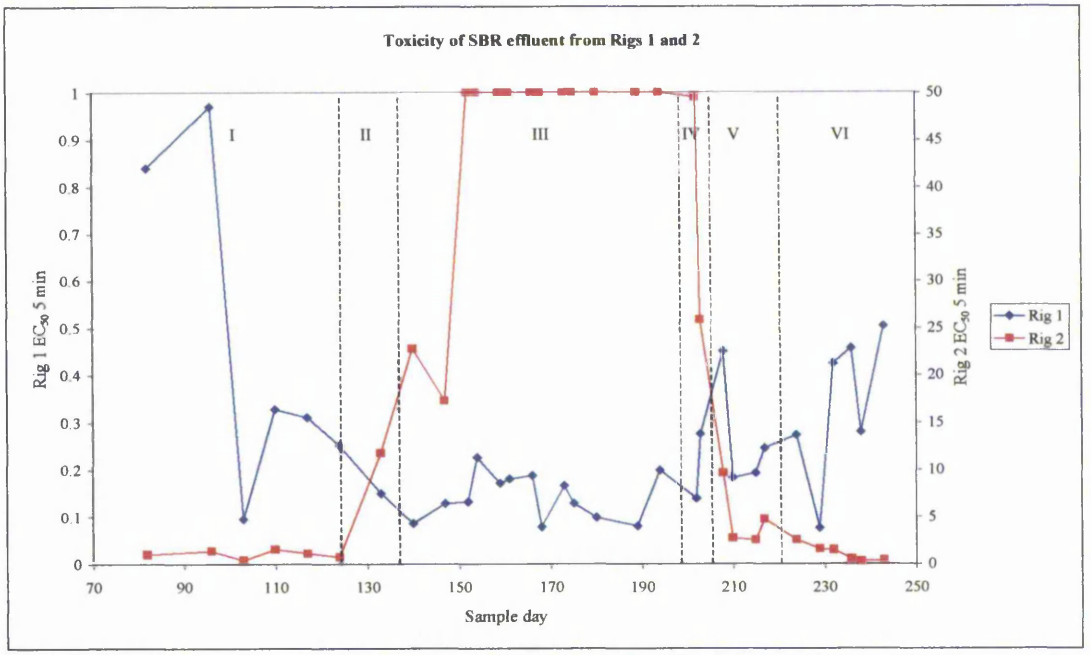
Table 3.10: Toxicity and genotoxicity of the STE with and without RBOH, and effluent after anaerobic and anaerobic-aerobic treatment in SBR Rigs 1 and 2

Sample	Toxicity (EC_{50} 5 min)	Genotoxicity (CS)
Feed		
STE with dye	11.47 ± 2.61 (14)	0.12 ± 0.08 (13)
STE without dye	>50.00 (8)	0.87 ± 0.37 (13)
Effluent		
Anaerobic (with dye)	0.24 ± 0.23 (21)	1.01 ± 0.41 (16)
Anaerobic-aerobic (with dye)	0.34 ± 0.16 (6)	0.93 ± 0.20 (6)
Anaerobic (without dye)	1.01 ± 0.48 (5)	0.98 ± 0.42 (7)
Anaerobic-aerobic (without dye)	>50.00 (13)	0.71 ± 0.36 (7)

N.B. Data with dye are for Rig 1, data without dye are for Rig 2. All toxicity values are expressed as percentage of original concentration, and values of >50% should be considered non-toxic. All genotoxicity values are shown for undiluted samples. Numbers in parenthesis are number of replicates of independent experiments.

The STE without the dye was non-toxic and non-genotoxic response (see Table 3.10). Inclusion of dye (RBOH) to the STE conferred both a toxic and genotoxic potential. Anaerobic treatment of the STE containing dye in the SBR increased toxicity (~48 times), and this toxicity was reduced ~1.5 times after anaerobic-aerobic treatment. It was also observed that the genotoxic potential of the STE containing dye was eliminated in both anaerobic and anaerobic-aerobic effluents. When the dye was absent, the anaerobic effluent had a toxic potential, which was eliminated in the anaerobic-aerobic effluent. The anaerobic effluent showed no genotoxicity, but a weak genotoxic response was observed in the anaerobic-aerobic effluent.

Figure 3.1: Toxicity of the effluent from SBR Rigs 1 and 2 during the period of operation



N.B. For stages of operation refer to Table 2.2. Toxicity values are expressed as percentage of original concentration. Values of 50% are representative of a value >50% of original concentration.

Analysis of the toxicity of the SBR effluents over time (Figure 3.1) showed that the toxicity of the effluent from the SBR decolourising RBOH (Rig 1) increased during stages I and II (anaerobic and anaerobic-aerobic operation) to settle at a stable level during stage III (anaerobic operation). When the aeration phase was reintroduced (stages V and VI) toxicity became more erratic but generally decreased. Toxicity of the effluent without the dye (Rig 2) remained at a fairly stable level during anaerobic operation (stage I), which quickly decreased during introduction of the aerobic phase (stage II) to become non-toxic during anaerobic-aerobic operation (stage III). As dye was introduced to the feed for Rig 2 (stages IV and V), toxicity rapidly increased again. When the dye was present at the maximum concentration treated (533 mgL^{-1}) (stage VI), toxicity continued to increase slightly over time from 2.52 to 0.39% of original concentration at the termination of the experiment.

3.2.2 Decolourising ability of the Sequencing Batch Reactor

3.2.2.1 Absorbance at visible wavelengths

The absorbance of the decolourised SBR effluents was measured over the visible range of wavelengths (400-700 nm) at 50 nm intervals after appropriate preparation. As

both Rig 1 and Rig 2 were used to decolourise RBOH under anaerobic-aerobic conditions (see Table 2.2 for operational conditions), data are given for both reactors.

Table 3.11: Absorbance of STE with RBOH, and effluent after anaerobic and anaerobic-aerobic treatment in SBR Rigs 1 and 2 at visible wavelengths

Sample	Wavelength (nm)													
	400		450		500		550		600		650		700	
	AU	%	AU	%	AU	%	AU	%	AU	%	AU	%	AU	%
Feed STE	5.8	-	5.1	-	6.6	-	12.5	-	16.0	-	6.6	-	0.6	-
Effluent														
Anaerobic	1.8	69	1.0	80	0.8	88	1.3	90	1.3	92	1.1	83	1.2	-100
Anaerobic -aerobic[a]	2.0	66	1.2	76	0.6	91	0.8	94	0.9	94	0.7	89	0.6	0
Anaerobic -aerobic[b]	1.9	67	1.3	74	0.7	89	0.9	93	0.9	94	0.8	88	0.7	-17

(Adapted from Shaw 2000)

N.B. AU columns show the average absorbance at the specified λ for the treatment phase. % columns show the percentage reduction of absorbance at the specified λ for the treatment phase compared with the STE. [a]Data for Rig 1, [b]data for Rig 2.

Reduction of absorbance at all measured wavelengths except 700 nm occurred after both anaerobic and anaerobic-aerobic treatment (see Table 3.11). After anaerobic-aerobic treatment, the percentage reduction in absorbance from the STE tended to be lower at wavelengths closer to the UV end of the spectrum (400 and 450 nm), higher at wavelengths around the wavelength exhibiting maximum absorbance (λ_{max}) of the dye (550, 600 and 650 nm), and remain unchanged at the remaining wavelengths (500 nm) in comparison to anaerobic treatment. An increase in absorbance at 700 nm (A_{700}) in the anaerobic effluent was observed, when compared to the STE. After anaerobic-aerobic treatment, the absorbance in both Rigs 1 and 2 returned to a similar level to that observed in the STE.

By construction of calibration curves, it was determined that the absorbance of RBOH at its λ_{max} (597 nm) was directly proportional to dye concentration (Shaw, pers. comm.). Therefore, A_{597} values were used to calculate the concentration of dye remaining in the treated effluents using the following formula: dye concentration (mgL^{-1}) = 34.023 × sample absorbance at 597 nm (Shaw, pers. comm.). From this the percentage of dye removal (decolourisation) was determined. As both Rig 1 and Rig 2 were used to decolourise RBOH under anaerobic-aerobic conditions, data are given for both reactors (Table 3.12).

Table 3.12: Percentage decolourisation of RBOH after anaerobic and anaerobic-aerobic treatment in SBR Rigs 1 and 2

Sample	Decolourisation (%)
Anaerobic	91.3 ± 0.8
Anaerobic-aerobic[a]	93.9 ± 1.3
Anaerobic-aerobic[b]	94.2 ± 0.6

(Adapted from Shaw 2000)

N.B. Data are average decolourisation values for the treatment phase, expressed as percentage reduction of dye concentration, showing standard deviation. [a]Data for Rig 1, [b]data for Rig 2.

Levels of RBOH decolourisation produced by both bioreactors were high under all atmospheric conditions (Table 3.12). Rigs 1 and 2 showed similar levels of decolourisation under anaerobic-aerobic conditions, both of which were slightly higher than the level observed under anaerobic conditions. Analysis by Student's t-test showed that the increase in decolourisation when the aerobic phase was introduced to Rig 1 was significant (t>tcrit = 10.2>2) (Shaw 2000).

3.2.2.2 Absorbance at UV, visible and IR wavelengths

The absorbance profiles of the SBR effluents were determined using a scanning spectrophotometer over the wavelength range 200-900 nm, which covers the UV, visible and IR regions of the spectrum. For comparison, the absorbance profiles of the STE both with and without inclusion of RBOH were obtained (see Figure 3.2), which exhibited high absorbance in the UV range (190-250 nm) with a peak at 198 nm (39 and 25 AU with and without dye respectively). Over the near UV and visible range (250-700 nm), absorbance of both STEs generally decreased, with absorbance of STE without dye dropping to below 0.1 AU after 600 nm and STE with dye reaching a similar level after 900 nm. It is apparent that STE with dye had two distinct absorbance peaks, at 308 and 590 nm, which were not present without dye.

It was discovered that the absorbance profiles of the SBR effluents showed a number of peaks at certain wavelengths (687, 754 and 972 nm), both after anaerobic and anaerobic-aerobic treatment. The absorbance values of these peaks, in addition to absorbance at 260 and 597 nm which correspond to the absorption maxima for aromatic amines formed by reduction of RBOH and the intact molecule respectively, were determined. As both Rig 1 and Rig 2 were used to decolourise RBOH under anaerobic-aerobic conditions, data are given for both reactors.

Figure 3.2: Absorbance profile of the STE with and without RBOH over the UV, visible and IR ranges of the spectrum

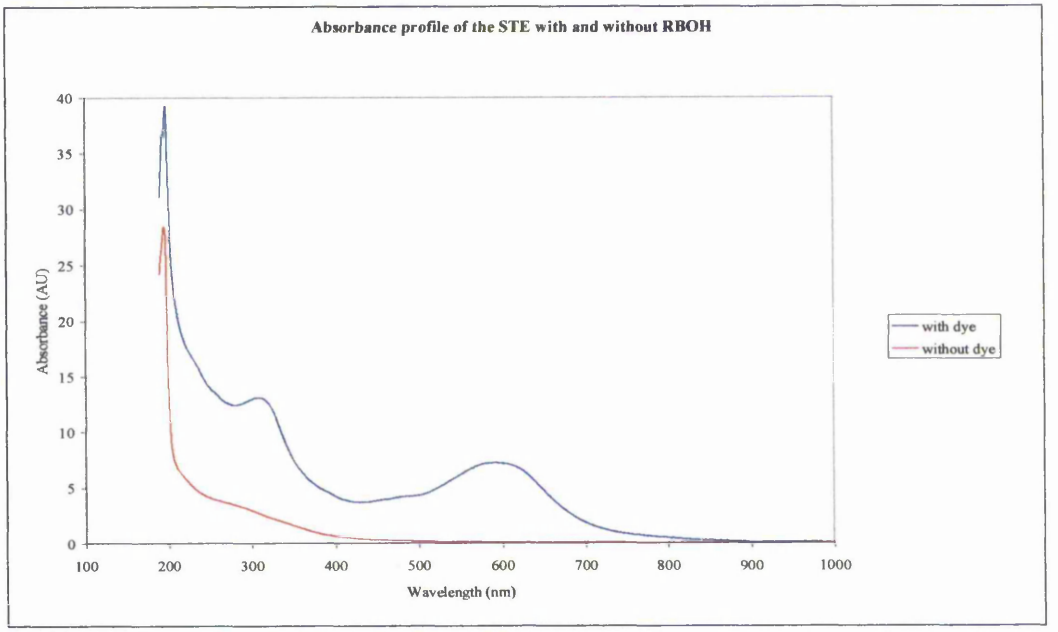


Table 3.13: Absorbance of STE with RBOH, and effluent after anaerobic and anaerobic-aerobic treatment in SBR Rigs 1 and 2 at observed wavelength peaks

Sample	Average absorbance at λ (nm) (approx. AU)				
	260	597	687	754	972
Feed					
STE	14.7	16.0	-	-	-
Effluent					
Anaerobic	24.0	1.4	-	1.5	1.5
Anaerobic-aerobic[a]	20.0	0.8	0.6	0.5	0.4
Anaerobic-aerobic[b]	22.0	0.8	0.7	0.4	0.4

(Adapted from Shaw 2000)

N.B. Data are average values for the treatment phase. [a]Data for Rig 1, [b]data for Rig 2.

During anaerobic operation, A_{260} increased with a concomitant decrease in A_{597} (corresponding to 91.3% decolourisation, see Table 3.12). A slight reduction in both A_{260} and A_{597} compared to anaerobic operation was observed under anaerobic-aerobic operation in both Rigs 1 and 2 (corresponding to 93.9 and 94.2% decolourisation in Rigs 1 and 2 respectively). The peak at 687 nm was only observed during anaerobic-aerobic treatment within the SBRs, and not when an anaerobic sample was left to oxidise in air. The peaks at 754 and 972 nm appeared after anaerobic treatment and were also seen to be unstable in air as shown by their reduction after anaerobic-aerobic treatment, and the fact that the peaks disappeared in oxidised samples with a concomitant increase in A_{597}.

3.2.3 Toxicity and genotoxicity of the Hybrid Anaerobic Baffled Reactor

Toxicity assays conducted on the hybrid anaerobic baffled reactor (HABR) samples were performed on the ToxAlert™ 100 instrument, using liquid-dried reagent, whilst assays conducted on the SBR samples were made on the Microtox®, using freeze-dried reagent. It was therefore necessary to conduct a comparative study of the two systems using reference toxicants, to determine whether the data produced from the HABRs and SBRs may be directly compared.

Table 3.14: Comparison of toxicity data determined by freeze-dried (Microtox®) and liquid-dried (ToxAlert™ 100) reagent

Compound	EC_{50} 5 min		EC_{50} 15 min	
	Microtox®	ToxAlert™ 100	Microtox®	ToxAlert™ 100
Phenol	19.2 ± 3.2 (3)	17.5 ± 2.7 (3)	21.2 ± 3.6 (3)	20.0 ± 2.4 (3)
Hydrolysed C.I. Reactive Black 5	11.4 ± 3.7 (2)	9.3 ± 1.2 (4)	7.8 ± 1.0 (2)	7.7 ± 0.5 (4)
1-amino-2-naphthol-6-sulphonate	369.3 ± 40.7 (3)	364.4 ± 92.6 (4)	321.7 ± 43.7 (3)	359.5 ± 103.1 (4)

N.B. All data are average EC_{50} at the specified contact time, expressed as mgL^{-1}, showing standard deviation. Numbers in parenthesis are number of replicates of independent experiments.

The data obtained for each of the reference toxicants, at both 5 and 15 min contact times, were found to be directly comparable for both instruments with all reference toxicants tested (Table 3.14), and therefore SBR and HABR data obtained using these instruments were directly comparable. It was observed that the standard deviation values for the ToxAlert™ 100 instrument were lower than the corresponding values for the Microtox® for the toxicants with lower EC_{50} values, such as RBOH and phenol, but higher for less toxic compounds. As the toxicity of bioreactor samples tested were in the same order of magnitude as the values obtained for pure RBOH and phenol, the ToxAlert™ 100 system was used to assess HABR samples. Additionally, more technical support was forthcoming from Merck Speciality Chemicals Ltd., the manufacturer of the ToxAlert™ 100 system.

Samples of STE and the effluent from the HABRs were obtained and assayed throughout the entire 229 day period of operation. After appropriate preparation, samples were assayed within 2 h post-sampling using the ToxAlert™ 100 toxicity assay and the *E. coli* differential kill genotoxicity assay.

Table 3.15: Toxicity and genotoxicity of STE with and without RBOH, and effluent after anaerobic, anaerobic-aerobic and anaerobic-anoxic-aerobic treatment in HABR Rigs A and B

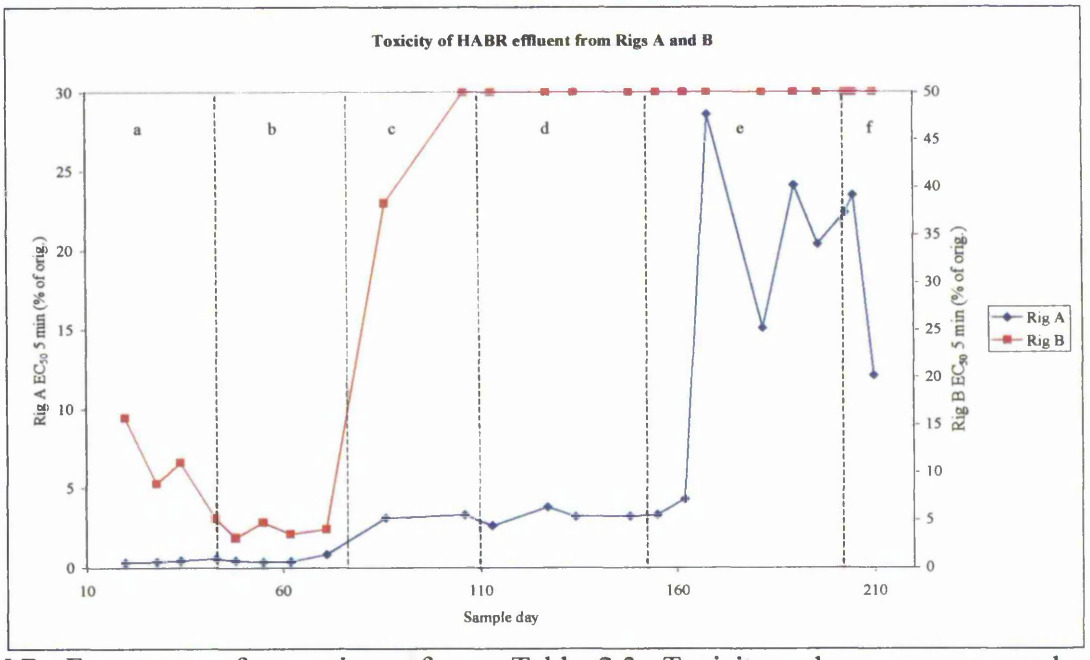
Sample	Toxicity (EC_{50} 5 min)	Genotoxicity (CS)
Feed		
STE with dye	4.97 ± 1.22 (22)	0.58 ± 0.19 (5)
STE without dye	>50.00 (16)	1.21 ± 0.29 (7)
Effluent		
Anaerobic (with dye)	0.46 ± 0.17 (8)	0.85 ± 0.27 (6)
Anaerobic-aerobic (unseeded) (with dye)	3.20 ± 0.38 (6)	0.58 (1)
Anaerobic-aerobic (seeded) (with dye)	16.89 ± 9.82 (7)	0.70 ± 0.05 (2)
Anaerobic-anoxic-aerobic (with dye)	17.80 ± 8.06 (2)	ND
Anaerobic (without dye)	6.98 ± 4.47 (8)	1.21 ± 0.52 (6)
Anaerobic-aerobic (unseeded) (without dye)	>50.00 (6)	0.85 (1)
Anaerobic-aerobic (seeded) (without dye)	>50.00 (7)	0.78 ± 0.17 (2)
Anaerobic-anoxic-aerobic (without dye)	>50.00 (2)	ND

N.B. Data with dye are for Rig A, data without dye are for Rig B. All toxicity values are expressed as percentage of original concentration, and values of >50% should be considered non-toxic. All genotoxicity values are shown for undiluted samples. Numbers in parenthesis are number of replicates of independent experiments. ND = not done.

The STE without the dye was found to be non-toxic and non-genotoxic (Table 3.15). Inclusion of RBOH to the STE conferred a toxic and a weakly genotoxic potential. It should be noted that these values were obtained on the ToxAlert™ 100 instrument, and were slightly different from those obtained on the Microtox® when testing the STE for use with the SBRs, although the composition of the STE was the same. When the dye was present in the HABR, the toxicity of the anaerobic effluent increased (~11 times) compared to the feed. Inclusion of aerated cells without seeding with biomass decreased toxicity by ~7 times. After seeding of the aerated cells, toxicity was further decreased (~5 times), Anaerobic-anoxic-aerobic treatment slightly reduced the toxicity further (~1.1 times), although this reduction was not significant in comparison to the alteration from anaerobic to anaerobic-aerobic treatment. The weakly genotoxic response observed by the STE with dye was eliminated after anaerobic treatment, but returned after anaerobic-aerobic treatment when the aerated cells were both unseeded and seeded.

When the dye was not present in the STE, the anaerobic effluent showed a toxic potential, which was again eliminated after anaerobic-aerobic treatment when the aerated cells were both unseeded and seeded, and when an anoxic phase was introduced. The anaerobic effluent showed no genotoxicity, but a weak genotoxic response was observed after anaerobic-aerobic treatment when the aerated cells were both unseeded and seeded.

Figure 3.3: Toxicity of the treated effluent with and without RBOH during the period of operation of the HABR



N.B. For stages of operation refer to Table 2.3. Toxicity values are expressed as percentage of original concentration. Values of 50% are representative of a value >50% of original concentration.

Analysis of the toxicity of the HABR effluents over time (Figure 3.3) showed that the toxicity of the effluent from Rig A remained at a stable level after anaerobic treatment (stages a and b) and thus a decrease in HRT, corresponding to an increase in OLR, did not affect toxicity. During anaerobic-aerobic treatment, when aerated cells were included but not seeded with biomass (stages c and d), toxicity decreased immediately and remained stable at this level. When the aerated cells were seeded with biomass (stage e), toxicity further decreased but the values were erratic. Under anaerobic-anoxic-aerobic conditions (stage f) toxicity appeared to increase slightly, but data is limited and the erratic nature of the values is still apparent. Toxicity of the effluent from Rig B increased during anaerobic operation at a longer HRT (stage a) and became stable with a decreased HRT (stage b) (see Figure 3.3). After anaerobic-aerobic

treatment, toxicity quickly decreased with the addition of one aerated cell (stage c), and became non-toxic during anaerobic-aerobic operation using three aerated cells, when the aerated cells were both unseeded and seeded (stages d and e). Under anaerobic-anoxic-aerobic conditions (stage f) the effluent was still non-toxic.

3.2.4 Decolourising ability of the Hybrid Anaerobic Baffled Reactor

3.2.4.1 Absorbance at visible wavelengths

The absorbance of the decolourised HABR effluents were measured over the visible range of wavelengths (400-700 nm) at 50 nm intervals after appropriate preparation.

Table 3.16: Absorbance of STE with RBOH, and effluent after anaerobic, anaerobic-aerobic and anaerobic-anoxic-aerobic treatment in HABR Rig A at visible wavelengths

Sample (operation stage)	Wavelength (nm)													
	400		450		500		550		600		650		700	
	AU	%	AU	%	AU	%	AU	%	AU	%	AU	%	AU	%
Feed STE	5.8	-	5.1	-	6.6	-	12.5	-	16.0	-	6.6	-	0.6	-
Effluent														
Anaerobic (a)	2.4	59	1.6	69	1.6	76	2.2	82	2.4	85	2.0	70	2.1	-250
Anaerobic (b)	1.5	74	1.1	78	1.3	80	1.8	86	2.1	87	1.6	76	1.5	-150
Anaerobic-aerobic (c)	1.3	78	1.1	78	1.2	82	1.9	85	2.4	85	1.8	73	0.6	0
Anaerobic-aerobic (d)	1.3	78	1.2	76	1.2	82	1.8	86	2.6	84	2.0	70	0.8	-33
Anaerobic-aerobic (e)	1.5	74	1.3	75	1.3	80	1.6	87	2.1	87	1.8	73	1.1	-83
Anaerobic-anoxic-aerobic (f)	1.4	76	1.0	80	1.0	85	1.5	88	2.1	87	2.1	68	2.2	-267

(Adapted from Shaw 2000)

N.B. For operation stage refer to Table 2.3. AU columns show the average absorbance at the specified λ for the treatment phase. % columns show the percentage reduction of absorbance at the specified λ for the treatment phase compared with the STE.

Reduction of absorbance at all wavelengths occurred after both anaerobic and anaerobic-aerobic treatment (Table 3.16), except absorbance at 700 nm (A_{700}). As HRT was decreased (stage b), the levels of percentage reduction from the STE increased at all wavelengths. The percentage reduction after anaerobic-aerobic treatment with the addition of one aerated cell (stage c) tended to be lower at wavelengths around the wavelength exhibiting maximum absorbance (λ_{max}) of the dye maximum (550, 600 and 650 nm) but higher at all other wavelengths. The addition of a further two aerated cells (stage d) did not significantly alter the absorbance profile. However, when the aerated cells were seeded with biomass (stage e), the opposite effect occurred and the

percentage reduction from the STE tended to be higher at wavelengths around the λ_{max} but lower at all other wavelengths. Anaerobic-anoxic-aerobic treatment (stage f) did not significantly alter the absorbance around the λ_{max}, but decreased the percentage reduction observed at other wavelengths especially the higher wavelengths (650 and 700 nm).

The absorbance of RBOH at its λ_{max} (597 nm) was found to be directly proportional to dye concentration (Shaw, pers. comm.). Therefore, A_{597} values were used to calculate the concentration of dye remaining in the treated effluents using the following formula: dye concentration (mgL^{-1}) = 34.023 × sample absorbance at 597 nm. From this, the percentage of dye removal (decolourisation) was determined.

Table 3.17: Percentage decolourisation of RBOH after anaerobic, anaerobic-aerobic and anaerobic-anoxic-aerobic treatment in HABR Rig A

Sample (operation stage)	Decolourisation (%)
Anaerobic (a)	85.1 ± 0.5
Anaerobic (b)	85.9 ± 0.6
Anaerobic-aerobic (c)	84.4 ± 0.6
Anaerobic-aerobic (d)	84.1 ± 0.5
Anaerobic-aerobic (e)	86.6 ± 1.5
Anaerobic-anoxic-aerobic (f)	87.6 ± 0.7

(Shaw 2000)

N.B. For operation stage refer to Table 2.3. Data are average decolourisation values expressed as percentage reduction of dye concentration showing standard deviation.

The levels of decolourisation of RBOH achieved were high, and similar during each stage of operation (see Table 3.17). However, during anaerobic operation, analysis by paired t test showed that the small increase in decolourisation caused by the decrease in HRT (stage b) was significant ($t>t_{crit}$ = 3.4>2.1) (Shaw 2000). After anaerobic-aerobic treatment with the addition of one aerated cell (stage c), a small decrease in decolourisation observed, which was also found to be significant ($t>t_{crit}$ = 5.9>2.1) (Shaw 2000). Inclusion of two further aerated cells (stage d) did not significantly alter the decolourising ability. When the aerobic cells were seeded with biomass (stage e), an increase in decolourisation was observed which was found to be significant ($t>t_{crit}$ = 6.3>2) (Shaw 2000). After anaerobic-anoxic-aerobic treatment (stage f) an increase in decolourisation was observed. This was found to be significant ($t>t_{crit}$ = 2.1>2) (Shaw 2000), although this significance is only marginal.

3.2.4.2 Absorbance at UV, visible and IR wavelengths

The absorbance profiles of the HABR effluents were determined using a scanning spectrophotometer over the wavelength range 200-900 nm, which covers the UV, visible and IR regions of the spectrum. It was discovered that the absorbance profiles of the effluents showed a number of peaks at certain wavelengths (687, 754 and 972 nm), both after anaerobic and anaerobic-aerobic treatment. The absorbance values of these peaks, in addition to absorbance at 260 and 597 nm which correspond to the absorption maxima for aromatic amines formed by reduction of RBOH and the intact molecule respectively, were determined.

Table 3.18: Absorbance of STE with RBOH, and effluent after anaerobic, anaerobic-aerobic and anaerobic-anoxic-aerobic treatment in HABR Rig A at observed wavelength peaks

Sample (operation stage)	Average absorbance at λ (nm) (approx. AU)				
	260	597	687	754	972
Feed STE	14.7	16.0	-	-	-
Effluent					
Anaerobic (a)	22.0	2.3	-	1.8	1.6
Anaerobic (b)	22.0	2.2	-	2.1	1.8
Anaerobic-aerobic (c)	20.0	2.5	-	0.4	0.1
Anaerobic-aerobic (d)	20.0	2.4	-	0.3	0.1
Anaerobic-aerobic (e)	10.0	1.7	1.6	0.4	0.1
Anaerobic-anoxic-aerobic (f)	10.0	2.0	2.3	0.4	0.1

(Adapted from Shaw 2000)

N.B. For operation stage refer to Table 2.3. Data are the average for the treatment phase.

During anaerobic operation (stages a and b), A_{260} increased with a concomitant decrease in A_{597} (Table 3.18). After anaerobic-aerobic treatment (stages c and d), A_{260} decreased whilst A_{597} slightly increased. When the aerated cells were seeded with biomass (stage e) A_{260} decreased by 50%, and remained at this level during anaerobic-anoxic-aerobic treatment (stage f). A decrease in A_{597} was observed during anaerobic-aerobic treatment when the aerated cells were seeded with biomass (stage e), which slightly increased during anaerobic-anoxic-aerobic treatment (stage f). It was observed that a peak at 687 nm appeared during anaerobic-anoxic-aerobic treatment (stage f) and intermittently during anaerobic-aerobic operation (stage e), which was also observed

during anaerobic-aerobic operation of the SBR (see Table 3.13). The peaks at 754 and 972 nm appeared after anaerobic treatment (stages a and b), and were also seen to be unstable in air as shown by their reduction after anaerobic-aerobic treatment (stages c and d). It is also interesting to note that the level of these peaks appeared to be unaffected by seeding of the aerated cells with biomass (stage e) or during anaerobic-anoxic-aerobic treatment (stage f). Analysis of the peak wavelengths in individual cells of the HABR showed that the biological processes that produce/reduce the compounds absorbing at these wavelengths occurred within the first cell of that treatment type. For cell numbering, refer to Figure 2.6 and Table 2.3 (cell 1 at the beginning of the reactor and subsequent cells numbered sequentially). The exception to this observation is absorbance at 597 nm, which was reduced in cell 1 but then further reduced in cell 2 during all operation stages.

3.3 Bioreactor microflora

3.3.1 Assessment of Hybrid Anaerobic Baffled Reactor microflora by traditional culturing techniques

The ability of selected colonies isolated under aerobic and anaerobic conditions from HABR samples taken on day 55 of operation to decolourise RBOH was assessed. Further to this, an identification of selected colonies including those with decolourising ability was made using biochemical techniques. For an explanation of the type of API identification kit used to identify isolates, see Table 2.6. Unfortunately, identification of aerobically isolated colonies using API STAPH, API 20NE or API 20E resulted in inconclusive identifications, possibly due to the logistical problems encountered with processing a large number of isolates simultaneously or impurity of the selected colonies. Therefore, aerobically isolated colonies were assigned to the genus or group *Staphylococcus* sp., *Micrococcus* sp., *Pseudomonas* sp. Enterobacteriacae or *Bacillus* sp. based on the results of the primary identification tests. Problems were also encountered with identification of anaerobically isolated colonies using API rapidID 32A, but here colonies could be assigned to four genera *Propionibacterium*, *Actinomyces*, *Prevotella* or *Bacteroides* spp.. Anaerobically isolated colonies that were assigned to the genera *Clostridium* sp. based on the results of primary tests were identified as such.

Table 3.19: Numbers and identification of aerobic and anaerobic isolates decolourising RBOH in HABR Rigs A and B on day 55 of operation

Rig	Cell no.	No. decolourising/ number isolates		Identification (no. decolourising/no. isolated)								
		Aerobic	Anaerobic	Aerobic				Anaerobic				
				St.	Ps.	Ent.	Bac.	Cl.	Pro.	Act.	Prv.	Bct.
A	1	0 / 6	1 / 5	0/2	0/3	0/1		0/2	0/1			0/1
	4	0 / 6	2 / 5	0/2	0/1			1/1	0/1	1/1		
	7	0 / 3	3 / 4	0/2				1/1				
B	1	0 / 7	1 / 4	0/1	0/4	0/1		1/1			0/1	
	4	0 / 8	3 / 4	0/2	0/2	0/1	0/1	0/1		0/1		
	7	0 / 7	3 / 5	0/3	0/3					1/2		1/1

Identification key: St. = *Staphylococcus* sp. Ps. = *Pseudomonas* sp. Ent. = Enterobacteriacae Bac. = *Bacillus* sp. Cl. = *Clostridium* sp. Pro. = *Propionibacterium* sp. Act. = *Actinomyces* sp. Prv. = *Prevotella* sp. Bct. = *Bacteroides* sp.. Not all isolates were identified.

It is apparent that there were no colonies isolated under aerobic conditions that were capable of decolourisation of RBOH under the same oxygen conditions, and that there were equal numbers of decolourising colonies isolated under anaerobic conditions in Rig A and Rig B (see Table 3.19). Identification of aerobic isolates showed that most isolates from both reactors were either *Staphylococcus* or *Pseudomonas* spp. (a total of 6 and 4 for Rig A and 6 and 9 for Rig B respectively). There were more isolates from more genera/groups at the beginning and middle of the reactors (cells 1 and 4) than at the end (cell 7). There were no Enterobacteriacae or *Bacillus* sp. at the end of either Rig A or B. Identification of anaerobic isolates showed that most were assigned to the genera *Clostridium* sp. and then *Propionibacterium* sp. in Rig A (a total of 4 and 2 respectively), whilst most isolates from Rig B were *Actinomyces* sp. and then *Clostridium* sp. (a total of 3 and 2 respectively). An anaerobic isolate identified as *Prevotella* sp. was observed in cell 1 of Rig B but not in Rig A, whilst anaerobic isolates identified as *Bacteroides* sp. were observed in both reactors. Decolourising colonies of *Clostridium* and *Actinomyces* spp. were isolated from both rigs, but contrary to expectations only the *Bacteroides* sp. isolate from Rig B was capable of decolourising RBOH.

3.3.2 Assessment of Sequencing Batch Reactor microflora diversity by Fluorescent *in situ* Hybridisation

Initial studies were conducted comparing the results obtained using SBR sludge samples fixed for both Gram positive and Gram negative organisms. A total of 5 random fields of view per sample were counted, and this amount has been shown to give a statistically meaningful representation of the sample by a nested ANOVAR

conducted on FISH data from an activated sludge (Davenport *et al.* 2000). As no differences in numbers or signal intensity were observed (data not shown), all subsequent FISH assays were conducted using samples fixed for Gram negative organisms, which was considered to be a more rigorous fixation procedure in common with the protocols in most other studies using FISH.

The positive control organisms *Escherichia coli* (bacteria) and *Haloferax denitrificans* (archaeum) showed excellent specificity of the probes used. *E. coli* showed positive signals from short rods with the bacterial probe EUB338 and no signal with the archaeal probe ARCH915, and *H. denitrificans* showed positive signals from small, irregular rods with ARCH915 and no signals with EUB338. Most studies utilising FISH assess cellular numbers stained using the non-specific DNA binding molecule 4',6-diamidine-2-phenylindole (DAPI) viewed using UV excitation, and then express numbers of domain or species specific cells observed as a percentage of the DAPI stained cells. However, DAPI stained fields showed an extremely high number of very small and irregular shaped points of bright yellow staining, some of which did not correspond to probed cellular structures and some of which were created by bleedthrough from intensely probed cells. It was therefore extremely difficult to determine cellular counts using DAPI staining, as many bright yellow points could not be ascribed to cellular structures or differentiated from acellular structures. Thus, only numbers of probed bacteria and archaea were determined, although it is appreciated that these numbers will not represent the total number of organisms present in the SBR sludge samples.

3.3.2.1 Numbers of bacterial and archaeal cells

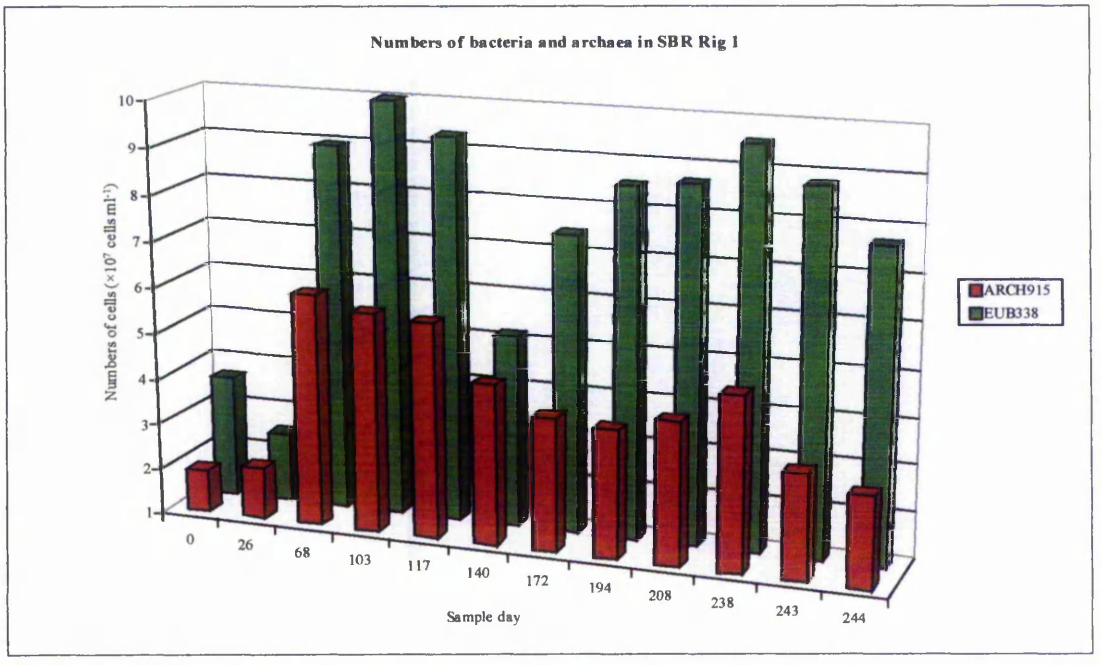
The cellular numbers of bacteria and archaea in the SBRs were assessed using the RNA bacterial probe EUB338 and the RNA archaeal probe ARCH915. Negative controls in the form of a DNA bacterial probe NONEUB and no probe were used to adjust the observed cellular counts with respect to specific binding of the probes to RNA and fluorescence produced by autofluorescent agents such as dust and chlorophyll. The ratios of archaeal to bacterial cells present within the SBRs were also calculated.

Table 3.20: Numbers of bacteria and archaea in SBR Rig 1 sludge assessed by FISH

Sample day	No. bacteria (cells ml^{-1})	% DNA binding	% auto-fluorescence	No. archaea (cells ml^{-1})	% auto-fluorescence	archaea: bacteria
0 (seed)	3.7×10^{7}	10.0	12.3	1.9×10^{7}	23.1	0.53
26	2.5×10^{7}	1.8	5.2	2.1×10^{7}	6.3	0.84
68	9.0×10^{7}	0.3	1.3	6.0×10^{7}	1.9	0.67
103	1.0×10^{8}	0.2	0.6	5.8×10^{7}	1.0	0.57
117	9.3×10^{7}	0.1	0.8	5.7×10^{7}	1.3	0.61
140	5.2×10^{7}	0	0.6	4.5×10^{7}	0.7	0.87
172	7.4×10^{7}	0	0.1	3.9×10^{7}	0.2	0.52
194	8.5×10^{7}	0	0.2	3.8×10^{7}	0.4	0.44
208	8.7×10^{7}	0	0.5	4.1×10^{7}	1.0	0.47
238	9.6×10^{7}	0	0.1	4.8×10^{7}	0.2	0.50
243	8.8×10^{7}	0	0	3.3×10^{7}	0	0.37
244	7.7×10^{7}	0	0	3.0×10^{7}	0	0.38

N.B. Number of bacteria are after adjustment for DNA binding and autofluorescence, whilst number of archaea are after adjustment for autofluorescence only. % DNA binding and autofluorescence are expressed as a percentage of unadjusted bacterial and archaeal counts. For oxygen conditions, refer to Table 2.4.

Figure 3.4: Changes in numbers of bacteria (EUB338) and archaea (ARCH915) in SBR Rig 1 over time



The number of bacteria in Rig 1, which was operated with the inclusion of RBOH, increased slightly over time from the initial seed, peaking after 68 days (see Table 3.20). There was then a slight fall in bacterial numbers at sample day 117, after which numbers again increased to approximately the same level. Archaeal numbers increased slightly from the initial seed, and then remained at a fairly steady level throughout the period of operation of the reactor, although they were always present at

lower numbers than the bacteria. The change in cellular numbers over time is graphically represented in Figure 3.4. With the exception of peaks in the ratio at sample days 26 and 140, where bacterial numbers are lower, the ratio was fairly constant at 0.5-0.6 until the end of the period of operation when the ratio decreased to approximately 0.4. The percentage of bacterial cells observed with EUB338 that were also non-specifically stained by NONEUB started at 10% in the initial seed, but then numbers rapidly decreased to a much lower level and were absent after 140 days. The amount of autofluorescent material determined by unprobed counts was initially just over 12 and 23% of total bacterial and archaeal counts respectively, but then decreased to around 1% for both after 26 days.

Table 3.21: Numbers of bacteria and archaea in SBR Rig 2 sludge assessed by FISH

Sample day	No. bacteria (cells ml^{-1})	% DNA binding	% auto-fluorescence	No. archaea (cells ml^{-1})	% auto-fluorescence	archaea: bacteria
0 (seed)	4.5×10^{7}	5.0	11.5	2.1×10^{7}	23.0	0.46
26	1.7×10^{7}	9.7	10.1	1.2×10^{7}	14.4	0.75
68	7.6×10^{7}	2.3	1.7	7.4×10^{7}	1.7	0.98
103	9.9×10^{7}	0.8	1.2	7.2×10^{7}	1.6	0.73
117	7.7×10^{7}	0	4.1	6.0×10^{7}	5.2	0.77
140	6.0×10^{7}	0	3.6	-	-	-
172	5.3×10^{7}	0	1.1	4.8×10^{7}	1.2	0.90
194	4.6×10^{7}	0.2	2.8	5.1×10^{7}	2.5	1.10
208	7.2×10^{7}	0	1.0	6.4×10^{7}	1.2	0.89
238	5.2×10^{7}	0	0.2	6.9×10^{7}	0.1	1.32
243	3.9×10^{7}	0	0	1.7×10^{7}	0	0.43
244	4.8×10^{7}	0	0.7	-	-	-

N.B. Number of bacteria are after adjustment for DNA binding and autofluorescence, whilst number of archaea are after adjustment for autofluorescence only. % DNA binding and autofluorescence are expressed as a percentage of unadjusted bacterial and archaeal counts. For oxygen conditions, refer to Table 2.4. - = data unobtainable.

The number of bacteria in Rig 2, which was operated initially without the inclusion of RBOH, decreased slightly at the next sample date after the initial seed (day 26), but then increased again peaking after 68 days (see Table 3.21). Levels then remained at a fairly steady level throughout the period of operation. Archaeal numbers followed approximately the same trend, although as in Rig 1 they were always present at lower numbers than the bacteria. Data on numbers of archaeal cells are missing from sample days 140 and 244, where only a large amount of background biomass was observed with no individual cellular structures visible. The change in cellular numbers over time in Rig 2 is graphically represented in Figure 3.5.

3.3.3 Assessment of Hybrid Anaerobic Baffled Reactor microflora diversity by Fluorescent *in situ* Hybridisation

3.3.3.1 Numbers of bacterial and archaeal cells

Comments concerning use of samples fixed for Gram negative organisms, number of data points, positive control cultures and DAPI stained samples given in section 3.4.1 also apply to assessment of HABR samples. The cellular numbers of bacteria and archaea in the HABRs were assessed using the RNA bacterial probe EUB338 and the RNA archaeal probe ARCH915. Negative controls in the form of a DNA bacterial probe NONEUB and no probe were used to adjust the observed cellular counts with respect to specific binding of the probes to RNA and fluorescence produced by autofluorescent agents such as dust and chlorophyll. The ratios of archaeal to bacterial cells present within the HABRs were also calculated.

Samples were collected from various compartments (referred to as cells), corresponding to the beginning, middle or end of the reactors, and were operated under various oxygen conditions. Samples on sample day 230 consisted of sludge taken from within the reactor sludge blanket, and biofilm taken from the sides of the perspex walls above the liquor level.

In Rig A, which was operated with the inclusion of RBOH in the STE, the number of bacteria in cell 1 increased over time from the initial seed, peaking on day 190, and then remained fairly constant (see Table 3.22). Numbers within cell 4 and especially 7 remained fairly steady throughout the period of operation, and were lower than those observed in cell 1. Bacterial numbers in cell 8 decreased from the initial seed by the first sample day, 34 days after inoculation, but then recovered to their original levels that were higher than those observed in cells 4 and 7. The same trend was observed with archaeal numbers, with the exception that archaea decreased in cell 8 from the initial seed by the first sample day and then stayed steady at that level for the remaining period of operation. The change in cellular numbers over time is graphically represented in Figure 3.8.

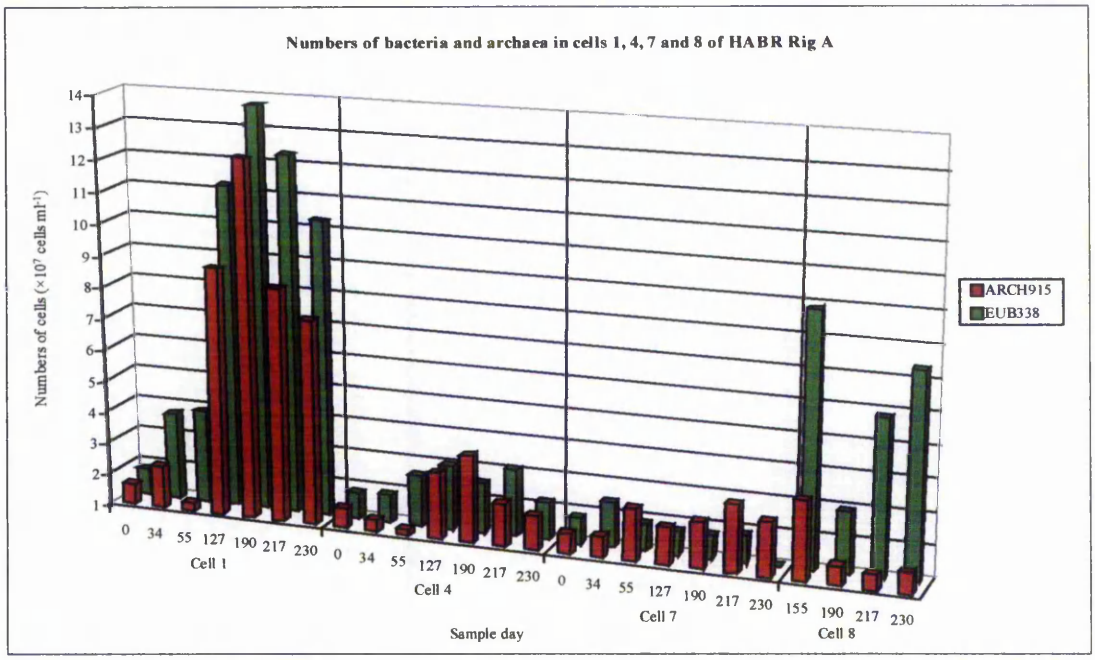
Table 3.22: Numbers of bacteria and archaea in HABR Rig A sludge assessed by FISH

Sample day	Cell no.	No. bacteria (cells ml^{-1})	% DNA binding	% auto-fluorescence	No. archaea (cells ml^{-1})	% auto-fluorescence	archaea: bacteria
0 (seed)	1-7	1.8×10^{7}	0.9	2.1	1.6×10^{7}	2.3	0.90
34	1	3.7×10^{7}	0.9	1.1	2.3×10^{7}	1.8	0.62
	4	1.8×10^{7}	3.0	1.9	1.4×10^{7}	2.6	0.74
	7	2.4×10^{7}	2.0	1.5	1.7×10^{7}	2.2	0.70
55	1	3.9×10^{7}	0.6	0.9	1.3×10^{7}	2.7	0.32
	4	2.6×10^{7}	3.0	1.3	1.2×10^{7}	2.9	0.46
	7	1.9×10^{7}	0.9	2.2	2.6×10^{7}	1.5	1.43
127	1	1.1×10^{8}	0.1	0.3	8.8×10^{7}	0.4	0.79
	4	3.0×10^{7}	0.5	1.1	3.1×10^{7}	1.1	1.04
	7	1.8×10^{7}	0.0	2.4	2.2×10^{7}	1.9	1.26
155 (seed)	8-10	8.9×10^{7}	0.5	0.5	3.5×10^{7}	1.3	0.39
190	1	1.4×10^{8}	0.3	0.2	1.2×10^{8}	0.2	0.90
	4	2.6×10^{7}	1.6	1.3	3.7×10^{7}	0.9	1.46
	7	1.8×10^{7}	2.6	2.0	2.5×10^{7}	1.5	1.41
	8	2.9×10^{7}	1.5	1.1	1.6×10^{7}	2.1	0.55
217	1	1.2×10^{8}	0.0	0.3	8.4×10^{7}	0.5	0.68
	4	3.1×10^{7}	1.4	1.0	2.3×10^{7}	1.4	0.76
	5	3.3×10^{7}	1.3	1.1	1.6×10^{7}	2.4	0.49
	7	1.8×10^{7}	2.6	2.3	3.1×10^{7}	1.4	1.69
	8	5.9×10^{7}	0.6	0.3	1.5×10^{7}	1.1	0.25
230 (sludge)	1	1.0×10^{8}	0.6	0.2	7.4×10^{7}	0.3	0.72
	4	2.2×10^{7}	2.1	1.6	2.1×10^{7}	1.8	0.94
	5	2.3×10^{7}	1.9	1.7	1.6×10^{7}	2.3	0.72
	7	9.6×10^{6}	4.1	3.5	2.7×10^{7}	1.3	2.81
230 (biofilm)	1	8.2×10^{7}	0.5	0.2	7.1×10^{7}	0.2	0.87
	4	3.2×10^{7}	2.5	1.1	2.2×10^{7}	1.7	0.69
	5	6.3×10^{7}	0.1	0.5	3.5×10^{7}	0.9	0.55
	7	4.1×10^{7}	2.5	0.8	1.4×10^{7}	2.3	0.34
	8	7.4×10^{7}	0.6	0.5	1.7×10^{7}	2.1	0.23
	10	7.1×10^{7}	0.6	0.5	1.5×10^{7}	2.3	0.21

N.B. Number of bacteria are after adjustment for DNA binding and autofluorescence, whilst number of archaea are after adjustment for autofluorescence only. % DNA binding and autofluorescence are expressed as a percentage of unadjusted bacterial and archaeal counts. For oxygen conditions of cells, refer to Table 2.5.

With the exception of a decrease in archaeal numbers on sample day 55 in cells 1 and 4, ratios were fairly constant in cells 1, 4 and 8 over the period of operation (an average of 0.66, 0.90 and 0.36 respectively). It was observed that the ratio in cell 7 increased steadily throughout the period of operation from 0.7-2.8. The percentage of bacterial cells observed with EUB338 that were also non-specifically stained by NONEUB varied between 0 and 3% (average 1.3%) throughout the period of operation. The amount of autofluorescent material determined by unprobed counts was initially just over 2 and 2.3% of bacterial and archaeal counts respectively, but then decreased to an average of 1.2 and 1.6% of total counts. It was observed that unprobed counts were generally lowest in cell 1, and increased in cells 4 then 7 and then 8.

Figure 3.8: Changes in numbers of bacteria (EUB338) and archaea (ARCH915) in HABR Rig A over time



In Rig B, which was operated without the inclusion of RBOH to the STE, the numbers of bacteria in cell 1 increased over time from the initial seed, peaking after day 127, after which numbers remained fairly steady at a slightly lower level for the remainder of the period of operation (see Table 3.23). Numbers within cell 4 slightly increased whilst those observed in cell 7 remained fairly steady throughout the period of operation, both of which were lower than those observed in cell 1. Bacterial numbers in cell 8 decreased from the initial seed by the first sample day, 34 days after inoculation, and remained steady. The same trend was observed with archaea, with the exception that numbers plateau in cell 1 after day 55. The change in cellular numbers over time is graphically represented in Figure 3.9.

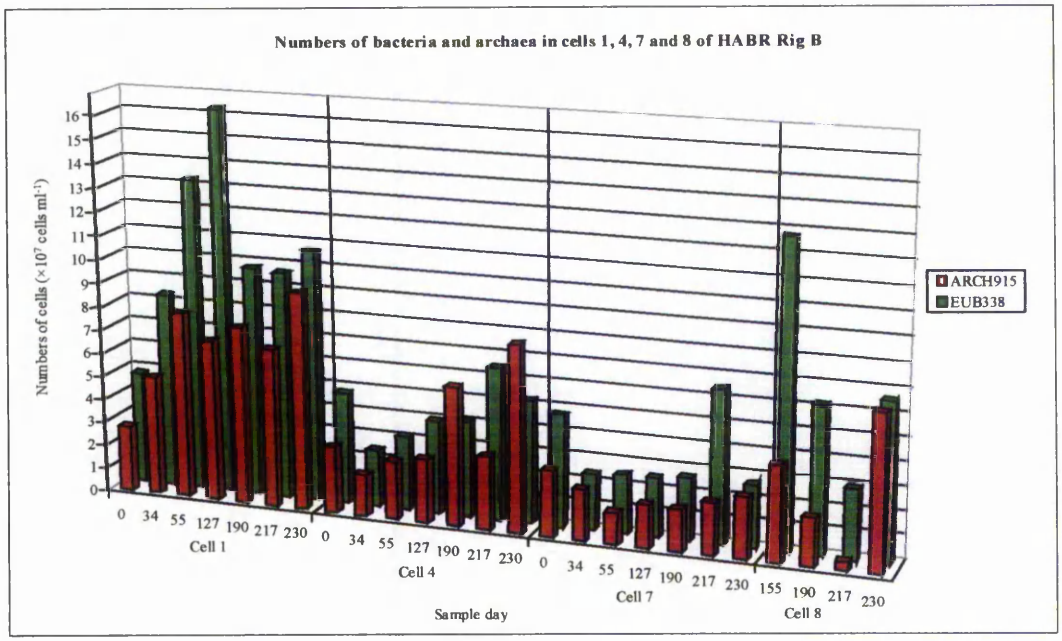
Ratios followed a similar trend to those in Rig A, except the increase in cell 7 was not observed (an average of 0.67, 0.94, 0.69 and 0.27 for cells 1, 4, 7 and 8 respectively). The percentage of bacterial cells observed with EUB338 that were also non-specifically stained by NONEUB varied between 1 and 4% (average 2.1%) throughout the period of operation. The amount of autofluorescent material determined by unprobed counts varied between 0.2 and 2.0% (average 1.0%) and 0.1 and 3.3% (average 1.6%) for total bacterial and archaeal counts respectively. As with Rig A, unprobed counts were generally lowest in cell 1, and increased in cells 4 then 7 and then 8.

Table 3.23: Numbers of bacteria and archaea in HABR Rig B sludge assessed by FISH

Sample day	Cell number	No. bacteria (cells ml^{-1})	% DNA binding	% auto-fluorescence	No. archaea (cells ml^{-1})	% auto-fluorescence	archaea: bacteria
0 (seed)	1-7	4.9×10^{7}	2.1	0.9	2.9×10^{7}	1.5	0.60
34	1	8.4×10^{7}	1.3	0.6	5.2×10^{7}	0.9	0.61
	4	2.6×10^{7}	3.7	1.8	1.8×10^{7}	2.7	0.72
	7	2.5×10^{7}	3.9	2.0	2.2×10^{7}	2.3	0.90
55	1	1.3×10^{8}	0.8	0.2	8.0×10^{7}	0.3	0.60
	4	3.2×10^{7}	3.0	1.6	2.6×10^{7}	2.1	0.79
	7	2.6×10^{7}	4.0	1.8	1.4×10^{7}	3.5	0.55
127	1	1.6×10^{8}	0.7	0.3	6.9×10^{7}	0.6	0.42
	4	4.0×10^{7}	3.0	1.3	2.8×10^{7}	1.9	0.70
	7	2.6×10^{7}	3.8	2.0	1.9×10^{7}	2.9	0.72
155 (seed)	8-10	1.3×10^{8}	0.8	0.4	4.1×10^{7}	1.1	0.32
190	1	9.8×10^{7}	1.1	0.1	7.6×10^{7}	0.1	0.78
	4	4.1×10^{7}	2.1	1.1	6.0×10^{7}	0.8	1.45
	7	2.8×10^{7}	3.8	1.9	1.9×10^{7}	2.9	0.68
	8	6.3×10^{7}	1.7	0.4	2.1×10^{7}	1.2	0.34
217	1	9.8×10^{7}	1.1	0.2	6.8×10^{7}	0.2	0.70
	4	6.5×10^{7}	1.1	0.7	3.2×10^{7}	1.4	0.49
	5	5.7×10^{7}	1.8	0.9	1.6×10^{7}	3.1	0.28
	7	6.5×10^{7}	1.6	0.8	2.3×10^{7}	2.3	0.35
	8	3.0×10^{7}	3.3	0.3	4.7×10^{6}	1.7	0.16
230 (sludge)	1	1.1×10^{8}	0.9	0.5	9.3×10^{7}	0.6	0.87
	4	5.3×10^{7}	1.9	0.8	8.0×10^{7}	0.5	1.51
	5	4.7×10^{7}	2.1	1.2	1.7×10^{7}	3.3	0.36
	7	2.8×10^{7}	3.8	2.0	$2.\times10^{7}$	2.2	0.96
230 (biofilm)	1	8.4×10^{7}	1.2	0.7	6.9×10^{7}	0.8	0.82
	4	3.8×10^{7}	2.7	1.5	6.1×10^{7}	1.0	1.61
	5	8.2×10^{7}	1.3	0.8	3.4×10^{7}	1.8	0.42
	7	6.9×10^{7}	1.5	0.8	1.9×10^{7}	2.8	0.27
	8	6.7×10^{7}	1.6	0.8	6.6×10^{7}	0.8	0.98
	10	5.5×10^{7}	1.9	0.9	4.9×10^{7}	1.0	0.89

N.B. Number of bacteria are after adjustment for DNA binding and autofluorescence, whilst number of archaea are after adjustment for autofluorescence only. % DNA binding and autofluorescence are expressed as a percentage of unadjusted bacterial and archaeal counts. For oxygen conditions of cells, refer to Table 2.5.

Figure 3.9: Changes in numbers of bacteria (EUB338) and archaea (ARCH915) in HABR Rig B over time



3.3.3.2 Morphology of bacterial and archaeal cells

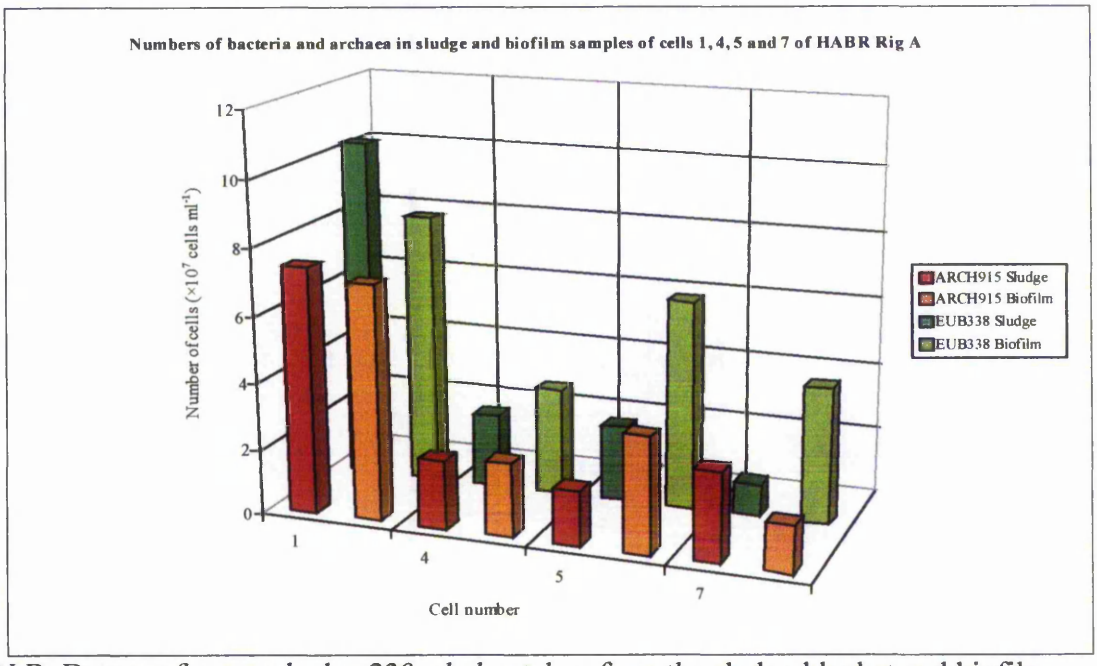
The morphology of the bacterial and archaeal cells was observed during FISH analysis. The most common bacterial morphology in the anaerobic sludge used to seed the HABRs was long rods, followed by short rods, filaments and cocci. By the first sample date after seeding, the most common morphology in the anaerobic cells was short rods, with long rods, cocci and occasionally filaments constituting variable percentages of the remaining morphologies. There were no obvious differences in morphologies between different anaerobic cells. The most common bacterial morphologies in the activated sludge used to seed the aerobic cells of the HABRs were short rods and cocci, followed by long rods and filaments. The abundance of these morphologies varied for the remainder of the period of operation in both HABRs in the aerobic cells.

The population composition appeared more stable for the archaeal cells. The most common archaeal morphology in the anaerobic sludge was short rods, followed by cocci, filaments and long rods. This morphological appearance did not obviously alter over the period of operation in all anaerobic cells. The most common archaeal morphologies in the activated sludge used to seed the aerobic cells of the HABRs were short rods and cocci, followed by filaments. This morphological appearance did not obviously alter over the remainder of the period of operation in Rig A, although long rods were observed in Rig B.

3.3.3.3 Numbers of bacterial and archaeal cells in sludge and biofilm samples

Sludge samples were taken from each HABR on day 230 from the sludge blanket as normal, and in addition biofilm samples growing on the side walls of the reactors were taken from the same cells. The numbers of bacteria and archaea in each sample were determined, which can be seen in Tables 3.27 and 3.28, in order to compare the influence of differences in community organisation.

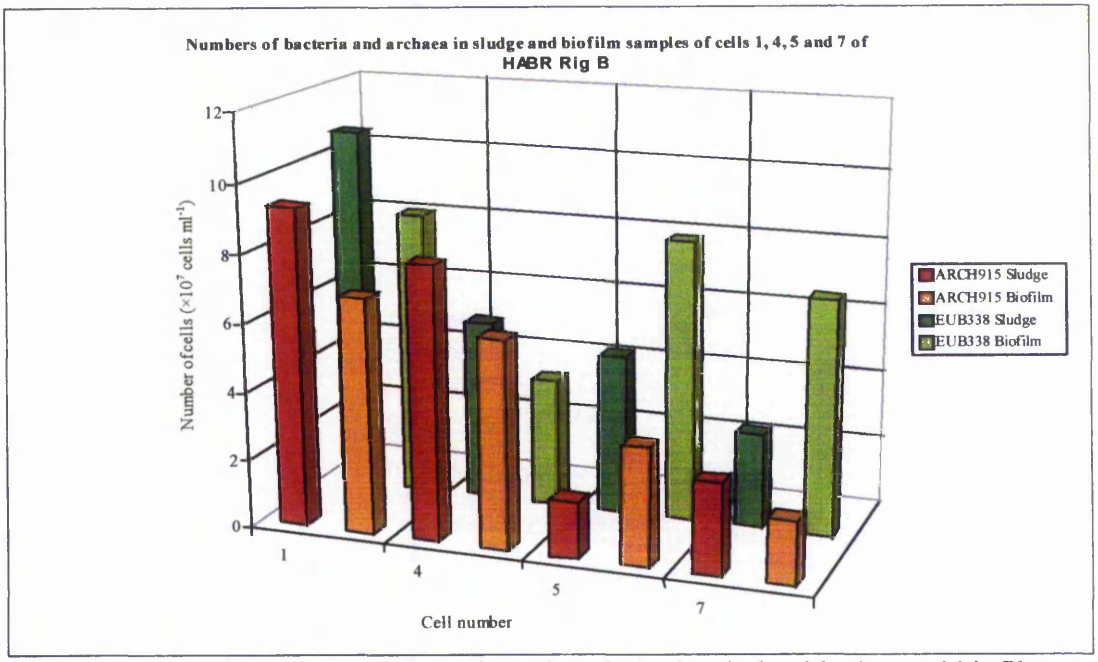
Figure 3.10: Comparison of bacterial (EUB338) and archaeal (ARCH915) numbers between sludge and biofilm samples of HABR Rig A



N.B. Data are for sample day 230, sludge taken from the sludge blanket and biofilm taken from the side walls above the liquor level.

The higher number of both bacterial and archaeal cells in cell 1 compared to the remaining cells observed during the whole period of operation was again apparent in these samples (see Figures 3.10 and 3.11). Numbers of archaea in sludge samples were not found to be significantly different to numbers observed within the biofilms in all cells in either reactor. Bacterial numbers in the sludge of cell 1 were slightly higher than numbers in the biofilm of that cell in both Rig A and Rig B, but these increases were not found to be significantly different. In contrast, in cells 4, 5 and 7 of Rig A and 5 and 7 of Rig B, there was an increase in the number of bacteria in biofilm samples compared with sludge samples. These increases were found to be significant ($p = 0.05$ and 0.02 for Rigs A and B respectively, paired t-test).

Figure 3.11: Comparison of bacterial (EUB338) and archaeal (ARCH915) numbers between sludge and biofilm samples of HABR Rig B

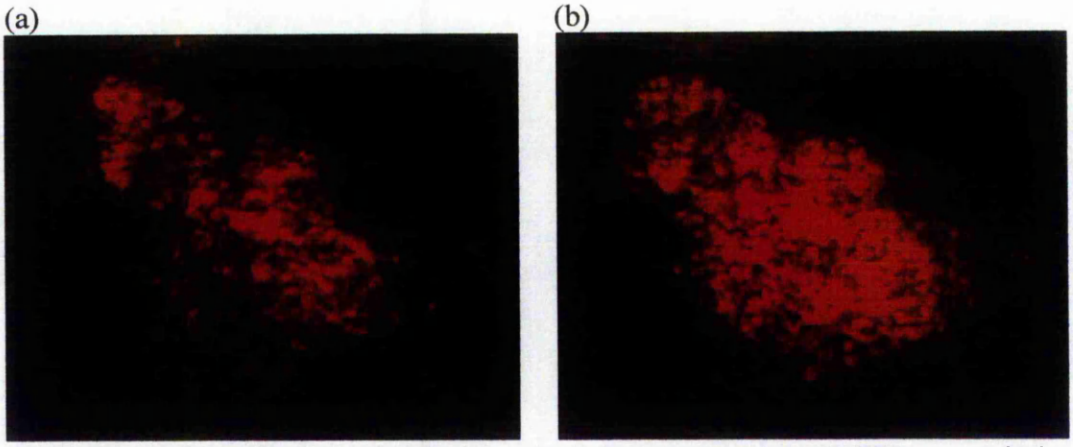


N.B. Data are for sample day 230, sludge taken from the sludge blanket and biofilm taken from the side walls above the liquor level.

3.3.3.4 Distinctive bacterial cells in sludges from the Sequencing Batch Reactor and the Hybrid Anaerobic Baffled Reactor

A point of interest was the appearance of distinctive bacterial cells in certain sludges of the two types of bioreactor studied. These cells were large cocci, approximately $^{1}/_{60}$ of the diameter of the microscope field of view and therefore approximately 3µm in diameter, forming tightly packed aggregates. Figure 3.12 shows these cells hybridised with the bacterial probe EUB338, taken using the confocal scanning laser microscope as a series of pictures by scanning in the z-position. Figure 3.12a shows the coccoid morphology of the individual cells, whilst Figure 3.12b clearly shows the density of the cells within the aggregate. The cells were observed in samples taken from the bioreactors operated both with and without the inclusion of RBOH, under anaerobic, anoxic and aerobic conditions (Rig 1 day 238, Rig 2 day 117, Rig A cell 5 day 217 and cell 4 day 230, Rig B cells 4-8 days 217 and 230).

Figure 3.12: CSLM image of large coccoid bacteria hybridised with EUB338



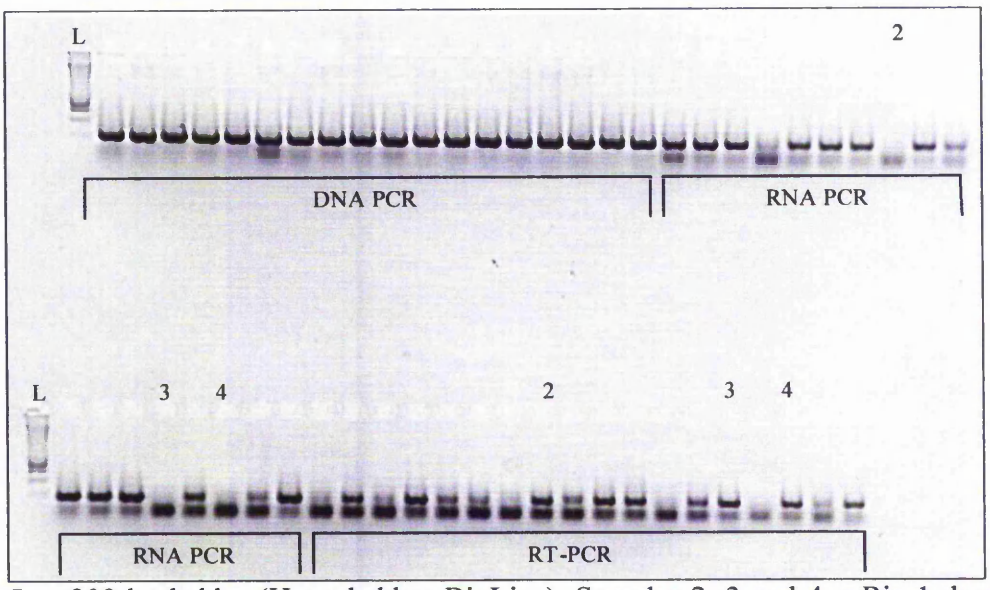
(a) First z-position (b) Second z-position. Both images excitation λ 550 nm, emission λ 565 nm, ×600 magnification.

3.3.4 Assessment of Sequencing Batch Reactor microflora diversity by Denaturing Gradient Gel Electrophoresis

3.3.4.1 Comparison of DNA and RNA derived DGGE profiles

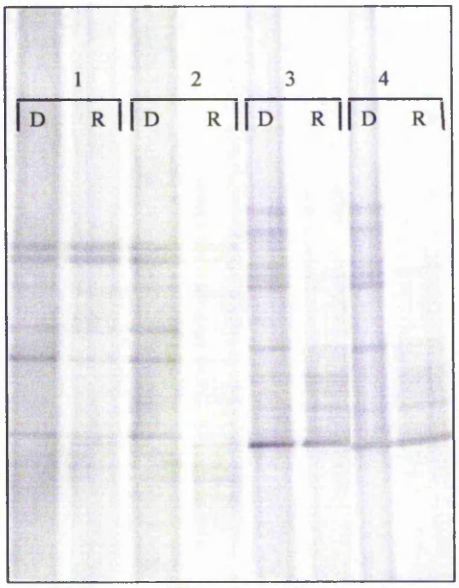
Of the sludge samples from the SBRs, only 4 were successfully amplified by RT-PCR i.e. did not produce a product from PCR of RNA but did from PCR of cDNA (see Figure 3.13). These samples were run in DGGE gels next to their corresponding DNA PCR products. It can be seen from Figure 3.14 that there is virtually no difference between the DNA and RNA profiles obtained, with the exception that the RNA profiles were weaker and thus some fainter bands within the DNA profile cannot be visualised in the RNA profile. Due to the problems encountered with obtaining pure RNA extracts, and the fact that there is no significant difference in the RNA profiles compared to the DNA profiles, it was decided to use only TNA extracts for amplification before DGGE analysis for all subsequent samples, which will give profiles of organisms based upon their 16S rRNA gene.

Figure 3.13: PCR of DNA, RNA and cDNA (RT-PCR) from SBR Rigs 1 and 2



Key: L = 200 bp ladder (Hyperladder, BioLine). Samples 2, 3 and 4 = Rig 1 day 172, Rig 2 day 238 and Rig 2 day 243. Sample 1 (Rig 1 day 140) data not shown.

Figure 3.14: DGGE of DNA and RNA from SBR Rigs 1 and 2



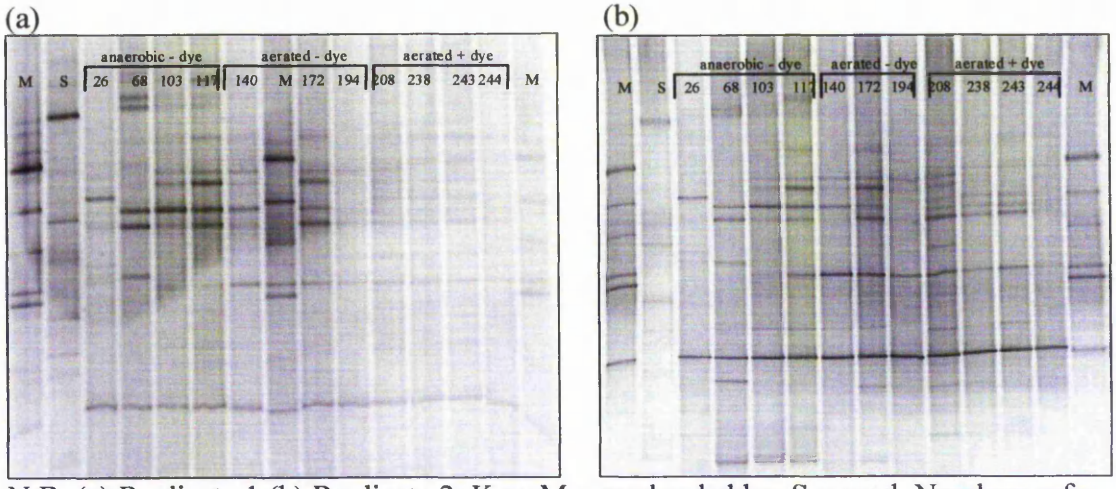
Key: D = DNA profile, R = RNA profile. Sample number as in Figure 3.13 key. Note that picture is a composite of two separate gels.

3.3.4.2 Bacterial DGGE profiles and analysis of divergence

The 16S rRNA genes of the bacterial population in both SBRs were amplified and separated in DGGE gels. All samples were amplified and separated in three independent experiments, showing essentially identical profiles. Figure 3.15 shows the DGGE profiles obtained from Rig 2 after two independent amplification and separation experiments. It can be seen that, although wider separation of the bands was achieved

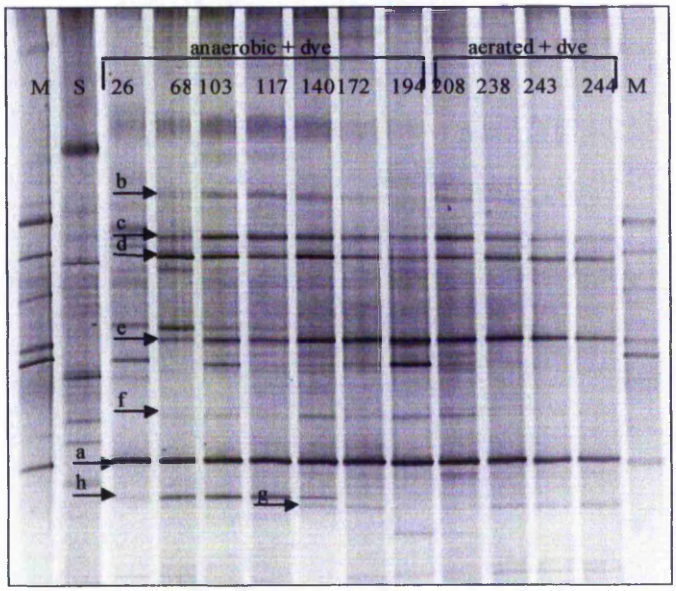
during the first replicate (Figure 3.15a), there were similar numbers of bands in similar positions relative to the marker ladder in the second replicate (Figure 3.15b). Analysis of divergence of the population over time and according to environmental parameters was conducted using gel analysis software (Phoretix 1D Advanced v4.01, Phoretix International) and construction of dendograms.

Figure 3.15: Comparison of the bacterial DGGE profiles obtained from Rig 2 after two independent experiments



N.B. (a) Replicate 1 (b) Replicate 2. Key: M = marker ladder, S = seed. Numbers refer to sample days.

Figure 3.16: DGGE profile of the bacterial population in SBR Rig 1

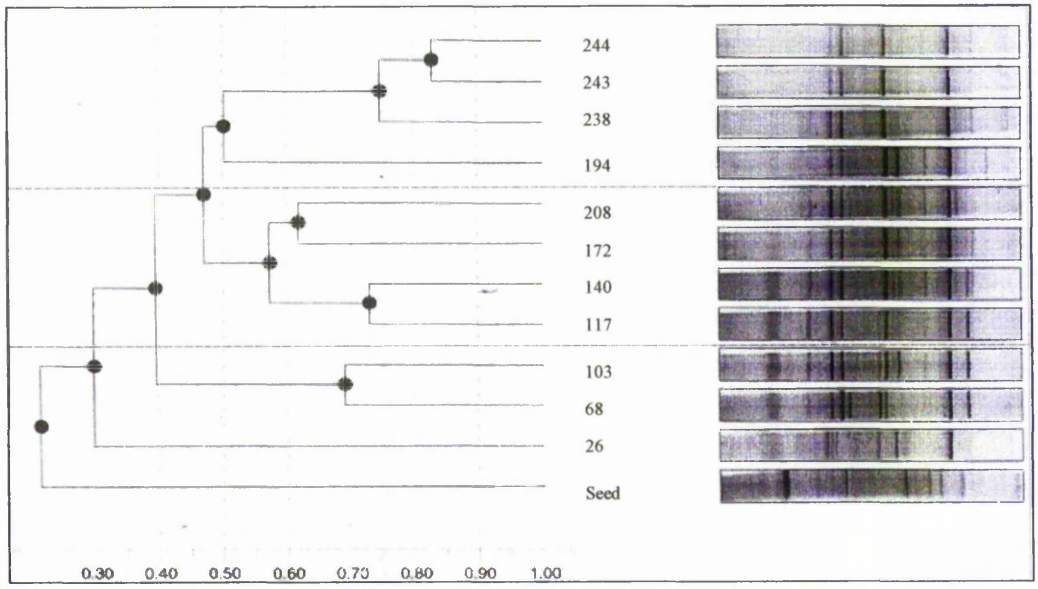


Key: M = marker ladder, S = seed. Numbers refer to sample days.

The profile for Rig 1 altered dramatically from the initial seed by day 26, and that this profile again changed at day 68 (see Figure 3.16). After this sample point, the profile changed very little for the remainder of the period of operation. There are some

significant bands within the profile. There was an extremely strong band that appeared at day 26 (indicated by arrow a) and persisted throughout the period of operation. There were at least 5 other bands that appeared at day 68 and persisted for the period of operation (indicated by arrows b-f). It is interesting that there was apparently only one band which was stimulated by aeration of the reactor (indicated by arrow g), which appeared when the reactor was aerated (from day 140), reduced when aeration was turned off (days 194 and 208), and then reappeared when aeration was begun again (day 238 onwards). There are also bands that seemed to disappear when aeration was initially begun on day 125 (such as that indicated by arrow h).

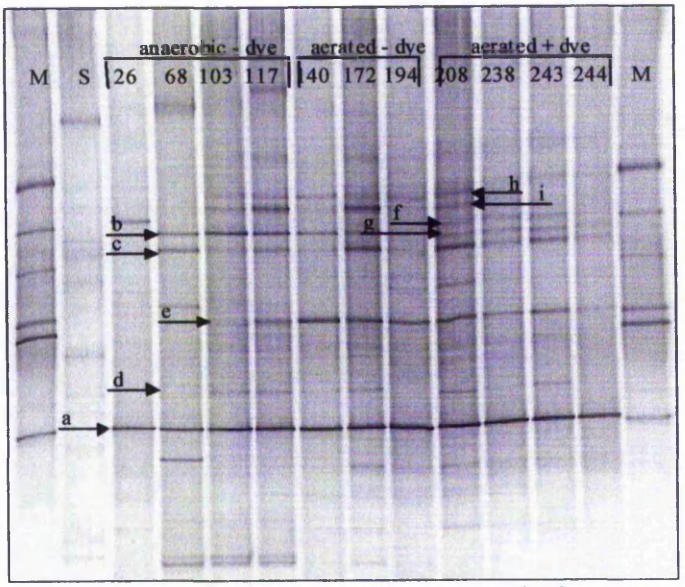
Figure 3.17: Dendogram of the bacterial population in SBR Rig 1 based on DGGE band matching



N.B. Numbers refer to sample days.

It can be seen from band matching and subsequent construction of a dendogram that SBR Rig 1 samples clustered into 3 main sets (Figure 3.17). The initial seed and sample from day 26 showed only ~20 and 30% similarity to the rest of the samples respectively, demonstrating the massive divergence of the bacterial population from the seed sludge that occurred over the first 68 days of operation. The first set comprised samples taken from the reactor operating under anaerobic conditions (days 68 and 103), showing ~40% similarity to the rest of the samples. The second set comprised samples from anaerobic conditions later in the period of operation (days 117-208 except day 194). The third set comprised samples taken from the reactor operating under anaerobic-aerobic conditions (days 194 and 238-244), showing ~47% similarity to the anaerobic set.

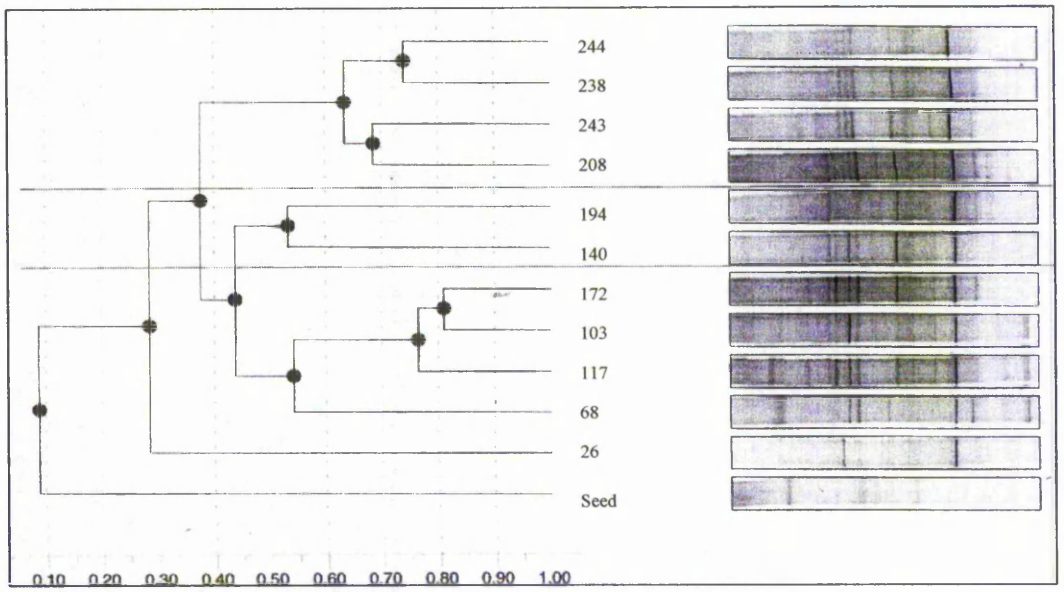
Figure 3.18: DGGE profile of the bacterial population in SBR Rig 2



Key: M = marker ladder, S = seed. Numbers refer to sample days.

As with Rig 1, the population in Rig 2 altered dramatically from the initial seed by day 26 and then further by day 68 (see Figure 3.18). However, it is apparent that the population within Rig 2 was far more diverse than in Rig 1, as there were many more bands present in the profile. There are some significant bands within the profile. There was an extremely strong band (indicated by arrow a) that appeared at day 26 and persisted for the remainder of the period of operation, although both oxygen conditions were altered and RBOH was added. There are also at least 4 other bands that appeared on days 68 or 103 and persisted for the remainder of the period of operation (indicated by arrows b-e). There are also bands that either appeared or disappeared when RBOH was introduced to the STE, during the anaerobic-aerobic operating stage of the reactor. Arrows f and g indicate bands that appeared during this stage, and bands h and i indicate bands that disappeared.

Figure 3.19: Dendogram of the bacterial population in SBR Rig 2 based on DGGE band matching



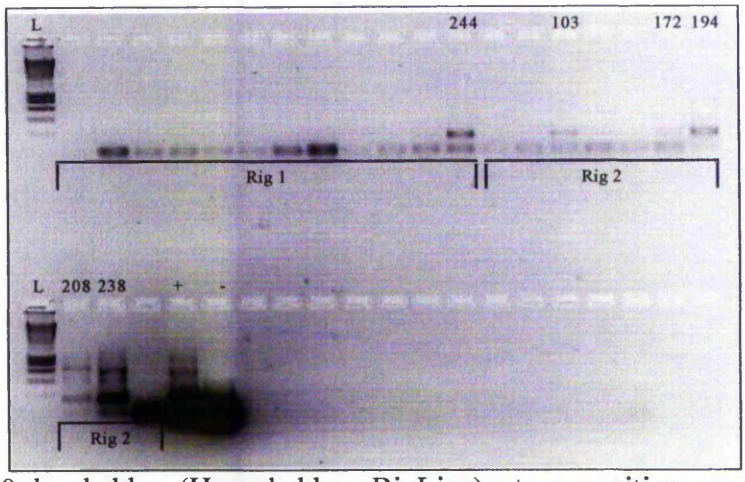
N.B. Numbers refer to sample days.

It can be seen from band matching and subsequent construction of a dendogram that samples from SBR Rig 2 clustered into 3 main sets (Figure 3.19). The initial seed and sample from day 26 showed only ~10 and 30% similarity to the rest of the samples respectively. The first set comprised samples taken from the reactor operating under anaerobic conditions (days 68-117). The second set comprised samples from the reactor operating under anaerobic-aerobic conditions without the inclusion of RBOH to the STE (days 140 and 194), showing ~43% similarity with the anaerobic set. The third set comprised samples taken from the reactor operating under anaerobic-aerobic conditions with RBOH added to the STE (days 208-244), showing ~38% similarity with the anaerobic-aerobic set when dye was not added.

3.3.4.3 Archaeal DGGE profiles and analysis of divergence

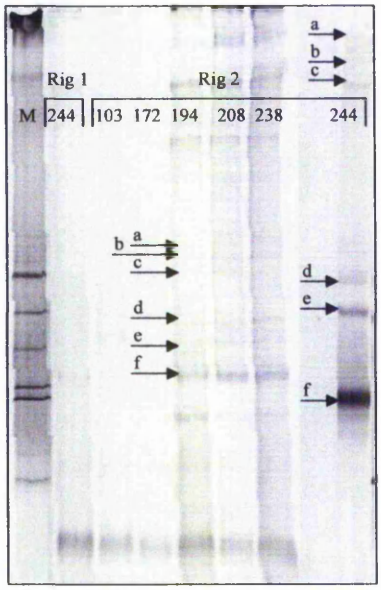
The 16S rRNA genes of the archaeal population in both SBRs were amplified and separated in DGGE gels. Archaea could only be amplified from Rig 1 on sample day 244 and from Rig 2 on days 103, 172-238 and 244 (see Figure 3.20). Therefore the analysis of divergence of the population over time and according to environmental parameters, conducted using gel analysis software (Phoretix 1D Advanced v4.01, Phoretix International) and construction of the dendograms was necessarily limited.

Figure 3.20: Amplification of archaeal 16S rRNA genes from SBR Rigs 1 and 2



Key: L = 200 bp ladder (Hyperladder, BioLine), + = positive control (*Acidanus brierleyi*, archaeum), - = negative control (*Arcobacter butzleri*, bacterium). Numbers refer to sample days, samples in consecutive lanes (see Table 2.4). Reduced exposure showed product in positive control and no product in Rig 2 day 243 and negative control. Rig 2 day 244 data not shown.

Figure 3.21: DGGE profile of the archaeal population in SBR Rigs 1 and 2



Key: M = marker ladder. Numbers refer to sample days. Note that picture is a composite of two separate gels.

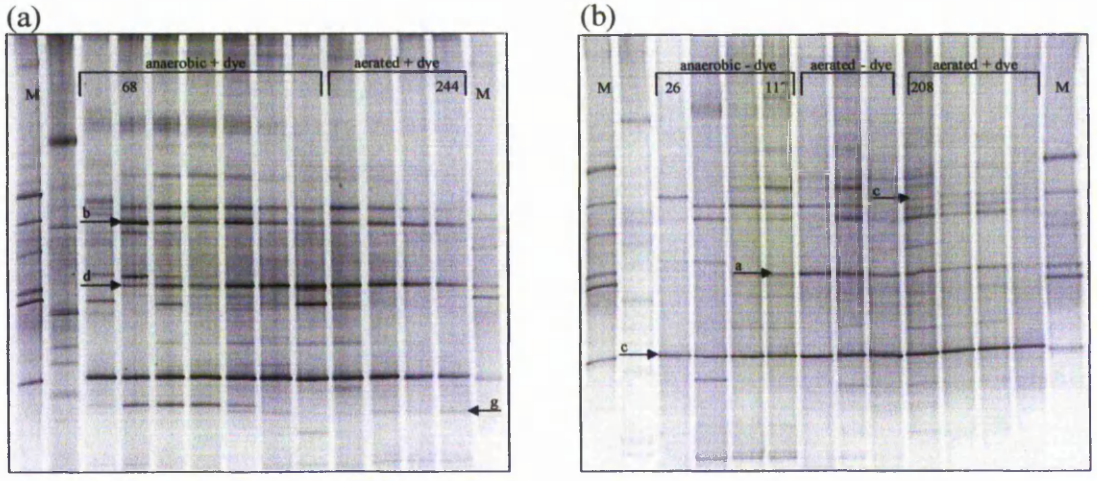
It can be seen that there was unfortunately not enough amplicon to produce visible DGGE profiles for samples taken from Rig 1 on day 244 or Rig 2 on days 103 and 172 (Figure 3.21). There were 6 main bands (indicated by arrows a-f) present within the profiles, relatively fewer in number compared with the bacterial profiles, and the profiles did not alter significantly in any of the samples. The bands in the profile for Rig 2 day 244 appear more widely spaced and further up the picture than in other profiles when compared to the marker ladder, but this is an image taken from a separate gel.

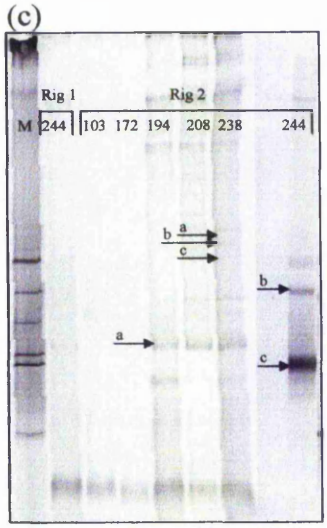
Band matching and subsequent construction of a dendogram was performed (data not shown) excluding the sample taken from Rig 2 on day 244, as this would require cross-comparison to a different gel which is not recommended, and excluding samples from Rig 1 on day 244 and Rig 2 on days 103 and 172. It was observed that the sample taken from Rig 2 on day 194, when no dye was included in the STE, showed ~60% similarity to samples taken on both days 208 and 238, when dye was included in the STE. The sample from day 208, when 53 mgL^{-1} of RBOH was included in the STE, showed ~90% similarity to day 238 when 533.3 mgL^{-1} of RBOH was included.

3.3.4.4 Sequencing of bands from bacterial and archaeal DGGE profiles

Bands of interest from the bacterial and archaeal SBR profiles, highlighted in Figure 3.22a-c, were excised and sequenced. Matches of the sequences produced using the forward and reverse primers and their consensus sequences to the nucleotides database held at the National Centre of Biotechnology Information (NCBI) were made, and the most likely identification of the band was determined based upon matches produced by each of the sequences.

Figure 3.22: DGGE profiles of the bacterial and archaeal populations in the SBRs, showing bands excised for sequencing





(a) Bacterial profile of Rig 1 (b) Bacterial profile of Rig 2 (c) Archaeal profile of Rigs 1 and 2. For all profiles, the number indicates the sample day, the band letter refers to the band on the sample day in question (see Table 3.24), and M is the marker ladder.

Table 3.24: Identification of 16S rRNA genes of microorganisms in the SBRs by sequencing

Rig	Sample day	Domain	Band	Reactor conditions	Identification	Database match
1	68	Bacteria	b	+ dye, anaerobic + aerated	*Clostridium* sp.	*Clostridium thermocellum* [AJ410280] (116/127)
1	68	Bacteria	d	+ dye, anaerobic + aerated	*Bacteroides* sp.	*Bacteroides uniformis* [AB050110] (120/129)
1	244	Bacteria	g	+ dye, anaerobic + aerated	Actinomycete	*Actinomadura aurantiaca* [AF134066] (22/22)
2	26	Bacteria	c	+/- dye, anaerobic + aerated	*Bacteroides* sp. / *Prevotella* sp.	*Bacteroides eggerthii* NCTC11185 [L16485] (61/61)
2	117	Bacteria	a	+/- dye, anaerobic + aerated	Invalid sequence	
2	208	Bacteria	c	+ dye, aerated only	Invalid sequence	
2	194	Archaea	a	+/- dye, aerated only	No sequence obtained	
2	238	Archaea	a	+/- dye, aerated only	No sequence obtained	
2	238	Archaea	b	+/- dye, aerated only	No sequence obtained	
2	238	Archaea	c	+/- dye, aerated only	No sequence obtained	
2	244	Archaea	b	+/- dye, aerated only	*Methanosaeta* sp. / *Methanothrix* sp.	*Methanosaeta concilii* [M59146] (35/37)
2	244	Archaea	c	+/- dye, aerated only	*Methanosaeta* sp. / *Methanothrix* sp.	*Methanosaeta concilii* [M59146] (59/61)

N.B. Database match shows best match of the sequences to the database, with NCBI Accession number in brackets and identities in parentheses. All database matches were to 16S rRNA genes (partial).

Of 12 excised bands, 4 did not produce any sequence data and 2 produced sequence data that did not show valid microbial matches to the databases (see Table

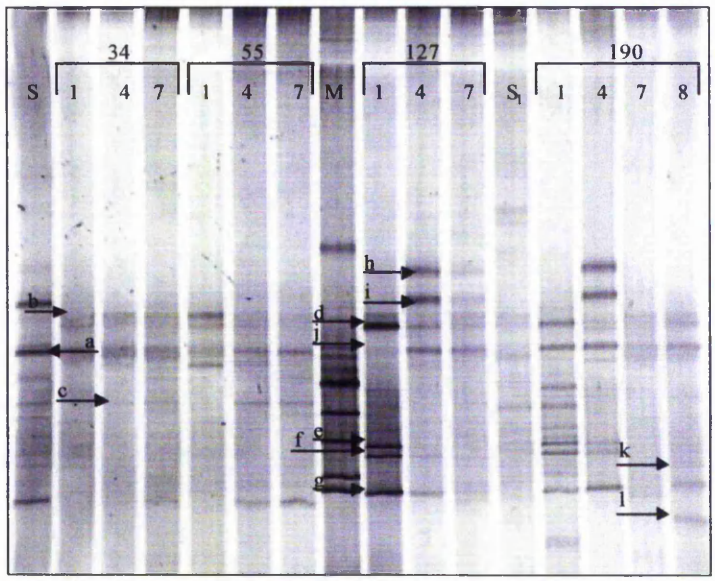
3.24). In Rig 1, which was operated with the inclusion of RBOH, two predominant bacterial bands that were persistent throughout the period of operation were identified as *Clostridium* and *Bacteroides* spp.. A band identified as the Actinomycete *Actinomadura* sp. was also present in some anaerobic and all aerated samples. It should be remembered that the anaerobic samples in which this band was observed were only those taken after aeration of Rig 1 was commenced, and that it disappeared some time after aeration was ceased and only reappeared after aeration was recommenced. Therefore, this band can be considered as present only under aerated conditions. In Rig 2, a persistent bacterial band was identified as *Bacteroides* or *Prevotella* spp. (although the closest match was to *Bacteroides eggerthii* NCTC 11185), whilst 2 archaeal bands only amplified at the end of operation were identified as *Methanosaeta* or *Methanothrix* spp..

3.3.5 Assessment of Hybrid Anaerobic Baffled Reactor microflora diversity by Denaturing Gradient Gel Electrophoresis

3.3.5.1 Bacterial DGGE profiles and analysis of divergence

The 16S rRNA genes of the bacterial population in both HABRs were amplified and separated in DGGE gels. All samples were amplified and separated in two independent experiments, showing essentially identical profiles. Analysis of divergence of the population over time and according to environmental parameters and spatial arrangement was conducted using gel analysis software (Phoretix 1D Advanced v4.01, Phoretix International) and construction of dendograms.

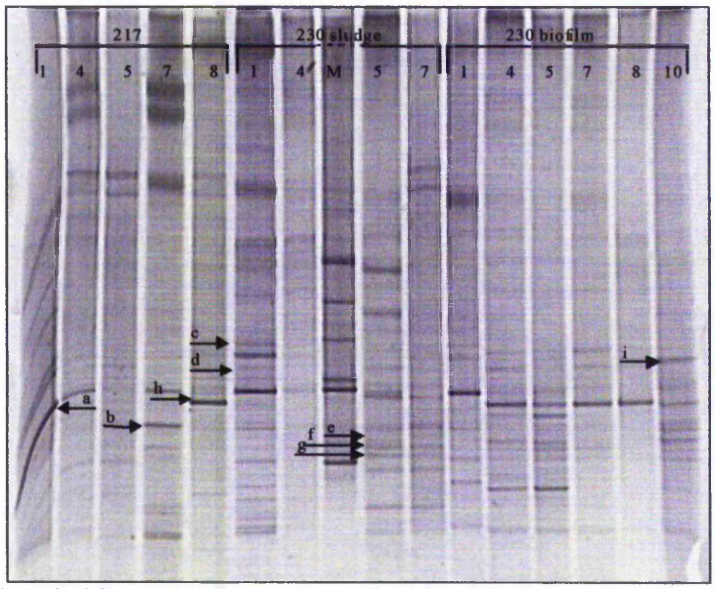
Figure 3.23: DGGE profile of the bacterial population in HABR Rig A up to day 190 of operation



Key: M = marker ladder, S = seed for cells 1-7, S_1 = seed for cells 8-10. Numbers above brackets indicate sample day, numbers in lanes refer to cells. Cells 1-7 operated under anaerobic conditions, cell 8 operated under aerobic conditions.

The number of main bands within the profiles of all cells analysed appeared similar to the seed for cells 1-7 until an increase by day 127 (see Figure 3.23). There are some significant bands within the profile. There was an extremely strong band (indicated by arrow a) present in the seed for cells 1-7 that persisted throughout the entire period of operation, including the sample taken under aerobic conditions. Another band (indicated by arrow b) not obvious in the seed persisted throughout the entire period in both anaerobic and aerobic cells, although its intensity was reduced in some samples. There was one obvious band (indicated by arrow c) that was only present in cells 4 and 7 of the reactor on days 34 and 55, whilst conversely there were also at least 4 bands (indicated by arrows d-g) present in samples taken on days 127 and 190 that were strongest in cell 1, reduced in cell 4 and not visible in cell 7. There were 2 bands (indicated by arrows h and i) that were strong in cell 4 on days 127 and 190, but were reduced or not visible in cells 1 and 7. Another band (indicated by arrow j) was present an all cells, both anaerobic and aerobic, only from day 127. Only 2 bands (indicated by arrows k and l) appeared to be unique to aerobic cell 8, which were not obvious in the seed for cells 8-10.

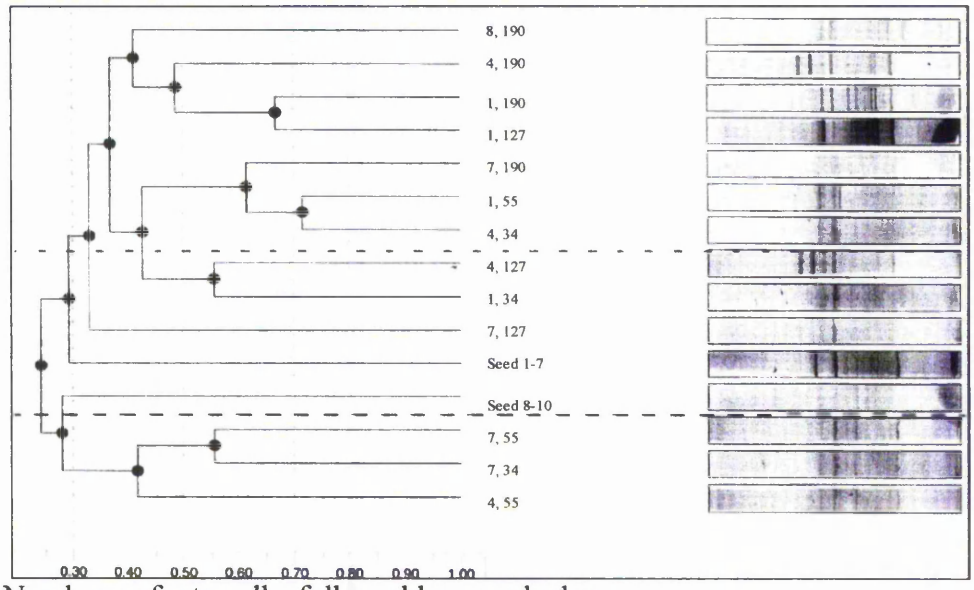
Figure 3.24: DGGE profile of the bacterial population in HABR Rig A up to the end of operation



Key: M = marker ladder. Numbers above brackets indicate sample day, numbers in lanes refer to cells. Cells 1 and 4 operated under anaerobic conditions, cells 5 and 7 operated under anoxic conditions, cells 8 and 10 operated under aerobic conditions.

The large number of bands that was observed in profiles up to day 190 continued until the end of operation of Rig A (Figure 3.24). There are some significant bands within the profile. A band (indicated by arrow a) was observed to be present in all sludge and biofilm samples taken from cells operating under anaerobic and anoxic conditions, and was especially strong in cell 1. A number of bands (indicated by arrows b-d) were present in sludge and biofilm samples taken from cells operating under anaerobic and anoxic conditions on day 230, although some were present in a few samples taken on day 217. There were at least 3 bands (indicated by arrows e-g) that were present in anoxic sludge and biofilm samples and anaerobic biofilm samples, but were not observed in anaerobic sludges. There was a strong band (indicated by arrow h) that was present in sludge and biofilm samples taken from cells operating under aerobic conditions, and also from anaerobic and anoxic biofilm samples. Only one band appeared to be unique to aerobic biofilms (indicated by arrow i), which was present in cell 10 on day 230, but this is extremely close to band d of the profile and highlights the importance of band matching by specialist software.

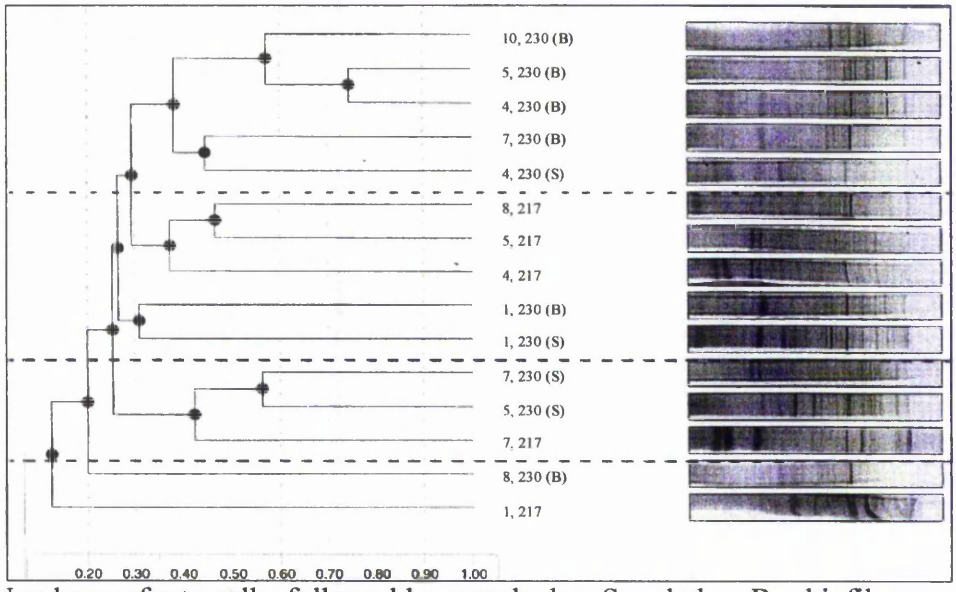
Figure 3.25: Dendogram of the bacterial population in HABR Rig A up to day 190 of operation, based on DGGE band matching



N.B. Numbers refer to cells, followed by sample day.

Band matching and subsequent construction of a dendogram showed that Rig A samples up to day 190 clustered into 4 main sets (Figure 3.25). The seed for cells 1-7 showed only 25-30% similarity to the rest of the samples, which demonstrates that the bacterial population diverged rapidly from the seed sludge within 34 days of operation. The first set comprised samples from cell 7 on day 34 and cells 4 and 7 on day 55, showing ~25% similarity to the rest of the samples and these cell 7 samples showing ~55% similarity to each other. The second set comprised samples from cell 1 on day 34 and cell 4 on day 127, showing ~35-42% similarity to different cells sampled on the same day or the same cells sampled on different days. The third set comprised samples from cell 4 on day 34, cell 1 on day 55 and cell 7 on day 190, with samples from cells 4 and 1 showing ~73% similarity to each other. The fourth set comprised samples from cell 1 on days 127 and 190, and cell 4 on day 190, showing~45-67% similarity to each other but only ~37% similarity to the third set. The sample taken from aerobic cell 8 on day 190, which clustered with the fourth set, showed ~42% similarity to samples from anaerobic cells 1 and 4 on this day and ~25-37% similarity to the rest of the samples or to the seed for cells 8-10.

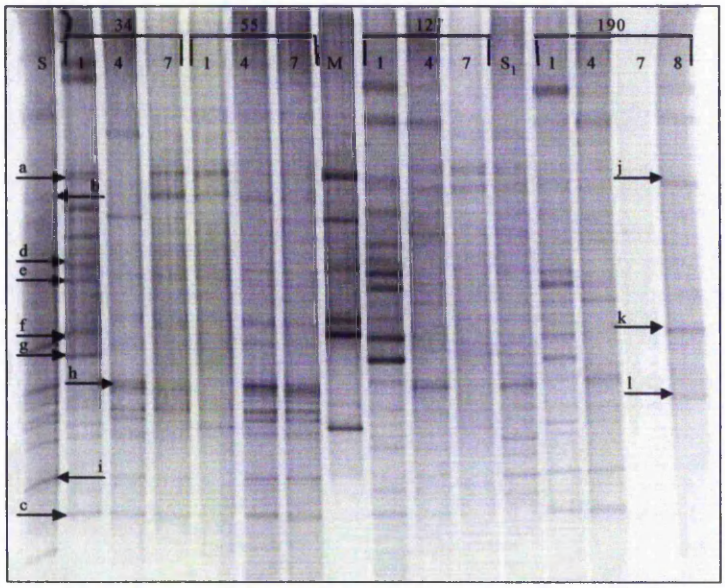
Figure 3.26: Dendogram of the bacterial population in HABR Rig A up to the end of operation, based on DGGE band matching



N.B. Numbers refer to cells, followed by sample day. S = sludge, B = biofilm.

Band matching and subsequent construction of a dendogram showed that Rig A samples up to the end of operation clustered into 4 main sets (Figure 3.26). The first set comprised sludge samples taken from cell 7 on day 217 and cells 5 and 7 on day 230 which were all operated under anoxic conditions, with day 230 samples showing ~57% similarity to each other. The second set comprised the sludge and biofilm samples taken from cell 1 on day 230 which were operated under anaerobic conditions, showing ~32% similarity to each other and ~25% similarity to the first set. The third set comprised samples taken from cells 4, 5 and 8 on day 217, which were operated under anaerobic, anoxic and aerobic conditions respectively, showing 25-30% similarity to the rest of the samples. The third set also showed ~25% similarity to the first set, which contained the sample from cell 7 on day 217, and only ~10% similarity to the sample from cell 1 on day 217. The fourth set comprised sludge and biofilm samples taken from cell 4 and biofilm samples from cells 5, 7 and 10, all on day 230. This set showed 25-27% similarity to the first and second sets, which contained sludge samples for this day.

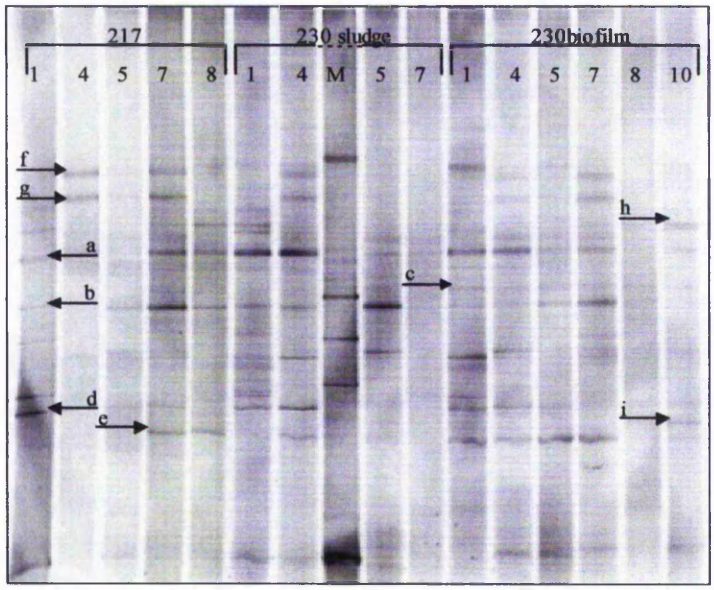
Figure 3.27: DGGE profile of the bacterial population in HABR Rig B up to day 190 of operation



Key: M = marker ladder, S = seed for cells 1-7, S_1 = seed for cells 8-10. Numbers above brackets indicate sample day, numbers in lanes refer to cells. Cells 1-7 operated under anaerobic conditions, cell 8 operated under aerobic conditions.

The profiles of all cells analysed appeared to differ to the profile for the seed for cells 1-7, although there were some similar bands, and the number of main bands increased by day 34 (see Figure 3.27). There are some significant bands within the profile. There were at least 3 bands (indicated by arrows a-c) that persisted in all anaerobic samples, although their intensity was reduced in certain samples. Bands b and c were also observed in the seed for cells 1-7. There were several bands observed in specific cells, such as 4 bands (indicated by arrows d-g) only present in cell 1, one band (indicated by arrow h) only present in cell 4, and one band (indicated by arrow i) only present in cells 4 and 7. The 3 main bands observed in cell 8 (indicated by arrows j-l), not obvious in the seed for cells 8-10, appeared to be unique to the aerobic cell.

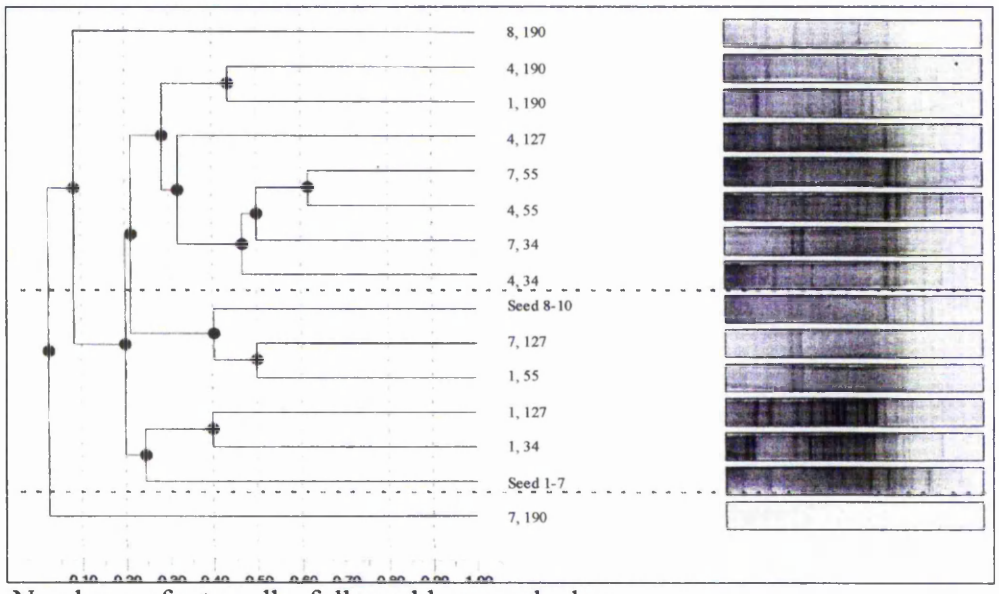
Figure 3.28: DGGE profile of the bacterial population in HABR Rig B up to the end of operation



Key: M = marker ladder. Numbers above brackets indicate sample day, numbers in lanes refer to cells. Cells 1 and 4 operated under anaerobic conditions, cells 5 and 7 operated under anoxic conditions, cells 8 and 10 operated under aerobic conditions.

The large number of bands that was observed within profiles up to day 190 continued up to the end of operation of Rig B (see Figure 3.28). There are some significant bands within the profile. There were at least 4 bands (indicated by arrows a-d) that were present in all samples, sludge and biofilm taken from cells operating under anaerobic, anoxic and aerobic conditions. At least one of these bands (indicated by arrow c) was observed in all biofilm samples but did not appear to be present in all sludge samples. There was one band (indicated by arrow e) that was present in all samples taken on day 230 only. At least 2 bands (indicated by arrows f and g) were present in sludge and biofilm samples taken from cells operated under anaerobic and anoxic conditions only. One of the main bands observed within the aerobic biofilm (indicated by arrow h) also appeared to be present anaerobic sludge samples. Only one band appeared to be unique to aerobic biofilm and possibly sludge samples (indicated by arrow i).

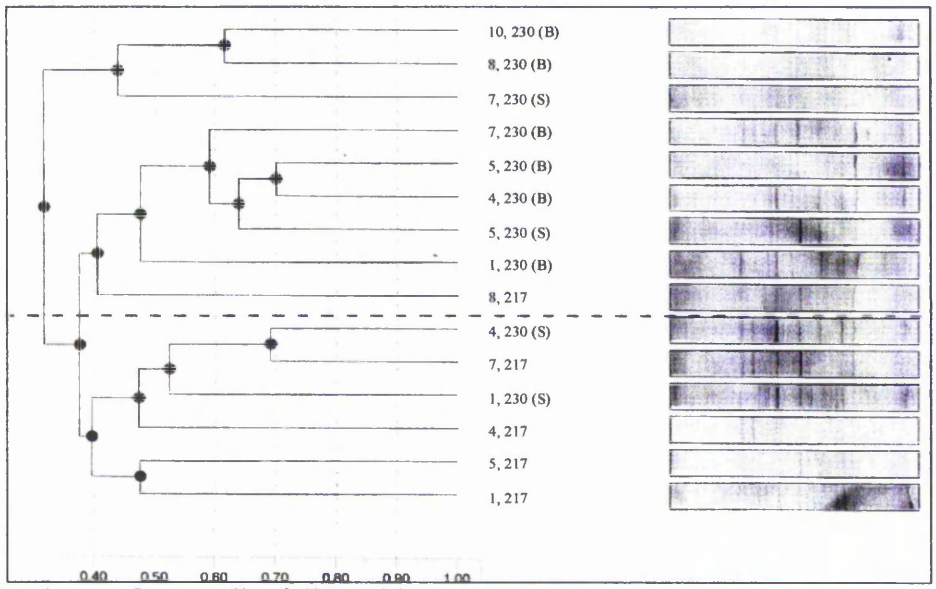
Figure 3.29: Dendogram of the bacterial population in HABR Rig B up to day 190 of operation, based on DGGE band matching



N.B. Numbers refer to cells, followed by sample day.

Band matching and subsequent construction of a dendogram showed that Rig B samples up to day 190 clustered into 4 main sets (Figure 3.29). The seed for cells 1-7 showed only ~10-25% similarity to the rest of the samples, which demonstrates that the bacterial population diverged rapidly from the seed sludge within 34 days of operation. The first set comprised samples from cell 1 on days 34 and 127, showing 20% similarity to the rest of the samples including those taken from different cells on the same days. The second set comprised samples from cell 1 on day 55 and cell 7 on day 127, again showing ~20% similarity to the rest of the samples. The third set comprised samples from cells 4 and 7 on days 34 and 55 and cell 4 on day 127, showing ~20% similarity to the first and second sets which contained samples from cell 1 on these days. The fourth set comprised samples from all anaerobic cells on day 190 for which a good profile was obtained, cells 1 and 4, showing ~43% similarity to each other and ~20-28% similarity to samples from all previous days. The sample taken from aerobic cell 8 on day 190 showed <10% similarity to all other samples, including samples from anaerobic cells on this day and to the seed for cells 8-10.

Figure 3.30: Dendogram of the bacterial population in HABR Rig B up to the end of operation, based on DGGE band matching



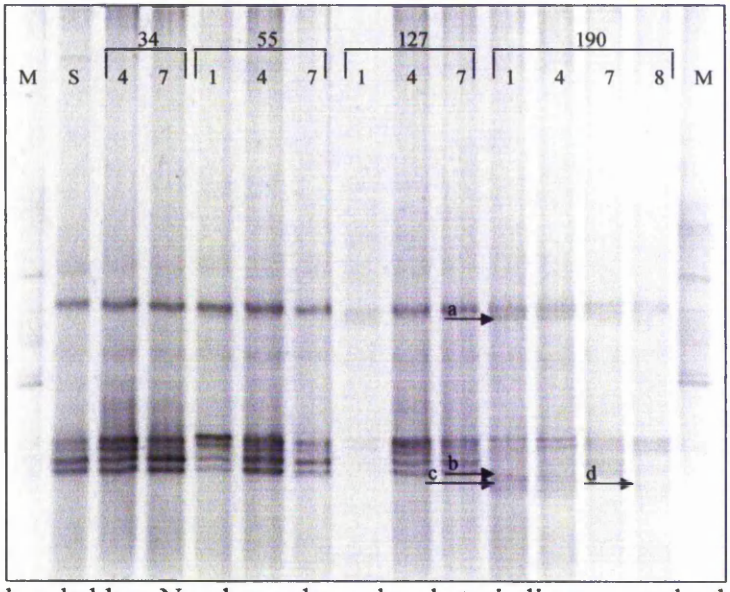
N.B. Numbers refer to cells, followed by sample day. S = sludge, B = biofilm.

Band matching and subsequent construction of a dendogram showed that Rig B samples up to the end of operation clustered into 4 main sets (Figure 3.30). The first set comprised samples taken from cells 1 and 5 on day 217, operated under anaerobic and anoxic conditions, showing ~47% similarity to each other. The second set comprised anaerobic and anoxic sludge samples taken from cells 4 and 7 on day 217 and 1 and 4 on day 230, showing ~47-70% similarity to each other and ~40% similarity to the first set. The third set comprised all anaerobic and anoxic biofilm samples taken on day 230 and also the sludge sample taken from cell 5 on day 230, showing ~59-70% similarity to each other (cell 1 shows lowest similarity). The fourth set comprised aerobic biofilm samples from cells 8 and 10 on day 230, showing ~62% similarity to each other and ~30% similarity to the anaerobic and anoxic biofilm samples in the third set, in addition to the sludge sample from cell 7 on day 230. The aerobic sludge from cell 8 on day 217 did not cluster with any other samples, but showed ~40% similarity to the anaerobic and anoxic samples taken on this day.

3.3.5.2 Archaeal DGGE profiles and analysis of divergence

The 16S rRNA genes of the archaeal population in both HABRs were amplified and separated in DGGE gels. Analysis of divergence of the population over time and according to environmental parameters and spatial arrangement was conducted using gel analysis software (Phoretix 1D Advanced v4.01, Phoretix International) and construction of dendograms.

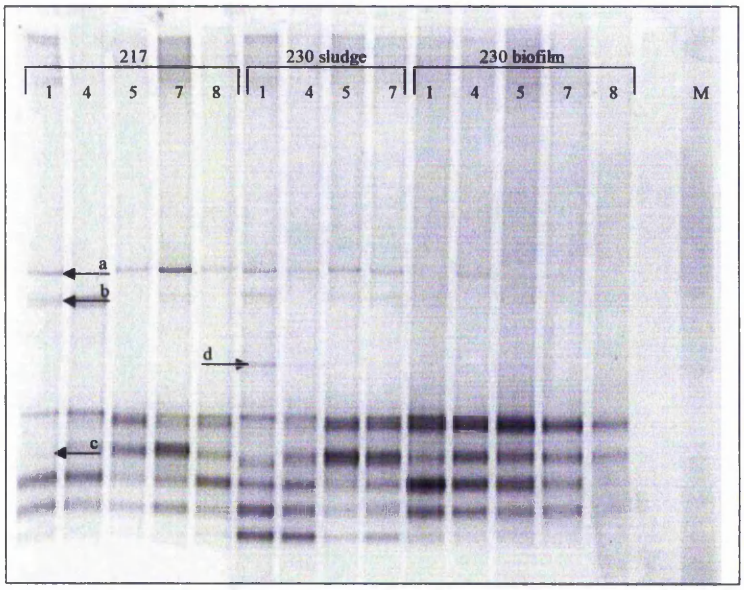
Figure 3.31: DGGE profile of the archaeal population in HABR Rig A up to day 190 of operation



Key: M = marker ladder. Numbers above brackets indicate sample day, numbers in lanes refer to cells. Cells 1-7 operated under anaerobic conditions, cell 8 operated under aerobic conditions.

Archaeal products could not be amplified from the sample taken from cell 1 on day 34 or the seed for cells 8-10. It can be seen that there were relatively few bands within the profiles compared with the bacterial profiles, and that the profiles did not alter significantly in any of the samples (see Figure 3.31). There were 5 main bands observed in the seed for cells 1-7 and nearly all samples, which appeared to be extremely similar for all of the profiles, and at least 3 additional bands (indicated by arrows a-c) that appeared on day 190 in cells 1 and 4. There appeared to be only one band (indicated by arrow d) that was unique to the aerobic cell.

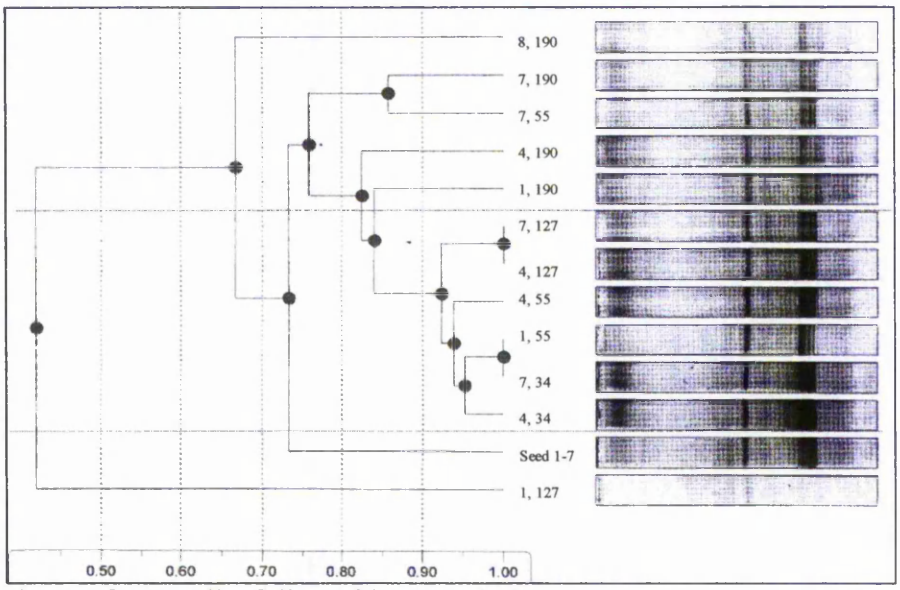
Figure 3.32: DGGE profile of the archaeal population in HABR Rig A up to the end of operation



Key: M = marker ladder. Numbers above brackets indicate sample day, numbers in lanes refer to cells. Cells 1 and 4 operated under anaerobic conditions, cells 5 and 7 operated under anoxic conditions, cell 8 operated under aerobic conditions.

Archaeal products could not be amplified from the biofilm sample taken from cell 10 on day 230. As previously observed for samples up to day 190 of operation, there were relatively few bands within the profiles compared with the bacterial profiles, and the profiles did not alter significantly in any of the samples (see Figure 3.32). There were 6 main bands observed in nearly all samples, which appeared to be extremely similar for all of the profiles. There was one band (indicated by arrow a) that appeared in all sludge samples, including the aerobic cells, that was not seen in the biofilm samples (except cell 4 biofilm on day 230). There was one band (indicated by arrow b) only present in sludge samples taken from cells 1 and 4 (although it was not easy to visualise in cell 4 on day 230). No bands were observed that appeared to be unique to aerobic sludges and biofilms.

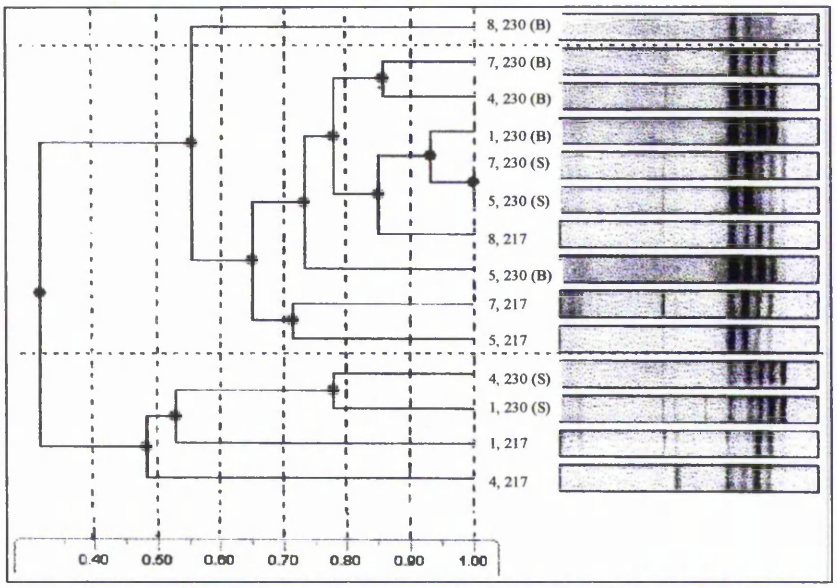
Figure 3.33: Dendogram of the archaeal population in HABR Rig A up to day 190 of operation, based on DGGE band matching



N.B Numbers refer to cells, followed by sample day.

Band matching and subsequent construction of a dendogram showed that Rig A samples up to day 190 clustered into 2 main sets, with high similarity values (~76-84%) between the sets (Figure 3.33). The seed for cells 1-7 showed ~73% similarity to the other anaerobic samples, which demonstrated that there was a slight alteration in the archaeal community composition within 34 days of operation. The first set comprised samples taken from all anaerobic cells on days 34, 55 and 127, except cell 7 on day 55 and cell 1 on day 127, showing ~93-100% similarity between samples. The profile of the sample from cell 1 on day 127 showed only ~40% similarity to any other sample, but this is most likely an artefact of the weak profile produced. The second set comprised samples taken from all anaerobic cells on day 190 and cell 7 on day 55, showing ~76-84% similarity to the first set. Samples from cells 1 and 4 on day 190 showed less similarity to the sample from cell 7 on this day (~76%) than to samples from the first set (~83-85%). It could also be seen that the sample taken from the aerobic cell 8 on day 190 showed ~67% similarity to all other samples.

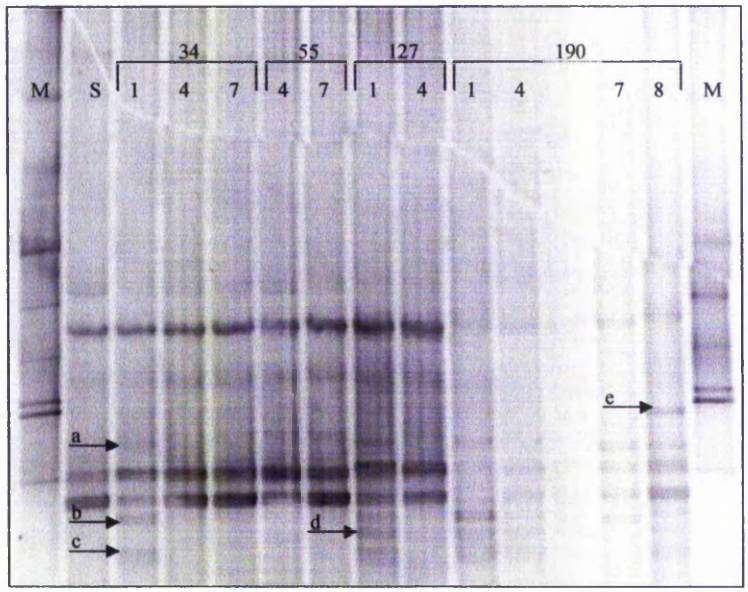
Figure 3.34: Dendogram of the archaeal population in HABR Rig A up to the end of operation, based on DGGE band matching



N.B. Numbers refer to cells, followed by sample day. S = sludge, B = biofilm.

Band matching and subsequent construction of a dendogram showed that Rig A samples up to the end of operation clustered into 3 main sets (Figure 3.34), with lower similarity values (~30-65%) between the sets than was observed up to day 190 of operation. The first set comprised sludge samples taken from cells 1 and 4 on days 217 and 230, which were operated under anaerobic conditions. The second set comprised samples taken from cells 5 and 7 on day 217 which were operated under anoxic conditions, showing ~30% similarity to the first set. The third set comprised sludge samples taken from cells 5 and 7 on day 230, biofilm samples taken from cells 1, 4, 5 and 7 on day 230 and cell 8 on day 217, showing ~65% similarity to the second set and only ~30% similarity to the first set. The anoxic sludge samples taken from cells 5 and 7 on day 230 were seen to be identical. All anaerobic and anoxic biofilm samples showed similarity values of ~70-90% to each other. The aerobic biofilm from cell 8 on day 230 showed ~55% similarity to anaerobic biofilms and anoxic biofilms and sludges, whilst the aerobic sludge from cell 8 on day 217 showed ~65-80% to these samples.

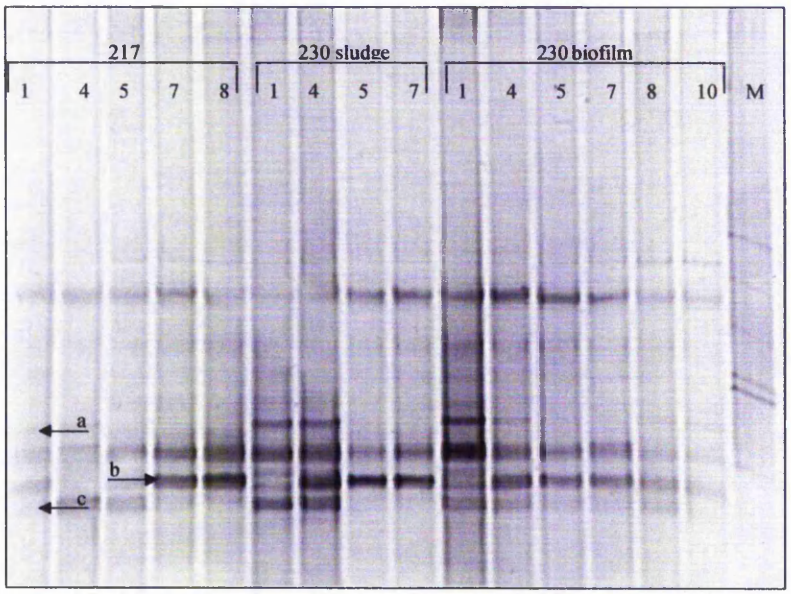
Figure 3.35: DGGE profile of the archaeal population in HABR Rig B up to day 190 of operation



Key: M = marker ladder, S = seed for cells 1-7. Numbers above brackets indicate sample day, numbers in lanes refer to cells. Cells 1-7 operated under anaerobic conditions, cell 8 operated under aerobic conditions.

Archaeal products could not be amplified from samples taken from cell 1 on day 55, cell 7 on day 127 or the seed for cells 8-10. It can be seen that there were relatively few bands within the profiles compared with the bacterial profiles, and that the profiles did not alter significantly in any of the samples (see Figure 3.35). There were only 3-4 main bands in all of the samples that appeared to persist from the seed for cells 1-7, in addition to other bands only observed in certain cells. There are some significant bands within the profile. There was at least one band (indicated by arrow a) that appeared only to be present in cell 1 up to day 127, but was then present in all anaerobic and aerobic cells on day 190. There were at least 2 bands (indicated by arrows b and c) that could be visualised in cell 1 from all samples, cell 4 from day 127, and in all cells (including the aerobic cell) on day 190. A further band (indicated by arrow d) was present in all anaerobic and aerobic samples from day 127, but was strongest in cell 1. There did not appear to be any bands that were unique to the aerobic cell, although some bands (such as that indicated by arrow e) were stronger in cell 8 on day 190 than the anaerobic cells on this day.

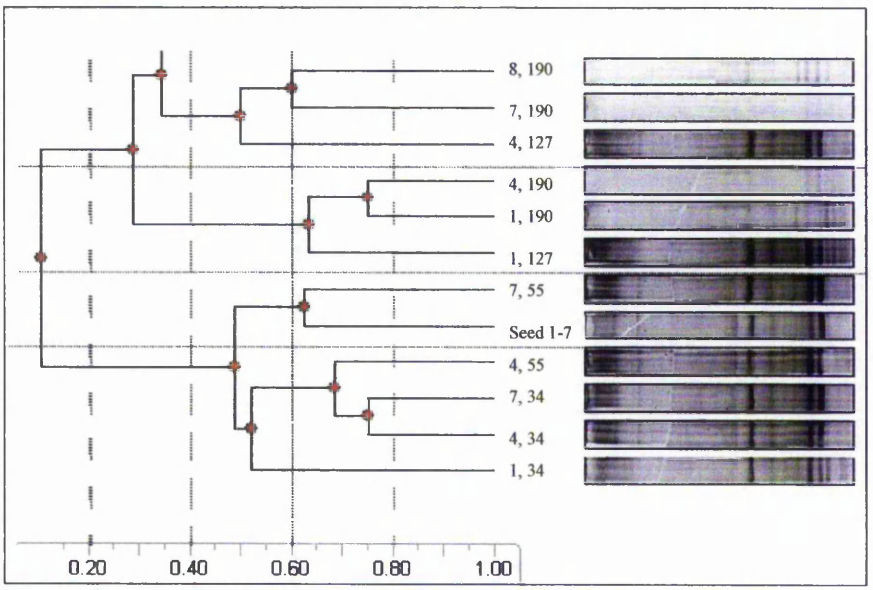
Figure 3.36: DGGE profile of the archaeal population in HABR Rig B up to the end of operation



Key: M = marker ladder. Numbers above brackets indicate sample day, numbers in lanes refer to cells. Cells 1 and 4 operated under anaerobic conditions, cells 5 and 7 operated under anoxic conditions, cells 8 and 10 operated under aerobic conditions.

As previously observed for samples up to day 190 of operation, there were relatively few bands within the profiles compared with the bacterial profiles, and the profiles did not alter significantly in any of the samples (see Figure 3.36). There were 4 main bands observed in nearly all samples, but also some additional bands only observed in certain cells. There was one band (indicated by arrow a) that was present in anaerobic sludge and biofilm samples taken from cells 1 and 4 in addition to aerobic biofilms from cells 8 and 10, but not in the aerobic sludge sample from cell 8 on day 217. There was one band (indicated by arrow b) present in all samples that was of higher intensity in anoxic sludges and biofilms and aerobic sludge samples. There was at least one other band (indicated by arrow c) that was of higher intensity in anaerobic sludges and biofilms than anoxic or aerobic samples.

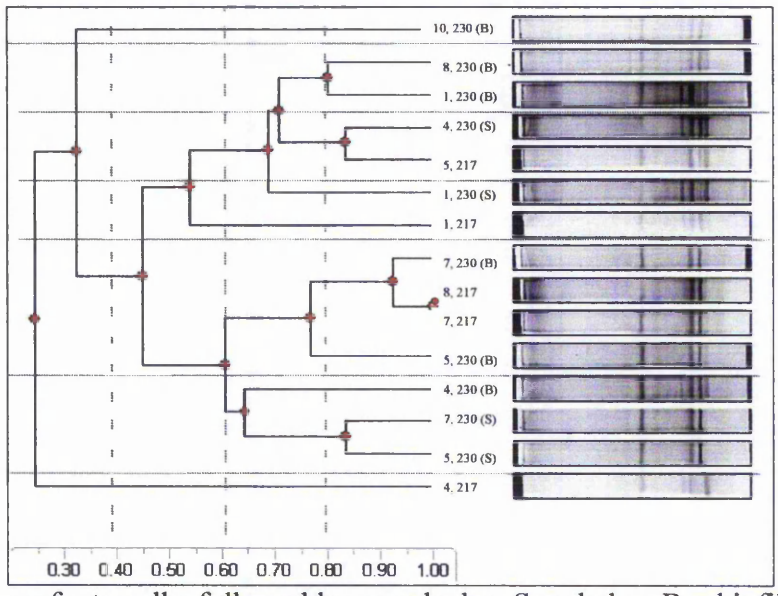
Figure 3.37: Dendogram of the archaeal population in HABR Rig B up to day 190 of operation, based on DGGE band matching



N.B Numbers refer to cells, followed by sample day.

Band matching and subsequent construction of a dendogram showed that Rig B samples up to day 190 clustered in to 2 main sets, showing only ~10% similarity between the sets, with each set split into 2 subsets (Figure 3.37). The first subset in the first set comprised samples taken from all cells on day 34 and cell 4 on day 55. The second subset, showing ~48% similarity to first, comprised the sample from cell 7 on day 55 and the seed for cells 1-7. The first subset in the second set comprised samples taken from cell 1 on days 127 and 190, and from cell 4 on day 190. The second subset comprised samples taken from cell 7 on days 127 and 190 and from aerobic cell 8 on day 190, and showed only ~28% similarity to the first subset. As the second set only showed ~10% similarity to the first set, it can be seen that the population composition altered dramatically after day 55.

Figure 3.38: Dendogram of the archaeal population in HABR Rig B up to the end of operation, based on DGGE band matching



N.B. Numbers refer to cells, followed by sample day. S = sludge, B = biofilm.

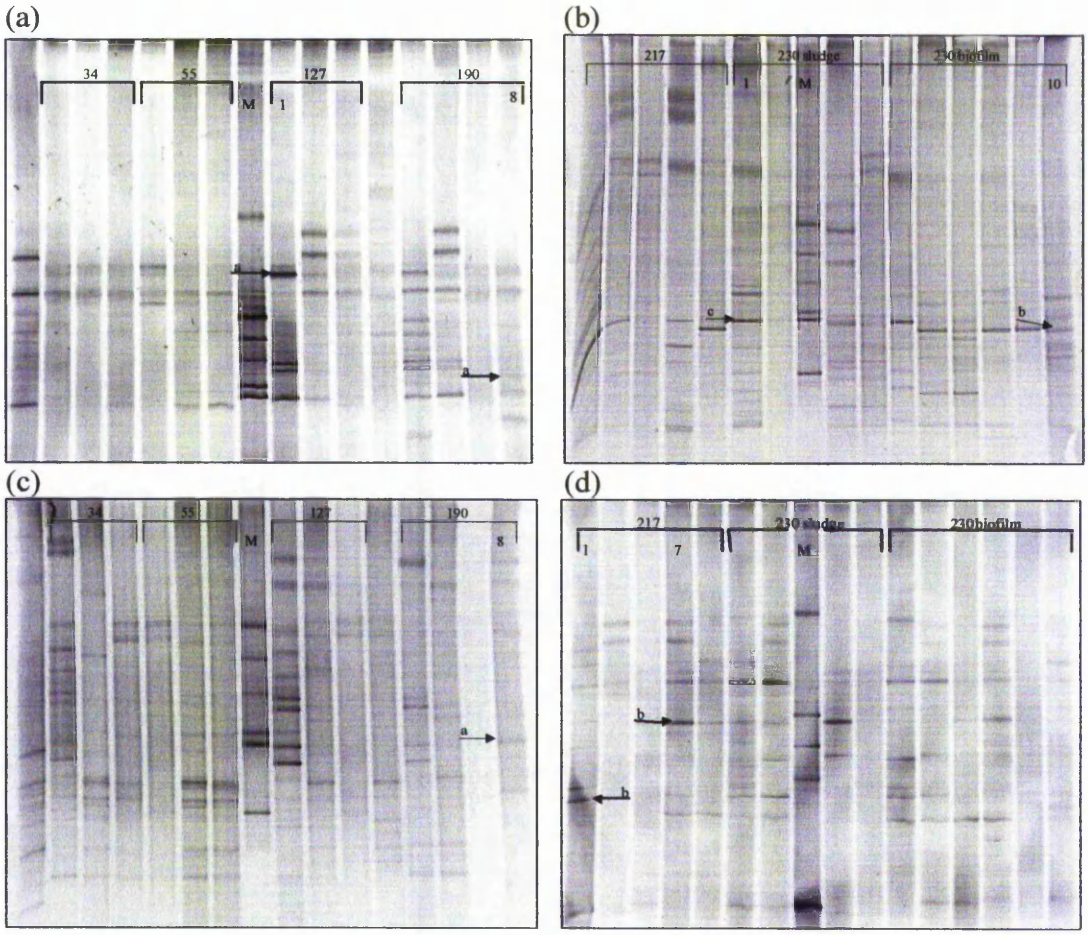
Band matching and subsequent construction of a dendogram showed that Rig B samples up to the end of operation clustered in to 2 main sets, showing ~45% similarity between the sets, with each set split into 2 subsets (Figure 3.38). The first subset in the first set comprised sludge samples taken from cells 5 and 7 on day 230, which were operated under anoxic conditions, and an anaerobic biofilm sample from cell 4 on day 230. The second subset, showing 60% similarity to first, comprised the anoxic sludge from cell 7 on day 217 and anoxic biofilm samples from cells 5 and 7 on day 230, in addition to the aerobic sludge from cell 8 on day 217 that was identical to the anoxic sludge. The first subset in the second set comprised sludge samples taken from cells 5 and 4 on days 217 and 230 respectively. The second subset, showing 70% similarity to the first, comprised biofilm samples taken from the anaerobic and aerobic cells 1 and 8 on day 230, which showed 80% similarity to each other. Of the remaining samples, sludges taken from cell 1 on days 217 and 230 showed ~53 and 70% similarity respectively to the second set containing sludges from different cells on these days. It can also be seen that the anaerobic sludge from cell 4 on day 217 and the aerobic biofilm taken from cell 10 on day 230 showed only ~25 and 30% similarity to the rest of the samples.

3.3.5.3 Sequencing of bands from bacterial DGGE profiles

Bands of interest from the bacterial HABR profiles, highlighted in Figure 3.39a-d, were excised and sequenced. Unfortunately, due to time and financial constraints,

sequencing of bands from archaeal profiles could not be completed. Matches of the sequences produced using the forward and reverse primers and their consensus sequences to the nucleotides database held at the National Centre of Biotechnology Information (NCBI) were made, and the most likely identification of the band was determined based upon matches produced by each of the sequences.

Figure 3.39: DGGE profiles of the bacterial populations in the HABRs, showing bands excised for sequencing



(a) Profile of Rig A up to day 190 of operation (b) Profile of Rig A up to the end of operation (c) Profile of Rig B up to day 190 of operation (d) Profile of Rig B up to the end of operation. For all profiles, the number above the brackets indicates the sample day, the number in the lane refers to the cell, the band letter refers to the band on the sample day in question (see Table 3.25), and M is the marker ladder.

Table 3.25: Identification of 16S rRNA genes of microorganisms in the HABRs by sequencing

Rig	Sample day	Cell no.	Band	Reactor conditions	Identification	Database match
A	127	1	a	+ dye, anaerobic + aerobic*	*Bacteroides* sp. / *Prevotella* sp.	*Bacteroides uniformis* [AB050110] (121/130)
A	190	8	a	+ dye, aerobic only	*Nocardia* sp. / *Prevotella* sp.	*Nocardia* sp. 86349 [AF227864] (21/21)
A	230	1 (sludge)	c	+ dye, anaerobic + anoxic, sludge + biofilm*	Invalid sequence	
A	230	10 (biofilm)	b	+ dye, anaerobic + anoxic biofilm, aerobic sludge + biofilm	*Rhodococcus* sp.	*Rhodococcus erythropolis* [AB046362] (25/25)
B	190	8	a	- dye, aerobic only	*Rhodopseudomonas* sp.	*Rhodopseudomonas palustris* [AB046735] (25/26)
B	217	1	b	- dye, anaerobic + anoxic, sludge + biofilm*	No sequence obtained	
B	217	7	b	- dye, anaerobic + anoxic + aerobic, sludge + biofilm	*Cytophaga* sp. / *Flexibacter* sp.	*Cytophaga* sp. strain BD1-16 [AB015525] (91/98)

N.B. Database match shows best match of the sequences to the database, with NCBI Accession number in brackets and identities in parentheses. All database matches were to 16S rRNA genes (partial). *Strongest at the beginning of the reactor. Note that sequencing of archaeal 16S rRNA genes was not completed.

Of 7 excised bands, 1 did not produce any sequence data and 1 produced sequence data that did not show valid microbial matches to the databases (Table 3.25). In Rig A, which was operated with the inclusion of RBOH, a predominant band present in anaerobic and aerobic sludge samples was identified as *Bacteroides* sp.. A band observed in all biofilm samples but only aerobic sludge samples was identified as the alpha-proteobacterium *Rhodococcus* sp., whilst a band unique to aerobic sludge was identified as *Nocardia* sp.. In Rig B, a predominant band present in sludge and biofilm samples under all oxygen conditions was identified as *Cytophaga* sp.. A band unique to aerobic sludge was identified as the alpha-proteobacterium *Rhodopseudomonas* sp..

Chapter 4: Discussion

The aim of this work was to evaluate the toxicity problems associated with decolourisation methods and to evaluate the efficacy of different bioreactor types in the decolourisation process. Our data have shown that hydrolysed C.I. Reactive Black 5 (RBOH) becomes highly toxic after decolourisation and this toxicity was traced to the vinyl sulphone component (2-(*p*-aminophenylsulphonyl) ethanol sulphate). In terms of bioreactor decolourisation efficiency, the most effective treatment regime for an STE containing RBOH was found to be a Hybrid Anaerobic Baffled Reactor operating under anaerobic-aerobic conditions. This reactor type operating under anaerobic-aerobic conditions allowed for the greatest increases in microbial diversity and speciation.

4.1 Decolourisation studies of azo dyes

A simple structure-activity evaluation was conducted in order to determine whether the toxicity and genotoxicity assays employed were suitable for detection of the sulphonated azo compounds under investigation. Agreement of the observations made during assessment of decolourised dyes and naphthol compounds related to the substituent aromatic amines with the literature demonstrates that the assays were suitable to detect changes made to the sulphonated azo dyes by biological reduction. Subsequently, the toxicological activities of 4 complex reactive azo textile dyes, C.I. Reactive Black 5 (RB), Procion Navy (PN), Yellow (PY) and Crimson (PC), were measured *in vitro*. Dyes were assessed both as whole molecules (unreduced) and after azo fission by biological decolourisation (reduced), and in both native (parent) and hydrolysed forms. It was observed that only RB showed significant increases in toxicity both after hydrolysis and decolourisation, and further work on this dye attributed this toxicity to the liberation of the vinyl sulphone (2-(*p*-aminophenylsulphonyl) ethanol sulphate) component. The only significant genotoxicity was demonstrated by unreduced hydrolysed RB (RBOH).

4.1.1 Structure-activity evaluation with simple azo dyes and related naphthol compounds

4.1.1.1 Toxicity and genotoxicity of unreduced dyes

Experiments conducted with Sunset Yellow (SY) and Orange II (O2) revealed that these sulphonated simple azo dyes exhibited toxic (not significant) potentials but were non-genotoxic (see Tables 3.1 and 3.2). The negative controls for the assays

showed neither toxicity nor genotoxicity, and thus the background matrix did not contribute to any toxic or genotoxic potentials observed. The toxicity of O2 is in agreement with previous studies, such as that of He and Bishop in 1994 who showed that O2 was inhibitory to all stages of the nitrification process in a continuously stirred tank reactor, the toxic effect being greater than that of some organic compounds (e.g. formalin, methanol, acetone) but less than that of some phenolic compounds. Using rotating drum biofilm reactors (RDBR), Harmer and Bishop (1992) and Zhang and co-workers (1995) determined that O2 caused a reduction in respiration of the microorganisms in the suspend phase, although no significant inhibition was observed in the biofilm at concentrations of up to 25 mgL^{-1}. It has also been observed that O2 was less toxic to the biofilm of a RDBR than the C.I. Acid Orange 10 and C.I. Acid Red 14, as measured by the amount of biomass present (Zhang *et al.* 1995) and inhibition of the respiration rate (Fu *et al.* 1994).

SY has previously been shown to be non-toxic in various bacterial assays (Combes & Haveland-Smith 1982), but none of those assays have used the systems utilised in this study. The toxicity assay employed in this study appears to be more sensitive to toxic potentials produced by azo dyes such as SY, as a toxic potential was observed. SY was also found to be non-genotoxic, which is in agreement with previous studies utilising several submammalian genotoxicity assays (including systems based upon bacterial DNA repair) (Combes & Haveland-Smith 1982). This observation is also supported by the fact that both SY and O2 have been shown to be non-mutagenic in the Ames test at high concentrations of up to 5 mg $plate^{-1}$ (Chung *et al.* 1981, Chung 1983, Brown & Dietrich 1983, Izbirak *et al.* 1990), and good correlations have been found between genotoxicity and mutagenicity.

The toxic potential observed from unreduced SY was probably due to the portion of the dye molecule equivalent to sulphanilic acid, due to their extremely similar toxicity values. It was also observed that the toxic potential of SY, which contains two sulphonic acid groups, is 1.4 times less than that of O2, which contains one sulphonic acid group (see Figure 2.1). There are numerous reports in the literature of sulphonation of compounds leading to a decrease in mutagenic activity (Combes & Haveland-Smith 1982, Lin & Solodar 1988, Rosenkranz & Klopman 1989, Chung & Cerniglia 1992, Puntener *et al.* 1993, Chung 2000, Moller & Wallin 2000), which is closely correlated to toxic and genotoxic activity (Benigni *et al.* 1989, Rao *et al.* 1995, White *et al.* 1997). A study of methanogenesis in an anaerobic sludge by Shcherbakova and co-workers in 1999 demonstrated that the toxicity of both benzene and toluene was eliminated by

sulphonation. Therefore the fact that increased sulphonation resulted in decreased toxicity is consistent with observations made in these previous studies.

4.1.1.2 Toxicity and genotoxicity of reduced dyes

The sulphonated naphthol compounds 1-amino-2-naphthol-6-sulphonic acid and sulphanilic acid, which are analogous to the substituent aromatic amines of SY, were non-genotoxic (see Tables 3.1 and 3.2). It has been shown that sulphonated naphthol compounds including these two compounds are non-genotoxic and non-mutagenic in the Ames test (Chung *et al.* 1981, Combes & Haveland-Smith 1982, Chung 1983, Lin & Solodar 1988, Rosenkranz & Klopman 1989, Chung & Cerniglia 1992, Puntener *et al.* 1993, Chung 2000, Moller & Wallin 2000). Therefore, it may be expected that reduced SY will not exhibit a genotoxic potential, if its action is dependent upon the toxicological responses to its constituent aromatic amines only. However, reduced SY did exhibit a genotoxic potential, which suggests that this effect is caused by an alternative mechanism to that normally associated with genotoxic aromatic amines.

Sweeney and co-workers demonstrated that reduced SY was genotoxic in a differential kill assay, and that the genotoxicity was due to superoxide radicals and hydrogen peroxide (Sweeney *et al.* 1994, Sweeney 1995). The differential kill genotoxicity assay employed in this study used the same strain of *Escherichia coli* (CM871) and experimental conditions as Sweeney and co-workers, and thus would be expected to detect any genotoxicity caused by oxygen radicals. Therefore, the genotoxic potential of reduced SY is not due to its substituent aromatic amines, but to the formation of oxygen radicals during the process of azo fission.

It was observed that the naphthol compound 1-amino-2-naphthol, which is analogous to a substituent aromatic amine of O2, was found to be extremely toxic and genotoxic. Concentrations of >5 mgL^{-1} rendered all test organisms non-viable in the genotoxicity assay, including the DNA repair proficient strain, and was still producing high percentage kills (>90%) at concentrations 2-fold lower than this. The concentration at which 1-amino-2-naphthol produced a CS value of 0.3 (i.e. the point at which the compound is classified as genotoxic) was determined to be between 0.61 and 1.23 mgL^{-1}. This observation agrees with previous studies demonstrating the potent carcinogenicity in tissue studies (Clayson *et al.* 1958, Daniel 1962, Chung *et al.* 1978) and toxicity to microorganisms (Chung *et al.* 1981, Chung 1983) of this compound.

Therefore, the highly toxic and genotoxic potential of reduced O2 is consistent with the toxicological response of one of its substituent aromatic amines.

4.1.1.3 Potential mechanisms of toxicity and genotoxicity of naphthol compounds

It was observed that the naphthol compounds tested, structurally similar to the aromatic amines that form the substituents of azo dyes released upon azo fission, varied in toxic and genotoxic potential (see Table 3.3). The structures of these compounds (see Figure 2.2) show that they are structurally related. 1-amino-2-naphthol-6-sulphonate is the sulphonated form of 1-amino-2-naphthol, and 1-naphthalene sulphonic acid and 2-naphthalene sulphonic acid have a sulphonic acid group on the aromatic ring on the 1' and 2' carbon respectively. 4-amino-1-naphthalene sulphonic acid and 4-amino-1-naphthalene sulphonic acid sodium salt have the same structure, but are prepared with the sulphonic acid ionically bonded to H^+ and Na^+ respectively.

1-amino-2-naphthol is extremely toxic and genotoxic. Sulphonation of this compound to form 1-amino-2-naphthol-6-sulphonate eliminates both the toxic and genotoxic potentials. This observation is in agreement with previous studies that indicate that this reduction may be mediated by a reduction in membrane permeability (Combes & Haveland-Smith 1982, Lin & Solodar 1988, Rosenkranz & Klopman 1989, Chung & Cerniglia 1992, Puntener *et al.* 1993, Chung 2000, Moller & Wallin 2000).

Whilst good correlations between mutagenicity and toxicity (Benigni *et al.* 1989), and between mutagenicity and genotoxicity (Rao *et al.* 1995, White *et al.* 1997) have been reported, it does not have to follow that a toxicant should exhibit genotoxicity. This was observed during assessment of 1- and 2-naphthalene sulphonic acid, when 2-naphthalene sulphonic acid was found to be more toxic than 1-naphthalene sulphonic acid but less genotoxic. This increase in toxicity but not genotoxicity may be due to differences in membrane permeability related to the position of the $-SO_3$ group, preventing 2-naphthalene sulphonic acid from entering whole cells and causing DNA damage whilst it still exerts a toxic pressure from outside the cells. It has also been shown that aminonaphthol compounds can exert genotoxic effects via other mechanisms, such as the production of oxygen radicals (Sweeney *et al.* 1994). A change in the position of the $-SO_3$ group may cause a decrease in O_2^- production, as the rate of radical production can be influenced by the properties of neighbouring structural groups. The fact that the toxic potential of 2-naphthalene sulphonic acid increases 3.5 times over its 1' carbon analogue may possibly be due to the presence of a structural determinant. A study by Rosenkranz and Klopman in 1989 showed the presence of a

structural determinant for carcinogenicity in the azo dye Butter Yellow. If such a structural determinant exists for toxicity within *n*-naphthalene sulphonic acid, it is probable that it encompasses the 2' carbon and not the 1' carbon.

It was also observed that ionic bonding of the sulphonic acid group to Na^+ as opposed to H^+ in the preparation of 4-amino-1-naphthalene sulphonic acid decreases the toxic potential. As there was no difference in the structure of the compounds, it may be considered that a difference in preparation method would not be expected to produce such a large alteration in toxicity. It was noted that the pH of the compounds was not neutralised prior to toxicity assessment. The pH of 4-amino-1-naphthalene sulphonic acid at 100 mgL^{-1} is 3.0, probably due to the dissociation of H^+ ions in solution, whilst the pH of its sodium salt is 7.0. It is also known that the Microtox® toxicity assay employed is sensitive to pH effects, and so the low toxic potential observed with 4-amino-1-naphthalene sulphonic acid is most likely to be due to the acidity of the background matrix rather than the action of the compound itself.

In summary, It has been demonstrated that the rapid bacterial toxicity and genotoxicity assays used are suitable tools for monitoring the toxicological activity of biologically decolourised sulphonated simple azo dyes. Whilst it is known that one toxicity or genotoxicity assay will not give an accurate description of all toxicological responses from all compounds and situations, the assays utilised in this study have shown advantages over other types. In addition to the advantages of rapid bioassays, these include specific advantages such as increased sensitivity of the toxicity assay to sulphonated azo dyes, and detection of genotoxicity via a different mechanism of action to that normally associated with aromatic amines.

4.1.2 Toxicity and genotoxicity of reactive azo textile dyes

4.1.2.1 Toxicological effects of hydrolysis of reactive azo textile dyes

Hydrolysis of PN, PY and PC only resulted in small increases or decreases (1.1 times) in toxic potential, whilst the toxicity of RB increased 2.4 times (see Table 3.6). Reasons for this variable effect of hydrolysis of the dyes on toxicity may well be related to structural differences between the compounds. The Procion dyes have monochlorotriazinyl reactive groups, as opposed to the vinylsulphonyl groups contained within RB (Dystar, pers. comm.), and as such hydrolysis of Procion dyes is likely to produce compounds with different structures to hydrolysed RB. These compounds do not appear to be as toxic as vinylsulphonyl-based compounds, due to the small increases

or decreases in toxicity observed with the Procion dyes. Thus, monochlorotriazinyl-based dyes may be more beneficial for the textile dyeing industry in producing wastewater that adheres to discharge consent limits. However, lower toxicity of the Procion dyes cannot definitely be ascribed to the monochlorotriazinyl groups, as the structure of the Procion dyes has not been released.

4.1.2.2 Toxicological effects of decolourisation of reactive azo textile dyes

Reduction of both the parent and hydrolysed dyes by both *Enterococcus faecalis* (Table 3.6) and *Clostridium butyricum* (Table 3.7) showed increases in toxic potentials from the corresponding unreduced dyes. These increases were higher when the dyes were assayed in their hydrolysed forms, which is most likely to be due to the liberation of less sulphonated aromatic amines. However, these apparent increases were attributed to the build-up of metabolic waste products from the bacterial cells used to decolourise the dyes in PN, PY and PC, as assays conducted in the absence of dyes (negative controls) showed similar toxic potentials to the reduced dyes (0.9-1.5 times). This is in contrast to the non-toxic response of the *E. faecalis* negative control during studies using simple azo dyes, and is most likely due to the longer period of incubation required for the reactive azo textile dyes. The negative controls were incubated for a time equal to the length of the longest dye decolourisation assay (0.17 h for simple dyes, 5/24 h for textile dyes with *E. faecalis*/*C. butyricum*, see Table 3.5), allowing time for build-up of any waste products. It was observed that RB was 3.7 times more toxic than the *C. butyricum* negative control, and the hydrolysed form of RB (RBOH) was 3.1 and 28.0 times higher than the *E. faecalis* and *C. butyricum* controls respectively. Thus RB in both parent and hydrolysed form was determined to be the only textile dye to produce "true" toxicity after decolourisation, and that toxicity was significantly higher from the hydrolysed form. This is of great environmental and industrial significance, as most reactive azo dyes are present in dyebath wastewater in their hydrolysed forms (see section 1.2.2) and RB is a very commonly used dye globally (Southern 1995), and as such it is expected that the toxic potential of decolourised textile dyehouse wastewater will be of significant concern.

Analysis of the relative toxicity values demonstrated that reduction of PC with *E. faecalis* did not significantly increase its toxicity. PC is a reactive azo dye with monochlorotriazinyl reactive groups, as are the other Procion dyes, and thus there is likely to be a small difference in the structure of PC that decreases the toxicity of its substituent aromatic amines that may be of benefit to textile dye manufacturers. As no

significant toxicity was observed with the Procion dyes under any set of experimental conditions, the use of monochlorotriazinyl groups in reactive azo dyes may aid the textile dyeing industry in reducing the toxicity of dyebath wastewater caused by hydrolysed reactive dyes. However, further work would be required to ascertain the benefits of these reactive groups.

4.1.2.3 Toxicity and genotoxicity of C.I. Reactive Black 5 decolourised by different methods

The extensive use of RB in the textile industry and the high toxicity values observed warranted further examination of this dye using different decolourisation methods, and identification of the toxic reduction product separated by HPLC. It was shown that the toxic potential of RB increased after reduction by each of *E. faecalis*, *C. butyricum* and sodium dithionite (see Table 3.8). Reduction by sodium dithionite produced less toxic products than reduction by *C. butyricum* (the level produced after reduction by *E. faecalis* was equivalent to the negative control). However, the method of decolourisation did not affect reduction of RBOH, where extremely similar toxic potentials (0.22-0.28 mgL^{-1}) were observed for each method (see Table 3.9). This suggests that the same products were being formed from fission of the azo bond in RBOH by each method, whereas chemical decolourisation resulted in the formation of different products to biological decolourisation. A study by Puntener and co-workers in 1996 demonstrated that reduction of the azo bond in azo dyes by sodium dithionite can result in the generation of new amines via a side reaction, which are neither part of the dye structure nor involved in the synthesis of the dye. If this effect occurred during reduction of RB by sodium dithionite during this study, then it appears that the amines produced are approximately half as toxic as those produced after biological reduction. That this effect was not observed during decolourisation of RBOH was most likely due to a masking of this effect, caused by the increased toxicity of the hydrolysed substituent aromatic amines.

It was noted that only unreduced RBOH exhibited a weak genotoxic potential, and that the genotoxicity values of reduced RB and RBOH did not differ significantly to those exhibited by the negative controls (1-1.3 times). Therefore, neither biological nor chemical reduction of RB or RBOH produces genotoxic products, and the weak genotoxic potential observed with unreduced RBOH is eliminated upon reduction of the dye molecule. A study by Rosenkranz and Klopman in 1989 showed that a structural determinant existed for the carcinogenicity of the azo dye Butter Yellow (*N,N*-

dimethylaminoazobenzene) that spanned the azo linkage of the intact molecule. Also, Razo-Flores and co-workers in 1997 observed that showed that several azo dyes exhibited far greater inhibitory effects on the activity of acetoclastic methanogens than their respective aromatic amine breakdown products, indicating that it was the intact azo group that exhibited the toxic effect (Razo-Flores *et al.* 1997*a*). Thus it can be proposed that RBOH contains a structural determinant for genotoxicity that spans the intact azo bond.

4.1.2.4 Toxicity and genotoxicity of HPLC fractionated decolourised hydrolysed C.I. Reactive Black 5

Two large fractions could be distinguished by analysis of *E. faecalis* reduced RBOH using HPLC. These fractions were identified as the 'vinyl sulphone' fraction, which derives from the auxochrome, and the 'diamino H-acid' fraction, which derives from the chromophore, and are highlighted in the structure of RBOH (see Figure 2.4). Whilst a specific toxicity value for the diamino H-acid fraction could not be established due to an inability to obtain sufficient concentrations, it is apparent that the diamino H-acid fraction is less toxic than the vinyl sulphone fraction (see Table 3.9). The toxicity of the vinyl sulphone fraction showed a similar toxic potential to a whole sample of reduced RBOH, and thus it can be suggested that the toxic potential of RBOH reduced by *E. faecalis* is due to the activity of the vinyl sulphone component. Genotoxicity potentials of the fractions could not be determined due to the inability to obtain sufficient concentrations from HPLC.

The lack of observable toxicity with the diamino H-acid fraction was confirmed using 1-amino-8-naphthol-3,6-disulphonic acid (H-acid), which is the commercially available equivalent to the diamino H-acid fraction. It was shown that this compound is essentially non-toxic (221 times less toxic than RBOH reduced by *E. faecalis*) and non-genotoxic, which agrees with previously published studies (Combes & Haveland-Smith 1982). This observation supports the suggestion that the diamino H-acid fraction is not the source of the toxicity of reduced RBOH. The attribution of toxicity of reduced RBOH to the vinyl sulphone fraction may have implications for the textile dye manufacturers. If alternative reactive groups could be used in azo textile dyes that did not show such large toxic potentials, such as the monochlorotriazinyl groups found in Procion dyes, then there would certainly be impetus for dyehouses to reduce the use of vinylsulphonyl-based dyes.

In summary, the toxicity and genotoxicity assays used were not only capable of identifying toxicological effects due to sulphonated azo dyes or their reduction products, but also were suitable to assess whole effluent toxicity taking into account complex background matrices produced by the negative controls for the assays. Additionally, it can be seen that the type of decolourisation method utilised can have a significant impact on the toxicity of the resultant decolourised dye solution, and is dependant not only upon the actual toxicity of the reduced dye but also on the toxic effect that the decolourisation method itself exerts. It would therefore be prudent to utilise decolourisation methods that only exert a small toxic potential in order not to exacerbate toxic hazards of decolourised azo dyes. In this study, it was noted that sodium dithionite exerted the smallest toxic potential, with biological waste products being 3.1 and 35.8 times more toxic (*C. butyricum* and *E. faecalis* respectively). However, *C. butyricum* required twice as long as *E. faecalis* to fully decolourise RB and the use of strictly anaerobic conditions, which increased the time, cost and technical ability required to utilise this organism for decolourisation. The cost of using sodium dithionite, which decolourises the dyes instantly after the addition of the chemical, would be higher than the running cost of using a biological reduction method. Thus, when the choice of dye decolourisation method is made, it should account for all factors including toxicity, cost, efficiency and technical difficulty.

4.2 Bioreactors

A synthetic dyehouse wastewater (STE) containing RBOH was treated in batch-fill and continuous flow types of bioreactor (an SBR and a HABR) under both anaerobic and anaerobic-aerobic conditions. The principle objective of treating textile dyehouse wastewater is to reduce the levels of polluting compounds in line with government regulations, such as the Environment Agency RQO limits for colour and future limits for toxicity. Therefore, the level of decolourisation of RBOH and the toxicity and genotoxicity of the decolourised effluents were assessed. It was concluded that the continuous flow HABR operated under anaerobic-aerobic conditions produced the 'best quality' effluent in terms of decolourisation, toxicity and stability of bioreactor performance.

4.2.1 Decolourisation of hydrolysed C.I. Reactive Black 5

4.2.1.1 Absorbance at visible wavelengths

Compliance with EA RQOs for colour involves adherence of effluents with a set of absorbance values measured over the visible wavelengths. Absorbance at all wavelengths measured between 400 (A_{400}) and 650 nm (A_{650}) reduced after anaerobic, anaerobic-aerobic and anaerobic-anoxic-aerobic treatment in both the SBR and HABR (66-94 and 59-88% respectively, see Tables 3.11 and 3.16). Inclusion of an aeration step in the SBR slightly decreased the A_{550}, A_{600} and A_{650} of the effluent, which are values around the wavelength exhibiting maximum absorbance (λ_{max}) for RBOH (597 nm). Absorbance values remained relatively similar under all oxygen conditions in the HABR. This demonstrates that aeration improved degradation of compounds absorbing around the λ_{max} for RBOH, including presumably the dye itself, but only in the SBR. This effect may be limited either by the design of the bioreactor (batch-fill versus continuous flow) or by the microbial populations adapted to the reactor environment. It was noted that A_{700} increased from the STE in both bioreactors, especially under anaerobic and anaerobic-anoxic-aerobic conditions. It is unclear from these experiments what this increase may be attributed to, although the effect is likely due to the breakdown of the dye molecule or its degradation products as this was not observed in the control reactors that did not contain dye.

The percentage decolourisation of RBOH under different treatment stages, calculated from the A_{597} of the effluents (λ_{max} for RBOH; Colour Index 1987), was 91.3-94.2 and 84.1-87.6% for the SBR and HABR respectively (see Tables 3.12 and 3.17). If data produced after anaerobic treatment only is compared to similar studies on decolourisation of RB in anaerobic bioreactors, these values are in the upper end of the range of what has been previously observed (see Table 4.1). Levels of decolourisation increased after anaerobic-aerobic treatment, and again after anaerobic-anoxic-aerobic treatment in the HABR. These increases, although relatively small, were determined to be significantly higher than those obtained after anaerobic decolourisation (Shaw 2000) (see Table 4.2). This increase in decolourisation produced by an aeration step is in accordance with the findings of similar studies on sequential anaerobic-aerobic decolourisation of reactive azo dyes (Lourenco *et al.* 2000, O'Neill *et al.* 2000, Panswad *et al.* 2001*a*), although in these studies the increases observed were commonly around 10%.

Table 4.1: Decolourisation of RB in bioreactors under anaerobic conditions

Inoculum	Dye conc. (mgL^{-1})	Decolourisation (%)	Time taken (h)	Reference
Anaerobic sludge	100	81	42	Brown & Laboureur 1983
Anaerobic sludge	100	80-85	4.5	Carliell *et al.* 1994
Anaerobic consortium	500	67	24	Nigam *et al.* 1996*a* & Nigam *et al.* 1996*b*
Anaerobic biofilm	500	>95	48	Oxspring *et al.* 1996
Anaerobic sludge	100	67	10.5	Setiadi & Loosdrecht 1997
Anaerobic sludge	300	79	10	Beydilli *et al.* 1998
Anaerobic sludge	10	53-72	24	Panswad *et al.* 2001*a*
Anaerobic granules/sludge	533	86-91	48-62.4*	This study

N.B. *HRT not time taken to decolourise dye

Table 4.2: Statistical analysis of decolourisation changes under anaerobic, anaerobic-anoxic-aerobic conditions in SBR Rig 1 and HABR Rig A

Atmospheric conditions	SBR			HABR		
	Decolourisation (%)	t	t_{crit}	Decolourisation (%)	t	t_{crit}
anaerobic	91.3	-	-	85.9	-	-
anaerobic-aerobic	93.9	10.2	2	86.6	6.3	2
anaerobic-anoxic-aerobic	-	-	-	87.6	2.1	2

(Adapted from Shaw 2000; analysis by paired t-test)

It has been suggested that increased decolourisation of reactive azo dyes should not be observed under anaerobic-aerobic conditions, as azo bond reduction only readily occurs under anaerobic conditions (Cripps *et al.* 1990, Zaoyan *et al.* 1992, Easton 1995, O'Neill *et al.* 1999, Chung 2000). However, it has been well documented that azo dye breakdown products, which are the constituent aromatic amines, build up in anaerobic systems (Kulla *et al.* 1983, Rafii *et al.* 1990, Banat *et al.* 1996, Chung 2000). Therefore it is not unreasonable to suggest that the build of toxic aromatic amines under anaerobic conditions inhibits the decolourising ability of microorganisms to a certain extent, and that mineralisation of at least some of these aromatic amines would decrease inhibition and cause decolourisation to increase. It has also been occasionally reported that azo dye decolourisation can be performed by aerobic microorganisms (Sarnaik & Kanekar 1995, Banat *et al.* 1996, Hao *et al.* 2000), and thus it is also possible that a small

decrease in A_{597} could be due to the action of the aerobic members of the bioreactor biomass.

The data demonstrated that both bioreactor types are efficient at decolourising a typical textile dyehouse effluent containing reactive dyes under both anaerobic and anaerobic-aerobic conditions. However, it should be noted that the EA RQO value for colour consent discharges on a typical sewage treatment plant is 0.04 AU at 600 nm (O'Neill *et al.* 1999). A_{600} values produced by the bioreactors under anaerobic and anaerobic-aerobic conditions were 0.9-1.3 and 2.1 AU for SBR and HABR respectively, which shows that anaerobic treatment in the two reactor types studied is insufficient to achieve the necessary colour removal required to meet EA RQO standards. Further decolourisation via an additional treatment phase or during bioremediation of the effluent at a sewage treatment plant would be essential if the problem of colour in water courses is to be alleviated.

4.2.1.2 Absorbance at UV, visible and IR wavelengths

Two distinct peaks in the absorbance profile of the STE over the UV-visible-IR range of the spectrum were observed after the addition of RBOH, a peak in the UV range (308 nm) and the visible range (590 nm) (see Figure 3.3). The observed peak at 590 nm corresponds to the λ_{max} of RB (597 nm). The UV peak observed may be explained by two factors. Firstly, commercial textile dyes only contain 25-60% pure colour, with the remainder comprised of salts, diluents and small quantities of other dyes (O'Neill *et al.* 1999). These impurities will exhibit absorbances at wavelengths other than the λ_{max} of the intended dye, and it is these impurities from the commercial dye used in this study that were likely to produce the UV peak. Secondly, the dye was added in its hydrolysed form, which involves hydrolysis of the vinyl sulphone side chains. RB has two of these side chains (see Figure 2.3) which require independent hydrolysis, and it is known that the process of hydrolysis may not always be complete for both side chains. Therefore structural variations of the dye molecule may be present in a hydrolysed sample, which would absorb at different wavelengths.

Additional peaks over the UV-visible-IR range were observed after treatment of the STE in the bioreactors. The peak at 260 nm is likely to correspond to aromatic amines formed by the reduction of the azo bonds in the dye. As expected, A_{260} increased with a corresponding decrease in A_{597} during anaerobic operation in both reactor types (see Tables 3.13 and 3.18). The accepted hypothesis is that a decrease in A_{597} with a

corresponding increase in A_{260} under anaerobic conditions is a result of dye molecules being reduced to the corresponding aromatic amines (Banat *et al.* 1996, Chung 2000). In addition, it is known that sulphonated dyes are not readily absorbed onto biomass (Hu 1992), and thus it was demonstrated that RBOH was degraded to its constituent aromatic amines under anaerobic conditions. Peaks at 754 and 972 nm previously undetected in the STE appeared after anaerobic treatment.

Both A_{260} and A_{597} decreased during anaerobic-aerobic operation in both reactor types (see Tables 3.13 and 3.18), although this effect was only observed in the HABR after additional biomass was introduced to the aerated cells. The accepted hypothesis is that a decrease in A_{260} under aerobic conditions is due to mineralisation of aromatic amines (Cripps *et al.* 1990, Zaoyan *et al.* 1992). Therefore, it was demonstrated that the anaerobic granules and activated sludge used to seed the SBR and aerated cells of the HABR respectively contained microorganisms that were capable of mineralising aromatic amines under aerobic conditions. It was observed that decreases in A_{260} only occurred to a maximum of 55% (from 22.0 to 10.0 AU in the HABR under anaerobic and anaerobic-aerobic respectively) which confirms that, although the level of aromatic amines had been reduced, there was still a considerable amount present after anaerobic-aerobic treatment. This is in accordance with the finding of O'Neill and co-workers in 2000, who treated an STE containing 450 mgL^{-1} hydrolysed C.I. Reactive Red 141 in an anaerobic UASB reactor and a subsequent aerobic vessel. It was observed that UV-absorbing by-products were formed during the anaerobic treatment of the STE, and that partial but not complete degradation to less aromatic, more polar compounds occurred in the aerobic stage.

The decreases in A_{597} after anaerobic-aerobic treatment demonstrated that further decolourisation of the STE occurred under aerobic conditions. Although most azo reduction is known to be performed by anaerobic microflora (Cripps *et al.* 1990, Zaoyan *et al.* 1992, Hao *et al.* 2000), decolourisation of azo dyes by aerobic microflora has also been occasionally reported (Banat *et al.* 1996, Hao *et al.* 2000). Therefore, it appears that the microflora were also decolourising the dye to a small extent under aerobic conditions, as shown by the decrease in A_{597}. The peaks at 754 and 972 nm were seen to be unstable in air, as absorbance values reduced after anaerobic-aerobic treatment and the peaks disappeared in oxidised samples left open to air. Reduction in A_{754} and A_{972} produced a concomitant increase in A_{597} in oxidised samples and in samples from the HABR when no additional biomass was added to the aerated cells.

This demonstrates that chemical oxidation may have played a role in decreasing A_{754} and A_{972} with the concomitant increase in A_{597}, suggesting that the compounds absorbing at 754 and 972 nm are dye molecules with partially reduced azo bonds.

A peak at 687 nm appeared during anaerobic-anoxic-aerobic operation (stage f) and intermittently during anaerobic-aerobic operation (stage e) in the HABR, and also during anaerobic-aerobic operation of the SBR. As anaerobic-aerobic operation of the SBR was within a single vessel, it was unavoidable that an anoxic stage would also be included during this treatment. Also during anaerobic-aerobic operation of the HABR, effluent from the aerated cells would occasionally recycle back into the previous anaerobic cell to produce anoxic conditions. This demonstrates that this peak was formed after anaerobic-anoxic-aerobic treatment of the STE, and accounts for its sporadic appearance during the anaerobic-aerobic stage. In addition, this peak was not observed when an anaerobic sample was left to oxidise in air nor after anaerobic-aerobic treatment in the HABR when no additional biomass was introduced (stages c and d). It can therefore be postulated that this peak corresponds to a biological biodegradation product formed by anaerobic-anoxic-aerobic degradation of RBOH. It was observed that the SBRs decolourising RBOH under anaerobic-aerobic conditions began to fail, as demonstrated by a reduction of TOC removal. Reduction in polyvinyl alcohol (PVOH) removal efficiency of the HABR decolourising RBOH occurred during stage e and especially stage f (anaerobic-aerobic and anaerobic-anoxic-aerobic conditions; Shaw 2000), which coincided with the appearance of this peak. It may therefore be considered that the product absorbing at 687 nm contributed to the failure of the SBRs under anaerobic-aerobic operation, possibly due to inhibition of the PVOH-degrading microorganisms, and may have reduced operating efficiency of the HABRs.

An individual analysis of the HABR cells revealed that most of the increases and decreases in absorbance values occurred in the first cell operating under the specified oxygen conditions, with the exception that the second anaerobic cell showed an decrease in A_{597} over the first cell. This effect was attributed to a lower biomass present in cell 1, which would be caused by washout of the biomass which cannot be replaced by any cells upstream of this. The implication of this observation appears to be that a single cell operating under each treatment type is adequate to produce the observed biological processes in terms of compound production/reduction, as long as the level of biomass can be retained at a suitable level. However, further parameters of bioreactor

efficiency, such as COD and toxicity reduction, would also need to be taken into account to assess the feasibility of this.

4.2.2 Toxicity and genotoxicity of the bioreactor effluents

It was observed that the STE utilised in this study exhibited a toxic and genotoxic potential only with the inclusion of RBOH (Tables 3.10 and 3.15), reflecting the toxicity and weak genotoxicity that was observed with unreduced RBOH in solution. It was noted that the toxicity and genotoxicity values obtained differed when analysing STE used to feed the SBRs and HABRs, although the composition of the STE remained unchanged. These differences are probably a function of the fact that the samples were collected at widely different time-points, and demonstrates batch-to-batch variation of the reagents used in the STE. Additionally, the toxicity analysis was conducted using different instruments and reagents (Microtox® for the SBRs and ToxAlert™ 100 for the HABRs), although comparative data showed that the instruments are comparable (see Table 3.14). Within the limits of the assays however, interpretation of these values still draws the same conclusion i.e. that the STE was toxic and genotoxic.

Anaerobic treatment of the STE in the SBR increased its toxicity 48 times and eliminated the genotoxicity. Toxicity data compare well with *in vitro* decolourisation data, in which the toxicity of RBOH was seen to increase 41-52 times after biological decolourisation. The increase in toxicity and elimination of genotoxicity was most likely to be due to production of sulphonated aromatic amines after the fission of the azo bond in RBOH, a process demonstrated by the reduction in absorbance at the λ_{max} of the dye. Most aromatic amines are toxic (Cripps *et al.* 1990, Zaoyan *et al.* 1992, Chung 2000), and sulphonated aromatic amines are known to be non-genotoxic (Combes & Haveland-Smith 1982, Puntener *et al.* 1993, Moller & Wallin 2000). A similar effect was observed in the HABR, although toxicity was only increased 11 times, which may be a function of the bioreactor designs or of interactions between different bioflora community associations. A toxic potential was also observed after anaerobic treatment of the STE that did not contain RBOH in both the SBR and HABR, but it was apparent that decolourised RBOH itself exhibited a toxic potential due to the higher toxicity of the decolourised effluent than effluent that did not contain RBOH.

Inclusion of an aeration step in the SBR or aerated cells in the HABR reduced toxicity (1.5 and 37 times respectively). This was most likely to be due to mineralisation of the aromatic amines which can be achieved by aerobic microorganisms (Kulla *et al.* 1983, Zaoyan *et al.* 1992), a process demonstrated by the reduction in absorbance at the wavelength associated with aromatic amines (260 nm). It was noted that, whilst toxicity was not eliminated in either reactor, only the effluent from the HABR was less toxic than the untreated STE, demonstrating the efficiency of this reactor type at treatment of an STE in terms of toxicity reduction. The toxic potential of the anaerobic effluent that did not contain RBOH was eliminated after anaerobic-aerobic treatment, suggesting that this toxic potential was due to products of anaerobic microbial metabolism, most of which are oxidised under aerobic conditions. The fact that this toxicity was eliminated in the HABR even when no additional biomass was introduced to the aerated cells that may have been adapted to utilise these by-products indicates that these toxic compounds were degraded by oxidation. One probable toxic waste product of anaerobic metabolism is hydrogen sulphide (H_2S), which would be formed as sulphate-reducing bacteria out-compete methanogenic bacteria in the high levels of sulphate present in the STE (Delée et al. 1998). The toxicity of the HABR effluent after anaerobic-anoxic-aerobic treatment (the most similar treatment phase to anaerobic-aerobic treatment in the SBR) was slightly further reduced, although not significantly.

It was observed that anaerobic-aerobic treatment of the STE both with and without RBOH produced a weak genotoxic potential in both reactors (except the dye-containing STE in the SBR). Although the source of this genotoxicity is unclear, it does not appear to be related to the aromatic amine substituents of RBOH as the effect occurs in the absence of RBOH, and thus may be related to an aerobic waste product or an oxidised anaerobic by-product.

Changes in the toxicity of the bioreactor effluents over time clearly demonstrated the rapid elimination of the toxic potential of the effluent without RBOH from both reactors under anaerobic-aerobic conditions (see Figures 3.1 and 3.3). This provided further evidence that the toxicity of these effluents was due to a compound that was oxidised rather than purely biodegraded, such as H_2S. A rapid increase in the toxicity of the effluent without dye was observed when RBOH was added to the feed of SBR Rig 2, reaching virtually the same values as Rig 1 by the end of operation. This demonstrated that the toxicity produced as a result of RBOH decolourisation was not significantly different between different enclosed bioflora communities.

4.2.3 Efficacy of batch-fill versus continuous flow type bioreactors at treatment of a simulated textile effluent

The batch-fill type SBR and the compartmentalised, continuous flow type HABR were compared in their ability to treat an STE with respect to mineralisation of RBOH and safety of the effluent. To facilitate this comparison, data previously presented concerning the mineralisation of RBOH, as shown by decolourisation and absorbance at 260 nm, and safety of the treated effluent, as shown by toxicity and genotoxicity, are collated in Table 4.3.

Table 4.3: Comparison of mineralisation of RBOH and safety of the effluents produced by the SBR and HABR

Parameter	Anaerobic		Anaerobic-aerobic		Anaerobic-anoxic-aerobic
	SBR	HABR	SBR	HABR	HABR
Decolourisation (%)[a]	91.3 ± 0.8	85.9 ± 0.6	93.9 ± 1.3	86.6 ± 1.5	87.6 ± 0.7
A_{260} (AU)[b]	24.0	22.0	20.0	10.0	10.0
Toxicity (EC_{50})[c]	0.24 ± 0.23	0.46 ± 0.17	0.34 ± 0.16	16.89 ± 9.82	17.80 ± 8.06
Genotoxicity (CS)[d]	1.01 ± 0.41	0.85 ± 0.27	0.93 ± 0.20	0.70 ± 0.05	-

N.B. SBR data are for Rig 1 stage I and III (anaerobic) and stage VI (anaerobic-aerobic). HABR data are for Rig A stage b (anaerobic), stage e (anaerobic-aerobic) and stage f (anaerobic-anoxic-aerobic). [a]percentage reduction in dye concentration measured by A_{597}; [b]average absorbance values; [c]EC_{50} 5 min values as % of original concentration; [d]CS values of undiluted sample.

The SBR gave significantly better decolourisation of the dye than the HABR, under both anaerobic ($t>t_{crit}$ = 22>2, Student's t-test) and anaerobic-aerobic ($t>t_{crit}$ = 17>2) conditions (Shaw 2000). However, the decolourisation values of 86-87% produced by the HABR are still good for decolourisation of RBOH in a bioreactor (see Table 4.1). It should be noted that the reduction of absorbance levels of the effluents were still insufficient to comply with EA RQO colour consent limits in any combination of bioreactor type and operating conditions, and thus further treatment to reduce absorbance (possibly a chemical method such as oxidation) would be necessary to achieve a fully treated effluent.

Inclusion of an aerobic phase in the SBR did not produce the level of A_{260} reduction that was observed in the HABR (17 and 55% in SBR and HABR respectively), demonstrating that aromatic amines were not degraded as efficiently in the SBR. The only significant decrease in toxicity of the effluents was observed when the aerobic phase was included in operation of the HABR, to levels that were 50 times less than that achieved during anaerobic-aerobic operation of the SBR. This confirms

the suggestion that the toxicity of the effluent from the SBR operated under anaerobic-aerobic conditions is due to the persistence of high levels of aromatic amines, and that the HABR was more effective in degradation of aromatic amines and thus reduction in toxicity. It is also of interest that the toxicity of the effluent produced after anaerobic-aerobic and anaerobic-anoxic-aerobic treatment in the HABR was 3 times less toxic than the untreated STE, and that this was the only combination of bioreactor type and operating conditions to produce a less toxic effluent after treatment. The genotoxic potential of the effluent produced after anaerobic-aerobic treatment in the HABR was higher than those observed after anaerobic treatment, or from the SBR under all operating conditions. However, a CS value of 0.7 is less genotoxic than the value of 0.58 observed in the untreated STE, and is on the border of CS values deemed to be non-genotoxic (Rosenkranz & Leifer 1980, Rosenkranz *et al.* 1981).

Whilst inclusion of an anoxic phase to the HABR slightly increased decolourisation and decreased toxicity, it was also noted that the levels of a compound absorbing at 687 nm produced during anaerobic-aerobic operation increased. It has been previously discussed that this compound possibly caused inhibition of PVOH-degrading microorganisms, causing a reduction in PVOH removal efficiency in the HABR under anaerobic-anoxic-aerobic conditions. Production of this compound in the SBRs decolourising the STE under anaerobic-aerobic conditions may also have contributed to the lack of operational stability of these reactors under these conditions, as evidenced by variation in physico-chemical parameters such as incomplete settling of bioreactor sludge and reduction in TOC removal (Shaw 2000). Therefore it can be concluded that the most beneficial and sustainable biotreatment process in terms of mineralisation of RBOH and the safety of the effluent is anaerobic-aerobic treatment in an HABR, although additional treatment would be required for compliance with some EA RQO consent limits.

4.3 Bioreactor microflora

In order to determine the interrelationships between treatment efficiency and microbial populations, Denaturing Gradient Gel Electrophoresis (DGGE) and enumeration by Fluorescent *in situ* Hybridisation (FISH) was used to assess the diversity of the microflora communities in the two bioreactors studied. Identification of species predominant under anaerobic and anaerobic-aerobic conditions was achieved by biochemical analysis of isolated colonies and sequencing of amplicons separated by DGGE. It was concluded that the continuous flow design of the HABR allowed greater

microbial diversity under all oxygen conditions than the batch-fill type SBR, and for speciation to occur in the aerobic compartments.

4.3.1 Assessment of Hybrid Anaerobic Baffled Reactor microflora by traditional culturing techniques

It was observed that the total number of distinct aerobic colonies isolated from HABR sludge was higher than the number of anaerobic isolates (see Table 3.19), which indicates a greater diversity in the organisms that could be cultured under aerobic conditions than those cultured under anaerobic conditions. No isolates were capable of decolourising RBOH under aerobic conditions, which is to be expected as decolourisation of azo textile dyes such as RBOH proceeds under anaerobic conditions via the use of the dye as an alternative electron acceptor. It was observed that there were an equal number of isolates capable of decolourising RBOH in Rig B, which was fed STE without RBOH, as there were in Rig A, which was fed STE with RBOH. Of two *Bacteroides* sp. colonies isolated from Rigs A and B, only that from Rig B was capable of decolourising RBOH. This showed that organisms with no prior exposure to RBOH can immediately begin decolourisation without a period of adjustment, and suggests that azo dye reduction is an inherent process to microorganisms that can be achieved by constitutively expressed proteins such as flavoproteins.

Identification of the isolated colonies showed that most aerobic isolates from both reactors were either *Staphylococcus* or *Pseudomonas* spp.. As both reactors were operated under anaerobic conditions at the time of sampling, it may be expected that the aerobic Pseudomonads would not be observed. This demonstrates that the design of the HABR allowed for the formation of microniches that could support bioflora communities that may not be able to survive in the given conditions. A difference in the most common types of anaerobic isolates from Rigs A and B was observed, with most isolates from Rig A identified as *Clostridium* and *Propionibacterium* spp. and most isolates from Rig B identified as *Actinomyces* and *Clostridium* spp.. As colonies were randomly selected for identification, this indicates that there was a difference in the predominant bacterial species between Rigs A and B, which would be due to the effect of inclusion of RBOH. As neither of the two *Propionibacterium* sp. colonies isolated from Rig A were capable of decolourisation of RBOH it is unclear why this species should be predominant in Rig A and not Rig B, although this may be a function of the very small number of this species isolated.

4.3.2 Assessment of Sequencing Batch Reactor microflora diversity by Fluorescent *in situ* Hybridisation

4.3.2.1 Numbers of bacterial and archaeal cells

The numbers of bacterial cells in Rig 1 were seen to slightly increase for the first 68 days of operation and then remain steady at 5.2×10^{7}-1.0×10^{8} cells ml^{-1} (Table 3.20). This demonstrates that bacterial numbers reached a critical level that could be supported by the conditions imposed by the bioreactor type. This level is slightly lower than those that have been reported in the literature, such as 1.5×10^{9} cells ml^{-1} identified by EUB338 in activated sludge by Snaidr and co-workers in 1997, indicating that the operating parameters of the SBR had a limiting effect on the total number of bacterial cells possible which was most likely due to large reductions in total numbers by high biomass washout. The ratio of archaea to bacteria was fairly constant at 0.5-0.7 (average 0.68) throughout the period of operation, demonstrating that archaea were present at just over half the abundance of bacteria. The observed decrease in this ratio to 0.4 at the end of the period of operation, when the reactor performance was declining, may be an indication of the fragility of the archaeal cells, as the inhibitory environmental parameters that were causing the decline of reactor performance were reducing the numbers of archaea faster than the numbers of bacteria. The amount of non-target staining of bacterial DNA, which was initially 10%, was rapidly eliminated. This demonstrates that the levels of RNA available for staining were very high, and is most likely due to the batch-fill type of operation selecting for rapidly growing bacterial cells. The rapid decrease in amount of autofluorescence was most likely due to selection against chlorophyll-containing cells such as cyanobacteria, as the bioreactors were operated in a closed room without a light source.

The numbers of bacterial cells in Rig 2 were seen to remain steady at 3.9×10^{7}-9.9×10^{7} cells ml^{-1} throughout the period of operation (Table 3.21). These levels were found to be significantly different to the levels of bacteria observed in Rig 1 ($p = 0.003$, paired t-test) and demonstrated that the inclusion of RBOH to the STE had a stimulatory effect upon bacterial growth, which may be due to the use of the dye as an alternative carbon and energy source. The stimulatory effect of RBOH on bacterial and archaeal growth was demonstrated by an increase in numbers of bacteria and archaea from day 208 onwards, which was most likely due to the addition of RBOH to the STE used to feed Rig 2 in increasing amounts from day 200 as this was the only operational change

made during this period. The ratio of archaea to bacteria was fairly constant at 0.7-0.8 (average 0.74) for the first 117 days of operation, meaning that archaea were present at just over three quarters the abundance of bacteria. This ratio then increased to an average of 0.93, meaning that there were equal numbers of bacteria and archaea, until a decrease in the ratio at the end of the period of operation when the reactor performance was declining. The average ratios of archaea to bacteria after day 140 of operation were found to be significantly different between Rigs 1 and 2 ($p = 0.01$). Aeration of the SBRs occurred from day 125, but the air supply to Rig 1 was turned off on day 137 and this reactor was returned to anaerobic operation. This demonstrates that the archaeal population was stimulated by anaerobic-aerobic conditions, at least in the absence of RBOH.

Data on numbers of archaeal cells are missing from sample days 140 and 244, where only a large amount of background biomass was observed with no individual cellular structures visible. It is possible that these dates coincided with major environmental events within the reactors, as bacterial and archaeal numbers were seen to decrease before both of these sample dates within both Rigs 1 and 2, although a more thorough analysis of toxicological, physico-chemical and microbial data is needed. As with Rig 1, the rapid elimination of non-target staining of bacterial DNA demonstrates the high levels of available RNA. It was observed that the residual level of autofluorescence in Rig 2 remained slightly higher than in Rig 1, suggesting that the inclusion of RBOH to the STE slightly inhibits the viability of chlorophyll-containing cells such as cyanobacteria.

4.3.2.2 Morphology of bacterial and archaeal cells

The most striking observation concerning the morphologies of the SBR microflora was that there was an obvious alteration in the morphological composition of both the bacterial and archaeal populations from the seed sludge by the first sample day. Whilst it is appreciated that morphological appearance does not adequately distinguish between different species, it can be suggested that the obvious alteration in morphological composition signified a shift in the population from the anaerobic granules, to a bacterial and archaeal population more adapted to the conditions in the SBRs. Reference to shifts in the DGGE profiles produced from these samples would confirm this suggestion. Additionally, the composition of the bacterial and archaeal populations of Rigs 1 and 2 did not show any significant differences, with the exception that long archaeal rods were not observed in Rig 1. This indicates that the microflora

populations were not greatly different between the reactors, although reference to the DGGE profiles from the SBRs would be required. As the major operating difference between Rigs 1 and 2 initially was the inclusion of RBOH to the STE, it is likely that the archaeal cells with long rod morphologies are susceptible to RBOH or its reduction products.

4.3.3 Assessment of Hybrid Anaerobic Baffled Reactor microflora diversity by Fluorescent *in situ* Hybridisation

4.3.3.1 Numbers of bacterial and archaeal cells

Bacterial cell numbers were seen to increase for the first 190 days of operation in cell 1 at the beginning of Rig A, and then remain steady at $1.0\text{-}1.4\times10^{8}$ cells ml^{-1} (see Table 3.22). Numbers observed in the remaining cells ranged from 9.6×10^{6} to 5.9×10^{7} cells ml^{-1}, and demonstrates that bacterial numbers reached a critical level in cell 1 that could be supported by the conditions imposed by the bioreactor type. This level is one order of magnitude lower than that reported by Snaidr and co-workers in 1997, indicating that the operating parameters of the HABR had a limiting effect on the total number of bacterial cells possible (most likely limited availability of nutrients, as numbers are highest at the start of the reactor).

The ratio of archaea to bacteria was fairly steady in cells 1 and 4 of Rig A at averages of 0.66 and 0.90 respectively, demonstrating that archaea were present at over two thirds the abundance of bacteria. Ratios were seen to increase steadily in cell 7 from 0.7 to 2.8, demonstrating that archaea began to predominate over the course of operation, whilst a steady ratio of 0.36 in cell 8 showed that aeration reduced the abundance of archaea to one third that of bacteria. This is in contrast to the ratios observed in SBR Rig 1, where the single vessel design resulted in an abundance of archaea of just over half the abundance of bacteria under both anaerobic and anaerobic-aerobic conditions. Although there are numerous reports of aerobic non-extremophile archaea in the literature, such as that of Gray and co-workers in 2002, it appears that aerated conditions limit archaeal numbers to some extent.

The amount of non-target staining of bacterial DNA in Rig A was steady at 0-3%, signifying high levels of RNA available for staining, and demonstrating that the HABR allows for high levels of bacterial activity. As with SBR Rig 1, the decrease in amount of autofluorescence to approximately 1-1.5% was most likely due to selection against chlorophyll-containing cells such as cyanobacteria, as the bioreactors were operated in a closed room without a light source.

Similar trends in bacterial cell numbers to those in Rig A were observed in Rig B, in that numbers remained steady at 9.8×10^{7}-1.6×10^{8} cells ml^{-1} in cell 1 after increasing for the first 127 days of operation and ranged from 2.5-6.7×10^{7} cells ml^{-1} in the remaining cells (see Table 3.23). As with Rig A, archaeal numbers in Rig B showed similar trends to bacterial numbers except numbers of archaea plateau earlier than bacteria, which is most probably due to out-competition of slow-growing archaea by faster growing bacteria for essential nutrients. Statistical analysis of the data showed that bacterial and archaeal numbers in cell 4 and bacterial numbers in cell 7 were significantly reduced in Rig A compared to Rig B ($p = 0.01$, 0.05 and 0.04 respectively, paired t-test). As a degree of separation of unreduced RBOH and aromatic amines from dye reduction can be achieved in the HABRs, these decreases were most likely due to inhibition of archaea and especially bacteria by toxic aromatic amines.

The ratio of archaea to bacteria was fairly steady in cells 1, 4 and 7 of Rig B at averages of 0.67, 0.94 and 0.69 respectively, demonstrating that archaea were present at over two thirds the abundance of bacteria. Similar trends were observed in Rig A, with the exception that ratios were seen to steadily increase over the period of operation in cell 7 of Rig A. The average ratios in cell 7 were found to be significantly higher in Rig A compared to Rig B ($p = 0.01$), which demonstrates preferential stimulation of the growth of archaeal cells by a degradation product of RBOH. Whether this stimulation is due to aromatic amines or mineralised products is unknown. As with Rig A, the ratio was reduced in cell 8 (average 0.27), again demonstrating the limiting effect of aerated conditions on archaeal numbers.

The amount of non-target staining of bacterial DNA in Rig B was similar to that observed with Rig A (average 2.1%), and again demonstrates that the operational parameters of the HABR allow for high levels of bacterial activity. The amount of autofluorescence within individual cells followed a very similar pattern as those observed with Rig A, generally lowest in cell 1, and increasing in cells 4 then 7 and then 8. As no RBOH was included in the STE used to feed Rig B, this demonstrates that RBOH does not affect numbers of chlorophyll-containing cells.

4.3.3.2 Morphology of bacterial and archaeal cells

The most striking observation concerning the morphologies of the HABR microflora was that there were no apparent trends in bacterial morphology in different cells or under anaerobic or aerobic conditions in either HABR, which is in contrast to the obvious alterations observed in the SBRs. Whilst it is appreciated that

morphological appearance does not adequately distinguish between different species, it can be suggested that the composition of the bacterial population varied greatly over time and did not seem affected by its relative position within the reactor (beginning, middle or end) or on oxygen availability. Reference to shifts in the DGGE profiles produced from these samples would confirm this suggestion. Additionally, the composition of the archaeal populations appeared to be more stable over time and unaffected by relative position within the reactor, with the exception that long rods seen in the activated sludge used to seed the aerated cells were not observed in Rig A. This indicates that the microflora populations were not greatly different between the reactors, although reference to the DGGE profiles from the HABRs would be required. As the major operating difference between Rigs A and B was the inclusion of RBOH to the STE, it is likely that the archaeal cells with long rod morphologies observed in the activated sludge are susceptible to RBOH or its reduction products, an observation which was made during analysis of the archaeal morphologies in SBR Rig 1.

4.3.4 Assessment of Sequencing Batch Reactor microflora diversity by Denaturing Gradient Gel Electrophoresis

4.3.4.1 Bacterial DGGE profiles and analysis of divergence

The main observation made from visual analysis of the bacterial DGGE profiles of the SBRs was that the profiles altered dramatically from those observed in the seed sludge up to day 68 of operation (Figures 3.16 and 3.18). This divergence was confirmed by band matching, where it was observed that samples from the initial seed and day 26 showed only ~10-20 and 30% similarity to the rest of the samples respectively in both SBRs (Figures 3.17 and 3.19). The large amount of divergence observed in both reactors may make the use of carefully selected consortia for seeding SBRs to decolourise dyehouse waste redundant. After day 68 the profiles appeared to change very little, with 4-7 main bands in the profile of each sample in Rig 1 and 6-9 main bands in Rig 2 profiles. The increased number of bands in Rig 2 indicated that its population was more diverse and heterogeneous than that of Rig 1, which is most likely due to a selective pressure exerted by the inclusion of RBOH and may be linked to the toxicity of this compound. However, the relatively few main bands observed demonstrated that the biomass of both SBRs included a small, stable bacterial population, which is most likely to be due to the daily microflora reductions by the high biomass washout level of this reactor type.

Several bands not apparent in the initial seed were observed to persist throughout the period of operation of both SBRs, which demonstrated that organisms were present that were equally capable of growth under both anaerobic and aerated conditions, and in the presence or absence of RBOH and its reduction products. Band matching of profiles from Rig 1 revealed that samples during the early stages of anaerobic operation (days 68 and 103) showed only 40% similarity to samples from later in the anaerobic stage (days 117-208). This showed that quite large changes in diversity could occur and not be detected by visual analysis, emphasising the importance of profile analysis software. Of the persistent bands not apparent in the seed, at least one appeared to be common to both Rigs 1 and 2, as a band was observed in both reactors in the same relative position on the same sample day. This indicated that there were at least some common bacterial species emergent in the SBRs.

Other bands were observed to appear or disappear upon commencement of aeration of the reactors or inclusion of RBOH to the STE used to feed Rig 2, demonstrating that alterations in these parameters affected the composition of the bacterial population. Band matching revealed that samples taken under anaerobic-aerobic conditions showed only 43-47% similarity to anaerobic samples in both reactors. This showed a large divergence in the bacterial population when aeration of the reactors was commenced, which did not appear to be affected by the presence of RBOH or its reduction products. Samples taken from Rig 2 under anaerobic-aerobic conditions after the addition of RBOH to the STE showed only 38% similarity to anaerobic-aerobic set without the inclusion of dye. This demonstrated that the inclusion of RBOH into the STE, as would be observed in real dyehouse waste, altered the composition of the bacterial population to a slightly greater extent than the divergence caused by a modification of the atmospheric parameters (aeration).

4.3.4.2 Archaeal DGGE profiles and analysis of divergence

The most striking observation concerning amplification of archaea from the SBRs was that amplicons could not be produced from most of the samples, at least in sufficient amounts to be visualised on agarose or DGGE gels (see Figures 3.20 and 3.21). This is in contrast to findings of FISH analysis of the SBR samples, which showed large numbers of archaea ($>1\times10^7$ cells ml^{-1}) in both reactors throughout the entire period of operation. It is unclear why this effect was observed, as the use of pure DNA extracts not subject to the effects of possible inhibition did not result in amplification from these samples.

Relatively few bands were observed in the profiles in comparison to bacterial profiles indicating that the archaeal population was far more homogenous than the bacterial population, which is likely due to both out-competition for nutrients by the faster growing bacteria and the effects of high biomass washout. The relatively homogenous bacterial and archaeal populations observed in the SBRs are probably characteristic of this type of bioreactor, as the continual alteration in nutrient levels and oxygen conditions (anaerobic, anoxic and aerobic) would be considered sub-optimal for microbial growth (Irvine *et al.* 1997). The profiles did not appear to alter over time by visual inspection, indicating a stable archaeal population, and no obvious differences in the profiles occurred upon addition of RBOH to the STE used to feed Rig 2. Band matching revealed that samples taken on days 194 and 208 (0 and 53 mgL^{-1} RBOH respectively) showed 60% similarity, whilst samples taken on days 238 and 244 (533.3 mgL^{-1} RBOH) showed 90% similarity to day 208. This showed that addition of a small amount of dye to the STE altered the composition of the archaeal population, although the change could not be detected by visual analysis and emphasised the importance of profile analysis software, but that a 10-fold increase in the concentration of the dye did not significantly alter the composition further. Thus, a specialised archaeal population was selected by the presence of RBOH, which was also able to tolerate high concentrations of its aromatic amine reduction products.

4.3.4.3 Sequencing of bands from bacterial and archaeal DGGE profiles

Excision, re-PCR and re-DGGE of bands of interest from bacterial and archaeal profiles prior to sequencing resulted in valid sequence data for 6 out of 12 (50%) of the excised bands (see Table 3.24). Of the 6 that did not produce valid data, 4 did not produce any sequence data, which was most likely due to dilution of the very faint target resulting in insufficient amplicon production in the sequencing reaction. The remaining 2 produced sequence data that did not show valid microbial matches to the databases, which was most likely due to dilution of the fairly faint bands resulting a concentration of target amplicon that was too low to be distinguished from the background produced by non-target amplicons.

Two predominant bacterial bands present under both anaerobic and anaerobic-aerobic conditions in the presence of RBOH were identified as *Clostridium* and *Bacteroides* spp.. Bands in the same relative position of the profiles from both Rigs 1 and 2 were identified as *Bacteroides* sp., although the closest matches were to different

species, demonstrating that some emergent species were common to the bioflora of the SBRs. *Clostridium* and *Bacteroides* spp. are commonly found in anaerobic sludges from bioreactors (Delbès *et al.* 2000), originating from the human gastrointestinal tract where they are also common (Cerniglia & Somerville 1995), and it has been reported that *Bacteroides* sp., members of the *Cytophaga-Flexibacter-Bacteroides* phylum, generally constituted the second largest group of organisms (~30% of all cells) in bioreactor sludges (Manz *et al.* 1996, Godon *et al.* 1997). Therefore, it could be expected that these species would be identified as forming predominant species in the SBR microflora. As these organisms were also tolerant to the toxicity due to residual aromatic amines produced after RBOH decolourisation, they may be potentially useful in bioreactors designed to decolourise textile dyehouse wastewaters containing reactive dyes. However, the large natural divergence from seed biomass observed in the SBRs may preclude this usefulness. In addition, although they are both anaerobic species they were also tolerant to aeration of the reactor, which may also make them potentially useful in decolourising bioreactors where an aeration step is considered necessary to mineralise the aromatic amines produced by anaerobic breakdown of reactive dyes.

A less predominant bacterial band only present under anaerobic-aerobic conditions (or in some anaerobic samples after aeration) in the presence of RBOH was identified as the Actinomycete *Actinomadura* sp.. Actinomycetes have also been identified in aerobic ecosystems such as soil (Heuer *et al.* 1997) and aerobic bioreactor sludge (Bramucci & Nagarajan 2000, Davenport *et al.* 2000), and thus it could be expected that this organism would be present under anaerobic-aerobic conditions. As this organism was tolerant to the toxic effects of residual aromatic amines, it also may be potentially useful in anaerobic-aerobic sequential bioreactors designed to reduce the toxicity of decolourised textile dyehouse wastewaters containing reactive dyes.

Archaeal bands identified as *Methanosaeta* sp. were only amplified from Rig 2 under anaerobic-aerobic operation in the presence of RBOH. *Methanosaeta* sp. (Plumb *et al.* 2001) and *Methanothrix* sp. (closely related to *Methanosaeta* sp.) (Boopathy & Tilche 1991, Boopathy & Tilche 1992) have been identified as dominant species in the archaeal microflora of bioreactors. Although these methanogens are obligate anaerobes, the existence of anaerobic microniches within sludge flocs as noted by Gray and co-workers in 2002 mean that archaea may be observed in sludges from aerobic environments. *Methanosaeta* sp. are known to be predominant in the aceticlastic core of anaerobic granules (Guiot *et al.* 1992, Sacks 1998), and thus its survival under

anaerobic-aerobic conditions is most likely due to a protective effect of the position of this species within the centre of sludge flocs which are likely to have lower oxygen levels than the outside layers. Assessment of the spatial arrangement of archaeal cells within sludge flocs by FISH analysis showed that archaea could be found in the centre of flocs, but also on the outside (see Figure 3.7a-f). In addition, it should be remembered that the SBRs were not fully aerobic as they were only aerated for 30 minutes in a 24-hour period. Methaogenic archaea of the *Methanosarcinales*, *Methanomicrobiales* and the *Methanobacteriales* groups have also been observed to be the predominant archaea in aerobic activated sludge samples, although they were shown not to be actively producing methane under aerobic conditions (Gray *et al.* 2002). This suggests that methanogens may commonly be found in aerobic sludges, but whether they are surviving or thriving is unclear.

4.3.5 Assessment of Hybrid Anaerobic Baffled Reactor microflora diversity by Denaturing Gradient Gel Electrophoresis

4.3.5.1 Bacterial DGGE profiles and analysis of divergence

The main observation made from visual analysis of the bacterial DGGE profiles of the HABRs was that there were significantly more bands in the profiles than in either SBR (Figures 3.23, 3.24, 3.27 and 3.28). Whilst SBR samples produced fewer, more predominant bands (maximum of 9 main bands), HABR samples produced more numerous, less predominant bands (maximum of 16 main bands and many faint bands). This demonstrated that the bacterial populations of the HABRs were far more diverse and heterogeneous than those observed in the SBRs. An alteration in the profiles from those observed in the seed sludges was visually observed from the first sample day in both reactors, indicating divergence from the seed sludge population as the microflora adapted to the reactor conditions. The immediate alteration in the profiles was confirmed by band matching, which demonstrated that samples from cells 1-7 showed only 25-30 and <10-25% similarity to the seed sludge in Rigs A and B respectively (Figures 3.25 and 3.29). The same observation was made with aerobic sludges, where samples from cell 8 were found to have 25 and <10% similarity to the seed sludge in Rigs A and B respectively. This dramatic divergence from the seed sludge was also observed in the SBRs, and emphasises the redundancy of using carefully selected consortia for seeding bioreactors to decolourise dyehouse waste irrespective of design.

The number of main bands was seen to increase from the number in the seed sludge by day 127 in Rig A and by the first sample day, day 34, in Rig B. These

increases demonstrated increasing diversity of the bacterial population over time, which was not observed in the SBRs. It was noted that numbers of main bands in the SBR samples were slightly reduced in the presence of RBOH, which corresponded to a lag in the time taken for diversity to increase in the HABRs. This demonstrated that the presence of RBOH or its reduction products in the STE decreased the diversity of the bacterial microflora, most likely due to inhibition of certain species by toxic aromatic amines.

Profiles for both HABRs demonstrated the presence of bands that were persistent in anaerobic, anoxic and aerobic cells, in addition to those only observed in anaerobic cells or aerobic cells, demonstrating the presence of ubiquitous facultative anaerobes and specialised anaerobes or aerobes. The large number of bands in the profiles indicated diverse bacterial populations, and there were no apparent trends influenced by time, atmospheric conditions or position within the reactor. Band matching of the profiles confirmed the presence of a highly divergent and dynamic population, as samples clustered into multiple sets in all of the HABR DGGE gels analysed showing only 20-42 and <10-40% similarity between any of the sets for Rigs A and B respectively. The lowest similarity values between sets in Rig B, which were lower than those observed in Rig A, occurred up to day 190 of operation, which demonstrates that the toxic effects of reduced RBOH limited the initial high divergence of the bacterial population.

Samples taken from different cells on the same day did not tend to cluster together at the beginning of operation of the reactors. However, similarity values between different cells sampled on the same day increased towards day 190 of operation and clusters tended to comprise all cells for a sample day by the end. In addition, similarity values between different sets in Rig B were initially <10-27%, which then increased to 32-40% towards the end of operation. This demonstrates that the populations in different parts of the reactors were becoming similar over time, especially in the absence of RBOH, thus highlighting the dynamic nature of the microflora.

Bands always present in specific cells of both reactors in consecutive samples were observed on several occasions. For example, bands were observed only in cells 4 and 7, or were strongest or only apparent in cell 1. As similar observations were made for both reactors, some of the differences between cells were not due to the effects of RBOH, but to other operating parameters of the HABRs. Bands unique to aerobic cells

were apparent in both reactors but in higher numbers in Rig B, indicating that reduction products of RBOH limited the diversity of aerobic species. Clustering of samples according to specific cells or atmospheric conditions was confirmed by band matching. Up to day 190 of operation, samples tended to cluster into sets containing samples from cell 1 and samples from cells 4 and 7 (with similarity values as low as 15-20%), but after day 190 samples tended to cluster into sets comprising anaerobic, anoxic and aerobic samples, demonstrating that the bioflora populations diverged according to varying environmental conditions. Similarity values between anaerobic and anoxic cells in Rig A were lower than in Rig B, which indicated that the presence of RBOH stimulated specialised organisms adapted to anoxic conditions, most likely due to utilisation of compounds such as methane and carbon dioxide from aerobic mineralisation of the aromatic amines produced by anaerobic degradation of RBOH. Sludge samples from the aerobic cells showed only 25-42 and <10-40% similarity to anaerobic and anoxic samples, showing divergence in the aerobically operated cells especially in the absence of RBOH. These observations demonstrate that the design and operational parameters of the HABRs encouraged increased diversity compared with the SBRs, and speciation of the bacterial population according to different environmental parameters.

Differences in the profiles produced from the sludge and biofilm samples were observed both visually and by band matching (Figures 3.24, 3.26, 3.28 and 3.30). A separate analysis of the samples taken on day 230 revealed that sludge and biofilm samples clustered together showing only 55% similarity to each other in both reactors (data not shown), which is most likely to be due to the differences in nutrient and oxygen availability between the sludge blanket and the exposed biofilm. Band matching revealed that anaerobic and anoxic biofilm samples showed 40-75 and 59-70% similarity to each other in Rigs A and B respectively, whilst aerobic biofilms showed 20-57 and 30% similarity to their respective anaerobic and anoxic biofilms, which demonstrated a divergence of the biofilm microflora with increasing oxygen availability. Bands in samples taken from anoxic sludges were also observed in anaerobic biofilms, whilst bands in samples taken from aerobic sludges were also seen in anaerobic and anoxic biofilms. These observations indicated that oxygen concentrations were higher in biofilms than sludges as organisms that could survive in biofilms taken from anaerobic and anoxic cells could not survive in the sludges of these

cells, which is to be expected as it is known that dissolved oxygen can penetrate biofilms (Bishop & Yu 1999).

4.3.5.2 Archaeal DGGE profiles and analysis of divergence

The main observation made from visual analysis of the archaeal DGGE profiles of the HABRs (see Figures 3.31, 3.32, 3.35 and 3.36) was that there were relatively few bands (3-6 main bands) observed in comparison to bacterial profiles (maximum of 16 main bands and many faint bands), indicating that the archaeal population was far more homogenous than the bacterial population. Archaeal species usually constitute a lower percentage of total microflora than bacterial species, which is most likely due to out-competition for nutrients by the faster growing bacteria.

There were 5-6 main bands present in nearly all samples from Rig A, which did not appear to alter over time by visual inspection. The stability of the population was confirmed by band matching, with low divergence demonstrated by high similarity between samples (67-100 and 30-100% for samples up to day 190 and up to the end of operation respectively). The decrease in similarity between the profiles at the end of operation demonstrated some divergence after day 217, although this is not as great as that observed during analysis of bacterial similarity values in these reactors (<10-73%). In contrast, visual inspection of the profiles from Rig B showed slightly more variation in the number of the 3-6 main bands present in any one sample. Band matching revealed that divergence in Rig B was higher than in Rig A, especially at the beginning of operation (10-75 and 23-100% similarity for samples up to day 190 and up to the end of operation respectively). The most dramatic change in the population composition occurred between days 55 and 127, as samples taken before and after these days showed only 10% similarity to each other. Reference to the operational parameters of the HABRs (Appendix 2) show that aerobic cells were added during this time, although the anaerobic cells should not have been affected by this, and so this divergence is most likely due to the establishment of slow growing organisms. The greater fluctuation of the archaeal population in Rig B combined with the decrease in numbers of main bands demonstrates that the inclusion of RBOH stimulated the growth of certain archaeal species but limited the natural fluctuations that occurred over time.

During analysis of bacterial profiles from both the SBRs and HABRs it was observed that the composition of the populations diverged rapidly from the seed sludges. In contrast, profiles from the HABRs revealed little divergence of the archaeal populations from the seed sludges (Figures 3.33 and 3.37). In Rig A, the seed sludge

produced 5 main bands which were seen to persist in nearly all samples and showed 73% similarity to all other samples analysed, which demonstrated that neither unreduced nor reduced RBOH exerted a significant selection pressure on the archaeal microflora. In Rig B, the 3-4 main bands in samples from days 34 and 55 appeared to persist from the seed sludge, which showed 48-63% similarity to those samples, although the population diverged greatly after day 55. As the bacterial but not the archaeal populations diverged dramatically from the anaerobic sludge used to seed the HABRs, it can be suggested that the predominant species in the archaeal population were capable of survival in the HABRs whilst the bacterial microflora required adaptation to the conditions imposed by them, including the presence of RBOH and its reduction products. No conclusions concerning the adaptation of the archaeal population of the activated sludge used to seed cells 8-10 could be made, as archaeal products could not be amplified this sample.

Profiles for both HABRs revealed the presence of bands that were persistent in anaerobic, anoxic and aerobic cells, in addition to those only observed in specific anaerobic cells, which demonstrated the presence of ubiquitous facultative anaerobes and specialised anaerobes adapted to specific conditions within the reactors. In both Rigs A and B, it was observed that some bands were either only present or were strongest in cell 1, which is most likely due to the higher levels of nutrients that would be present at the beginning of the reactors. Clustering of samples according to specific cells or atmospheric conditions was confirmed by band matching. In Rig A, samples tended to cluster into sets comprising anaerobic, anoxic and aerobic samples, especially after day 190. However, in Rig B samples did not exhibit such a strong tendency to cluster according to atmospheric conditions, and similarity values between anaerobic and anoxic cells in Rig A were lower than in Rig B, which is most likely due to the presence of methane and carbon dioxide from mineralisation of RBOH stimulating specialised organisms adapted to anoxic conditions. Only one band appeared to be unique to aerobic samples (in cell 8 of Rig A on day 190 only), and sludge samples from the aerobic cells showed 55-85 and <20-100% similarity to anaerobic and anoxic samples (Rigs A and B respectively). This demonstrated there was little difference in the archaeal populations that derived from the anaerobic and activated sludges used to seed the HABRs, and that any aerobic archaeal species that may have been present in the activated sludge were unable to adapt to the conditions prevalent in the aerobic cells of the HABRs, but that differentiation was more acute in the absence of RBOH. The

stability and low diversity of the archaeal population is similar to that observed in the SBRs, although no differences in the populations according to atmospheric conditions were observed in the SBRs. This demonstrates that the archaeal community remained relatively homogenous irrespective of bioreactor design, but that the design and operational parameters of the HABR allowed for a small amount of speciation of the archaeal population according to different environmental parameters.

Visual analysis of the profiles produced from sludge and biofilm samples indicated very little difference in archaeal population between the two sample types, as only one band present in all sludge samples in Rig A was obviously absent from biofilms. However, some differences between the sample types were observed by band matching (Figures 3.34 and 3.38). Anaerobic and anoxic biofilms clustered together in Rig A, showing 30 and 73-87% similarity to their respective sludges, with the greater divergence in the anaerobic samples most likely due to higher nutrient availability in the sludge blanket and higher oxygen concentrations in the biofilm layer. In contrast, anaerobic biofilms tended to cluster with anoxic sludges and anoxic biofilms with anoxic and aerobic sludges in Rig B, reiterating the observation that oxygen concentrations were higher in biofilms than sludges which was made during analysis of bacterial microflora in HABR biofilms. Anaerobic and anoxic biofilms showed 73-93 and 45-60% similarity to each other whilst aerobic biofilms showed 55 and 33-80% similarity to these samples, in Rigs A and B respectively. These observations demonstrated that archaeal populations in the biofilm samples diverged due to atmospheric conditions, and that this divergence was more apparent in the presence of RBOH and its reduction products. This divergence caused by aeration was more apparent in biofilms than sludges, as anaerobic and anoxic biofilm and sludge samples showed 55-65 and 67-80% similarity respectively to their corresponding aerobic samples, and was most likely due to higher levels of oxygen in biofilms.

4.3.5.3 Sequencing of bands from bacterial DGGE profiles

Excision, re-PCR and re-DGGE of bands of interest from bacterial profiles prior to sequencing resulted in valid sequence data for 5 out of 7 (70%) of the excised bands (see Table 3.25), in comparison to 50% of excised bands for the SBRs. It is known that excising bands of interest from DGGE gels, re-amplifying and then sequencing has yielded poor quality data in other studies (Ishii *et al.* 2000, Gray *et al.* 2002), and even the additional purification attempted in this study by a second round of DGGE prior to

amplification and sequencing has not been completely successful. Dilution of the target amplicon resulting in no sequences or high background from non-target amplicons presents a greater problem for identification of less predominant species, and thus the direct sequencing of bands is not recommended when analysis of less dominant species that show interesting ecological patterns such as those found in the HABRs is required.

A predominant band present in anaerobic and aerobic sludges in Rig A was identified as *Bacteroides* sp., whilst a predominant band in sludge and biofilm samples from all oxygen conditions in Rig B was identified as *Cytophaga* sp.. Both of these species form part of the *Cytophaga-Flexibacter-Bacteroides* (CFB) phylum. These observations demonstrate that the presence of RBOH and its reduction products did not significantly affect the predominant microflora of the HABRs. *Bacteroides* sp. have been reported to be very common in bioreactor sludges (Manz *et al.* 1996, Godon *et al.* 1997, Delbès *et al.* 2000), and a study by Plumb and co-workers (2001) using an anaerobic baffled reactor of similar design to the HABRs utilised in this study to treat wastewater containing food azo dyes found that members of the CFB phylum were predominant members of the bacterial population. A predominant band identified as *Bacteroides* sp. (also showing the closest match to *Bacteroides uniformis*) was also observed in SBR Rig 1, which indicates that this species was predominant in the bioreactors decolourising RBOH irrespective of their design. In addition, an anaerobic species identified as *Bacteroides* sp. was also isolated from both Rigs A and B by culturing, which showed that both traditional media-based and sequence based methods of bacterial identification can distinguish species (or at least members of a phylum) that form predominant members of a bioreactor microflora. Although *Bacteroides* sp. are anaerobic this band was also observed in aerobic sludges, possibly protected in anaerobic microniches. This demonstrates that *Bacteroides* sp. was tolerant to the presence of RBOH and its reduction products and to aeration of the reactor, as was also observed in the SBRs, and may make this species potentially useful in anaerobic-aerobic decolourising bioreactors.

Bands present in sludge samples only under aerobic conditions in both Rigs A and B were identified as *Nocardia, Rhodococcus* and *Rhodopseudomonas* spp.. These aerobic organisms belong to the group of mycolic acid-containing actinomycetes called mycolata, which have previously been reported in activated sludges from wastewater treatment plants such as that used to seed the aerobic cells of the HABRs (Davenport *et*

al. 2000). Small amounts of aromatic amines were present in the aerobic cells of Rig A, as evidenced by a toxic potential and absorbance at 260 nm of the effluent, which indicates that mycolata may be a significant species in anaerobic-aerobic bioreactors used to decolourise azo dyes. The band identified as *Rhodococcus* sp. was also observed in anaerobic and anoxic biofilms in Rig A. This indicates that the formation of biofilms (which is possible in a continuous-flow reactor such as the HABR) was beneficial to the survival of aerobic species in cells operating under anaerobic and anoxic conditions, which demonstrates one way in which the design of the bioreactor can influence the diversity of the bacterial microflora. As organisms were observed to be unique to aerobic cells in the HABRs, it was apparent that separated aerobic compartments allowed for the persistence of some introduced aerobic microflora.

4.3.6 Relationships between bioreactor microflora and the safety of effluents

4.3.6.1 Relationships in the Sequencing Batch Reactors

The interrelationships between STE treatment efficiency (in terms of toxicity, genotoxicity and decolourisation) and microbial populations were considered. To facilitate elucidation of these relationships, data previously presented concerning the mineralisation of RBOH and safety of the effluent, in addition to diversity and identification of the microflora from the dye-decolourising bioreactors are collated in Table 4.4.

In Rig 1 of the SBRs, bacterial numbers were substantially higher than archaeal numbers under both anaerobic and anaerobic-aerobic conditions, an effect that was not observed in Rig 2 when RBOH was not used in the STE. Using species abundance curves, Curtis and co-workers (2002) have demonstrated that the number of species in a population increases in direct proportion with increases in the total number of individuals in that population. Therefore, an increase in microbial numbers as measured by FISH indicates an increase in the diversity of that population. However, the number of main bands in Rig 1 was actually slightly lower than Rig 2, which demonstrated that there was slightly less diversity in Rig 1, and so it appears that the presence of RBOH merely stimulated the growth of bacteria in the SBRs.

Table 4.4: Influence of numbers, diversity and types of bacteria and archaea on mineralisation of RBOH and safety of the effluent produced by the SBR

Parameter	Anaerobic	Anaerobic-aerobic
Decolourisation (%)[a]	91.3 ± 0.8	93.9 ± 1.3
A_{260} (AU)[b]	24.0	20.0
Toxicity (EC_{50})[c]	0.24 ± 0.23	0.34 ± 0.16
Genotoxicity (CS)[d]	1.01 ± 0.41	0.93 ± 0.20
No. of Bacteria[e] **($\times 10^7$ cells ml^{-1})**	8.2 ± 1.7	8.7 ± 1.0
No. of Archaea[e] **($\times 10^7$ cells ml^{-1})**	4.5 ± 1.4	3.7 ± 1.0
Bacterial diversity[f]	5-7 main bands	4-5 main bands, 47% similarity to anaerobic
Archaeal diversity[f]	-	6 main bands
Bacterial species[g]	*Bacteroides* sp., *Clostridium* sp.	*Bacteroides* sp., *Clostridium* sp., *Actinomadura* sp.
Archaeal species[g]	-	*Methanosaeta* sp.

N.B. Data are for Rig 1 stage I-III (anaerobic) and stage VI (anaerobic-aerobic). [a]percentage reduction in dye concentration as measured by A_{597}; [b]average absorbance values; [c]EC_{50} 5 min values as % of original concentration; [d]CS values of undiluted sample; [e]number of bacteria/archaea as determined by FISH; [f]numbers and similarities of bacterial/archaeal bands as determined by DGGE and band matching; [g]bacterial/archaeal species identified by sequencing.

Anaerobic treatment of the STE produced high decolourisation and an effluent that was toxic, due to high levels of aromatic amines (determined by absorbance at 260 nm), and non-genotoxic. Aeration of the SBR slightly increased decolourisation and genotoxicity, and slightly decreased toxicity due to a small decrease in the levels of aromatic amines, although none of these alterations were considered to be significant. A minor increase in the numbers of bacteria was observed whilst conversely the diversity of the bacterial population was slightly decreased, as demonstrated by a reduction in the numbers of strong bands observed, which indicates that aeration merely stimulates bacterial growth. The composition of the bacterial population appeared to alter significantly after aeration, as demonstrated by a 53% divergence of the DGGE profiles, but the predominant species identified by sequencing persisted from anaerobic operation and only one species considered to be unique to anaerobic-aerobic operation was observed and identified. Aeration resulted in a decrease in the numbers of archaea, which is most likely due to inhibition of obligate anaerobic archaea that were present on the surface of the sludge flocs. The diversity of the archaeal population under anaerobic-aerobic conditions appeared to be similar to that of the bacterial population under

anaerobic conditions, but the effects of aeration on diversity and population composition could not be ascertained due to a lack of amplification from anaerobic-aerobic samples.

Thus it was demonstrated that an SBR operated under anaerobic conditions decolourised RBOH and supported a relatively diverse, thriving, stable bacterial population predominated by *Bacteroides* and *Clostridium* spp., and produced a non-genotoxic effluent which had a toxic potential due to the persistence of aromatic amines. Operation under anaerobic-aerobic conditions did not significantly alter either the mineralisation of RBOH or the safety of the effluent, and no substantial difference in bacterial diversity was observed. However, aeration resulted in divergence of the bacterial population and the appearance of *Actinomadura* sp. although the predominant species were unchanged, and an archaeal population of similar diversity to the bacterial population predominated by *Methanosaeta* sp. was now observed. Therefore, operation of the SBR under anaerobic conditions produced an effluent that was not effectively treated by the microflora, and the few alterations in the microflora observed were not sufficient to produce a safe effluent.

4.3.6.2 Relationships in the Hybrid Anaerobic Baffled Reactors

The interrelationships between STE treatment efficiency (in terms of toxicity, genotoxicity and decolourisation) and microbial populations were considered. To facilitate elucidation of these relationships, data previously presented concerning the mineralisation of RBOH and safety of the effluent, in addition to diversity and identification of the microflora from the dye-decolourising bioreactors are collated in Table 4.5, which also gives data for individual compartments (cells) where appropriate.

In Rig A of the HABRs, both bacterial and archaeal numbers are significantly higher in cell 1 during anaerobic-aerobic operation than any other samples, which corresponded to higher numbers of strong bands in the profiles. Similar observations were made with Rig B when RBOH was not used in the STE, and thus the compartmentalisation of the HABRs allowed for differential adaptation of the microflora within different compartments due to the differing environmental conditions present across the reactors. Whilst archaeal diversity increased in cell 4 under anaerobic-aerobic conditions, bacterial numbers did not. Bacterial diversity, including number of organisms, did increase in Rig B under these conditions, which demonstrated

that the toxic reduction products of RBOH inhibited the growth of bacteria that were likely to have adapted to all other environmental conditions.

As with the SBR, anaerobic treatment of the STE produced high decolourisation and an effluent that was toxic, due to high levels of aromatic amines (determined by absorbance at 260 nm), and non-genotoxic. Addition of an aerobic cell (cell 8) to the HABR slightly increased decolourisation and genotoxicity, and significantly decreased toxicity and the levels of aromatic amines present. Bacterial diversity, demonstrated by numbers of both organisms and strong bands, increased in cells 1 and 4 but remained relatively unchanged in cell 7. The diversity of the bacterial population in the aerobic cell appeared to be similar to that of the anaerobic cells, whilst the composition was significantly different as demonstrated by a 63-75% divergence of the DGGE profiles. The predominant species in anaerobic cells identified by sequencing persisted in the aerobic cells, but several species considered to be unique to the aerobic cells were observed and identified. Archaeal diversity increased in all cells (demonstrated by the number of strong bands) whilst numbers in cell 7 were unchanged, which indicates inhibition of archaeal growth and is likely due to the proximity of this cell to the aerated cell and the incomplete separation of atmospheric conditions. The diversity of the archaeal population in the aerobic cell was reduced in comparison to the anaerobic cells, most likely due to the absence suitable microniches for some obligate anaerobes, and the composition was more similar to the anaerobic cells as demonstrated by a 20-35% divergence of the DGGE profiles.

Thus it was demonstrated that a HABR operated under anaerobic conditions decolourised RBOH and supported an increasingly diverse, thriving, dynamic bacterial population predominated by *Staphylococcus* and *Bacteroides* spp. and a less diverse, more stable archaeal population, and produced a non-genotoxic effluent which had a toxic potential due to the persistence of aromatic amines. Compartmentalisation of this reactor allowed differential adaptation of the microflora. Addition of aerated compartments significantly decreased the levels of residual aromatic amines and the toxicity of the effluent whilst not significantly affecting its genotoxic potential, which corresponded to the appearance of *Nocardia* and *Rhodococcus* spp. adapted to the aerobic environment. It is most probable that the significant decreases in the levels of toxicity and residual aromatic amines were due to the presence of these species, which

was made possible by the differential adaptation that occurred in this compartmentalised bioreactor.

4.3.7 General observations on the microflora of the Sequencing Batch Reactors and Hybrid Anaerobic Baffled Reactors

There were some interesting observations made about the microflora of the SBRs and HABRs during FISH and DGGE analysis. During FISH analysis of the spatial arrangements of bacteria and archaea in sludge flocs in a sample taken from the SBR operating under anaerobic conditions in the presence of RBOH, it was observed that bacterial and archaeal cells appear to be evenly distributed throughout the length and breadth of the sludge floc, and that both bacterial and archaeal cells were present at the surface of the floc (see Figure 3.7a-f). This is contrary to the observation generally made about anaerobic granules (flocs), in which there is distinct layering of facultative fermenters at the surface and obligate anaerobic archaea at the core (Guiot *et al.* 1992, Sacks 1998). This demonstrates that under these specified conditions, operating characteristics of the SBR such as the high levels of mixing and biomass washout concomitant with batch filling alter the normal spatial arrangements of archaea within the sludge flocs and allow them to be present at the surface as well as in the centre. It was noted that far more archaeal cells than bacterial cells could be visualised in the flocs, especially at the surface. The bacterial probe used for dual probing, EUB927, does not hybridise with as large percentage of bacterial cells as EUB338 which was used in other FISH assays. This would decrease the numbers of bacterial cells that could be visualised with the probe used, some of which may have been present on the surface of the sludge flocs. Further work would need to be conducted using a bacterial probe that hybridises to a greater percentage of bacterial cells, such as EUB338.

Table 4.5: Influence of numbers, diversity and types of bacteria and archaea on mineralisation of RBOH and safety of the effluent produced by the HABR

Parameter	Anaerobic			Anaerobic-aerobic			
	cell 1	cell 4	cell 7	cell 1	cell 4	cell 7	cell 8
Decolourisation (%)[a]	-	-	85.9 ± 0.6	-	-	-	86.6 ± 1.5
A_{260} (AU)[b]	-	-	22.0	-	-	-	10.0
Toxicity (EC_{50})[c]	-	-	0.46 ± 0.17	-	-	-	16.89 ± 9.82
Genotoxicity (CS)[d]	-	-	0.85 ± 0.27	-	-	-	0.70 ± 0.05
No. of Bacteria[e] **($\times 10^7$ cells ml^{-1})**	3.9	2.6	1.9	13.7	2.6	1.8	2.9
No. of Archaea[e] **($\times 10^7$ cells ml^{-1})**	1.3	1.2	2.6	12.3	3.7	2.5	1.6
Bacterial diversity[f]	4 main bands	5 main bands	5 main bands	11 main bands	7 main bands	4 main bands	5 main bands, 25-37% similarity to cells 1-7
Archaeal diversity[f]	5 main bands	5 main bands	5 main bands	7 main bands	7 main bands	7 main bands	5 main bands, 65-80% similarity to cells 1-7
Bacterial species[g]	*Staphylococcus* sp., *Pseudomonas* sp., *Clostridium* sp., *Propionibacterium* sp., *Bacteroides* sp.	*Staphylococcus* sp., *Pseudomonas* sp., *Clostridium* sp., *Propionibacterium* sp.	*Staphylococcus* sp., *Bacteroides* sp., *Clostridium* sp.	-	-	-	-
Bacterial species[h]	*Bacteroides* sp.	*Bacteroides* sp.	*Bacteroides* sp.	*Bacteroides* sp.	*Bacteroides* sp.	*Bacteroides* sp.	*Nocardia* sp., *Rhodococcus* sp., *Bacteroides* sp.

N.B. Data are for Rig A stage b (anaerobic) and stage e (anaerobic-aerobic). All cells 1-7 anaerobic, cell 8 aerobic. Decolourisation, A_{260}, toxicity and genotoxicity data refer to the final effluent from the HABR. [a]percentage reduction in dye concentration as measured by A_{597}; [b]average absorbance values; [c]EC_{50} 5 min values as % of original concentration; [d]CS values of undiluted sample; [e]number of bacteria/archaea as determined by FISH; [f]numbers and similarities of bacterial/archaeal bands as determined by DGGE and band matching; [g]bacterial species identified by culturing (not performed on anaerobic-aerobic samples); [h]bacterial species identified by sequencing.

A comparison of the influence of differences in community organisation, either as sludge flocs or biofilms, on the numbers of bacteria and archaea identified by FISH showed that there was no significant difference in archaeal numbers between sludge and biofilm samples (Figures 3.10 and 3.11). Whilst bacterial numbers were seen to be slightly higher in sludges than in biofilms from cell 1, these differences were not significant, and were most likely due to the higher level of nutrients present in the sludge liquor of cell 1. However, in cells 5 and 7 of both HABRs (and cell 4 of Rig A), there was a significant increase in the number of bacteria in biofilm samples compared with sludge samples ($p = 0.05$ and 0.02 for Rig A and B respectively, paired t-test). This indicates that bacterial growth is enhanced when organisms form part of biofilm communities as opposed to flocs. DGGE analysis of biofilms also demonstrated divergence in bacterial community compositions, as biofilm profiles showed 55% similarity to sludge profiles, but there was very little divergence between archaeal biofilm and sludge profiles especially under anoxic and aerobic conditions. It was noted that bands observed in aerobic sludges were also seen in anoxic and anaerobic biofilms, and similarly that bands observed in anoxic and anaerobic biofilms were not present in their corresponding sludges. As it is known that dissolved oxygen can penetrate biofilms (Bishop & Yu 1999), these observations indicate that the levels of oxygen in the biofilms were higher than those in the sludge blanket, which may be stimulatory to bacterial growth. It is appreciated that the significance of the increases in bacterial numbers is attached to very few data points, and so further work would be required to establish the true influence of community organisation on numbers of microorganisms.

A point of interest made during FISH analysis of the bioreactors was the appearance of distinctive bacterial cells in both the SBRs and HABRs (see Figure 3.12a-b). These cells were large cocci, approximately 3μm in diameter, forming tightly packed aggregates. These cells were not seen in all samples and, as it is reasonable to assume some heterogeneity of the population due to mixing, it is probable that these cells were not ubiquitous in the sludge and were an emergent population. These cells were observed in sludge samples taken from bioreactors operated under anaerobic, anoxic and aerobic conditions, and are therefore facultative anaerobes. They did not appear until at least day 117 of operation, and often not until day 217, and are therefore slow growing. In addition, they were observed in bioreactors operated both with and without RBOH, and are therefore tolerant to reduction products of RBOH and possibly also the intact dye. However, the cells were absent from cell 1 of both HABRs for the

entire period of operation, and so appear to be inhibited by operating conditions prevalent at the beginning of the HABRs.

During a limited study of the difference between bacterial DGGE profiles of RNA derived from RT-PCR and DNA derived from PCR, it was observed that there was virtually no difference between the DNA and RNA profiles obtained with the exception that the RNA profiles were weaker (Figure 3.14), demonstrating reduced extraction and amplification efficiency of RNA using the RT-PCR method. Some studies comparing DGGE or Temperature Gradient Gel Electrophoresis (TGGE) profiles of RNA and DNA from bacterial 16S genes have found them to be similar, such as that of Felske and Akkermans in 1998 analysing bacteria in soil. Conversely, others have reported that more bands could be observed in RNA than DNA profiles, such as that of Teske and co-workers in 1996 analysing sulphate-reducing bacteria in aquatic environments and that of Felske and co-workers in 1996 analysing bacteria in soil. The most likely reason for differences in RNA and DNA profiles is the percentage of active species in the microflora population. Therefore it is unsurprising that the RNA and DNA profiles of the SBRs were similar, as high levels of active growth would be made possible by the high biomass washout from the SBRs.

Chapter 5: Conclusions

There were three major conclusions that could be drawn from this study. Firstly, during pure culture decolourisation studies of sulphonated azo dyes using toxicity and genotoxicity assays suitable for use with these dyes, it was observed that only RB showed significant increases in toxicity both after hydrolysis and decolourisation. Further work on this dye attributed this toxicity to the liberation of the vinyl sulphone (2-(*p*-aminophenylsulphonyl) ethanol sulphate) component. The only significant genotoxicity was demonstrated by unreduced hydrolysed RB (RBOH).

Secondly, using batch-fill and continuous flow types of bioreactor (an SBR and a HABR) under both anaerobic and anaerobic-aerobic conditions to treat a STE containing RBOH, it was concluded that the continuous flow HABR operated under anaerobic-aerobic conditions produced the 'best quality' effluent in terms of decolourisation, toxicity and stability of bioreactor performance.

Thirdly, using FISH, DGGE and sequencing to determine microflora abundance, diversity, divergence and identification, it was concluded that the continuous flow design of the HABR allowed greater microbial diversity under all oxygen conditions than the batch-fill type SBR. Compartmentalisation of the HABR allowed for speciation to occur under different atmospheric conditions, and supported different species adapted to the aerobic compartments.

Interesting observations were also made during the course of this study. During decolourisation studies using microorganisms isolated from the HABRs, it was observed that there were an equal number of isolates capable of decolourising RBOH in Rig B which was fed STE without RBOH as there were in Rig A which was fed STE with RBOH, which showed that organisms with no prior exposure to RBOH can begin decolourisation without a period of adjustment. FISH analysis of the spatial arrangements of bacteria and archaea in SBR sludge flocs demonstrated that bacterial and archaeal cells appear to be evenly distributed throughout the length and breadth of the sludge floc, and that both bacterial and archaeal cells were present at the surface of the floc. Bacterial microflora in biofilms were more abundant and communities divergent from sludge flocs, whilst no significant differences in archaeal microflora were observed. It was concluded that levels of oxygen in the biofilms were higher than

those in the sludge blanket, which enhanced bacterial growth. An emergent population of distinctive bacterial cells was observed during FISH analysis of both the SBRs and HABRs. These cells were large cocci, approximately 3μm in diameter, forming tightly packed aggregates, were facultative anaerobes, slow growing and tolerant to RBOH and its reduction products.

Chapter 6: Future Work

6.1 Decolourisation studies with reactive azo textile dyes

During analysis of the 3 Procion dyes studied, it was observed that toxicity either increased (as in the case of PY) or decreased (as in the case of PN and PC) after hydrolysis (see Table 3.5). Reference to the literature indicates that hydrolysis of a sulphonated azo dye should increase its toxic potential, as hydrolysis removes the sulphonic acid groups (Weber & Stickney 1993, Hao *et al.* 2000) that would contribute to decreased toxicity (Combes & Haveland-Smith 1982, Lin & Solodar 1988, Rosenkranz & Klopman 1989, Chung & Cerniglia 1992, Puntener *et al.* 1993, Chung 2000, Moller & Wallin 2000). In addition, lower toxicity values were observed with decolourised Procion dyes. It was suggested that differences in the levels of toxicity after hydrolysis may be due to differences in the number of sulphonic acid groups present, and that the lower toxicity of the Procion dyes may be ascribed to the presence of monochlorotriazinyl groups. However, the structure of the Procion dyes has not been released at the current time, and therefore further assessment on the toxicological effects of hydrolysis and decolourisation should be conducted either on other reactive azo textile dyes of known structure or at a time when the structure of these Procion dyes is released.

Decolourisation of both RB by pure cultures of *Clostridium butyricum* resulted in a genotoxic potential. Reference to the literature indicates that electron donating groups produced by anaerobic metabolism of microorganisms, such as would have been produced during assays utilising *C. butyricum*, are less toxic than oxygen-based electron withdrawing groups (Mahmud *et al.* 1997, Börnick *et al.* 2001), and that a mechanism of genotoxicity observed during biological reduction of azo dyes concerns the generation of oxygen radicals (Sweeney *et al.* 1994, Sweeney 1995). Further work on decolourisation of RB using *C. butyricum* should focus on determination of the reduction products, probably including fractionation of the products and detection of oxygen radicals.

6.2 Bioreactors

A significant problem encountered by the SBR was that failure of the rigs occurred after a period of operation under anaerobic-aerobic conditions when decolourising RBOH, as indicated by variability in the toxicity levels and other physico-

chemical parameters. Problems also occurred with incomplete settling of bioreactor sludge during this period (Shaw 2000). This incomplete settling is most probably due to an alteration in the characteristics of the anaerobic granules used to seed the SBRs. A study by Shen and Guiot in 1996 reported that fluffy biolayers appeared on the surface of anaerobic granular sludge in a UASB reactor treating a synthetic wastewater after three months of operation at dissolved oxygen levels of 8.1 mgL^{-1}, which imposed a negative impact on the settleability of the granules and caused a slightly higher sludge washout. It seems apparent that granular sludge is an inappropriate choice of biomass seed for anaerobic-aerobic conditions, and further work on a method of sludge immobilisation or retention would have to be considered if the SBR is to be used to decolourise textile dyehouse wastewaters under anaerobic-aerobic conditions to increase mineralisation of the dye. This highlights one of the distinct advantages of the HABR over the SBR, in that biomass with high settling properties is not required (Sacks 1998), which decreases the difficulty in obtaining a suitable reactor seed.

In addition, the biogases produced by the SBR were collected in the same headspace (Shaw 2000), which included methane produced by the *Methanosaeta* sp. identified in the sludge and air from aeration of the reactor. This combination is highly explosive, and thus shows a potentially dangerous problem in the use of this type of reactor for anaerobic-aerobic treatment. Therefore, further work should be conducted on a method of biogas separation if a single tank bioreactor is operated under anaerobic-aerobic conditions.

It was concluded that the most efficient treatment of RBOH in terms of decolourisation and safety of the resultant effluents was anaerobic-aerobic operation in a HABR. Further work on treatment of STEs containing other reactive azo dyes, such as the Procion dyes, and other classes of textile dyes, such as acid or direct dyes, should be conduct to assess the suitability of this treatment technique to other textile wastewaters.

It was noted that there was a flaw in the design which allowed recycling of the aerated effluent back into the anaerobic cells (Shaw 2000). This may cause problems in terms of gas mixing, and also in the production of anoxic conditions which were shown to be detrimental to the quality of the effluent in terms of production of novel compounds absorbing at 687 nm (see Table 3.17) and a slight increase in genotoxic potential (see Table 3.14). Further work should be conducted on a method to prevent backwash of bioreactor liquor if a compartmentalised reactor is operated under anaerobic-aerobic conditions.

During analysis of the individual compartments (cells) of the HABR, it was noted that most of the increases and decreases in absorbance values at peak wavelengths (see paragraph underlying Table 3.17) and most of the increases in bacterial and archaeal numbers occurred in the first cell operating under the specified oxygen conditions (see Table 3.26). It was suggested that a single cell operating under each oxygen condition is adequate to produce the observed biological processes in terms of compound production/reduction, as long as the level of biomass can be retained at a suitable level. However, further parameters of bioreactor efficiency, such toxicity and genotoxicity reduction, were not assessed for individual cells. Therefore, further work on compartmentalised bioreactors may concentrate on using reduced numbers of compartments, but the indicators of reactor efficacy should be determined on each individual cell.

It was noted that the EA RQO value for colour consent discharges on a typical sewage treatment plant is 0.04 AU at 600 nm (O'Neill *et al.* 1999), and the A_{600} values of the effluents after anaerobic-aerobic treatment were 0.9 and 2.1 AU for the SBR and HABR respectively (see Tables 3.10 and 3.15 respectively). This shows that anaerobic-aerobic treatment in the two reactor types studied is insufficient to achieve the necessary colour removal required to meet EA RQO standards. Further work on decolourisation of textile dyehouse wastewaters may need to include an additional treatment phase or an increase in the efficiency of absorbance reduction by bioreactors if EA RQO standards are to be met using bioremediaion technology.

6.3 Bioreactor microflora

During decolourisation studies using microorganisms isolated from the HABRs, it was observed that organisms with no prior exposure to RBOH can begin decolourisation without a period of adjustment. Further work should be conducted to elucidate the molecular mechanisms of biological decolourisation of RBOH and other reactive azo dyes.

During determination of the spatial arrangement of bacteria and archaea in sludge flocs, it was noted that the bacterial probe used (EUB927) does not hybridise with as large percentage of bacterial cells as EUB338 that was used in other FISH assays. It was suggested that this would decrease the numbers of bacterial cells that could be visualised in sludge flocs, some of which may have been present on the surface, and thus may give a distorted picture of the relative prevalence of bacteria and

archaea on the surface of the flocs. Further work on determination spatial arrangement of bacteria and archaea in sludge flocs should be conducted using a bacterial probe that hybridises to a greater percentage of bacterial cells, such as EUB338.

During comparisons of the number of bacteria and archaea between samples of sludge and biofilm from the HABRs, it was observed that there was a significant increase in bacterial numbers in the biofilms of cells 5 and 7 ($p = 0.02$) (see Figure 3.11). It was suggested that bacterial growth is enhanced when organisms form part of biofilm communities as opposed to flocs, which is most likely due to higher oxygen levels in the biofilms. However, caution should be given to this observation as it was made using only two data points, and further work on bioreactors capable of supporting biofilm structures, such as the HABR or RBC, should concentrate on the influence of community structure on growth of microflora.

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Appendices

Appendix 1: Chemical composition of the STE used in bioreactor experiments

Component	Compound	Concentration (mgL^{-1})
Mineral	$MgCl_2.6H_2O$	120
	KCl	86.7
	$CaCl_2.6H_2O$	24.9
	$CoCl_2.6H_2O$	2
	$MnCl_2.4H_2O$	1.33
	H_3BO_3	0.38
	$CuCl_2.2H_2O$	0.18
	$Na_2MoO_4.2H_2O$	0.17
	$ZnCl_2$	0.14
Vitamin	Pyridoxine hydrochloride	0.01
	Riboflavin	0.005
	Thiamin	0.005
	Nicotinic acid	0.005
	Pantothenic acid	0.005
	p-Aminobenzoic acid	0.005
	Thioctic acid	0.005
	Biotin	0.002
	Folic acid	0.002
	B_{12}	0.0001
Auxiliary	$FeCl_3.6H_2O$	460
	$NH_2CO.NH_2$ (Urea)	259.4
	$MgSO_4.7H_2O$	128.3
	$NaH_2PO_4.2H_2O$	95.3
Alkali	NaCl	26670
	Na_2CO_3	1000
	NaOH	164.5
Size	Potato starch	2670
	Polyvinyl alcohol	400
	Carboxymethyl cellulose	270
Dye	Remazol Black B (133%)	533.3

(Shaw 2000)

N.B. For control reactors requiring no dye, 6.66 ml NaOH was substituted for Remazol Black B which had been hydrolysed in 1:10 66.6 ml NaOH.

Appendix 2: Detailed description of operational changes and events in both types of bioreactor utilised in this study

SBRs

Day	Date	Operational change or event
0	23.04.98	SBR start up. Anaerobic sequencing only. Rig 1 with dye, Rig 2 control.
11	04.05.98	Add 1 gL^{-1} $NaHCO_3$ to synthetic feed.
13	06.05.98	Add 2 gL^{-1} $NaHCO_3$ to synthetic feed.
29	22.05.98	Add 1.75 gL^{-1} $NaHCO_3$ to synthetic feed.
32	25.05.98	Add 1.5 gL^{-1} $NaHCO_3$ to synthetic feed.
36	29.05.98	Add 1.25 gL^{-1} $NaHCO_3$ to synthetic feed.
43	05.06.98	Add 1 gL^{-1} $NaHCO_3$ to synthetic feed.
60	22.06.98	Add 0.75 gL^{-1} $NaHCO_3$ to synthetic feed.
62	24.06.98	Add 0.5 gL^{-1} $NaHCO_3$ to synthetic feed.
64	26.06.98	Add 0.25 gL^{-1} $NaHCO_3$ to synthetic feed.
67	29.06.98	Add no $NaHCO_3$ to synthetic feed.
74	06.07.98	Replaced dye (C.I. Reactive Black 5 from Aldrich) with Remazol Black B 133% grains (from DyStar UK) at the same concentration.
85	17.07.98	Changed nitrogen and phosphate sources from old recipe to new one described in current methods.
125	26.08.98	Work towards stage 2 operation. 5 minutes air at 10 $Lmin^{-1}$ ending at 0500.
126	27.08.98	Work towards stage 2 operation. 10 minutes air at 10 $Lmin^{-1}$ ending at 0500.
127	28.08.98	Work towards stage 2 operation. 15 minutes air at 10 $Lmin^{-1}$ ending at 0500. Rig 2 (control) had blocked diffuser so drained reactor and unblocked.
129	30.08.98	Work towards stage 2 operation. 20 minutes air at 10 $Lmin^{-1}$ ending at 0500.
130	31.08.98	Work towards stage 2 operation. 30 minutes air at 10 $Lmin^{-1}$ ending at 0500.
133	03.09.98	Air delivered at 5 $Lmin^{-1}$.
137	07.09.98	Air supply turned off to Rig 1 (dye).
142	14.09.98	Add 2 gL^{-1} $NaHCO_3$ to Rig 1 synthetic feed.
149	21.09.98	Add 1.75 gL^{-1} $NaHCO_3$ to Rig 1 synthetic feed.
151	23.09.98	Add 1.5 gL^{-1} $NaHCO_3$ to Rig 1 synthetic feed.
153	25.09.98	Add 1.25 gL^{-1} $NaHCO_3$ to Rig 1 synthetic feed.
156	28.09.98	Add 1 gL^{-1} $NaHCO_3$ to Rig 1 synthetic feed.
158	30.09.98	Add 0.75 gL^{-1} $NaHCO_3$ to Rig 1 synthetic feed.
162	02.10.98	Add 0.5 gL^{-1} $NaHCO_3$ to Rig 1 synthetic feed.
169	09.10.98	Add 0.25 gL^{-1} $NaHCO_3$ to Rig 1 synthetic feed.
172	12.10.98	Add no $NaHCO_3$ to Rig 1 synthetic feed.
193	02.11.98	Hot room electricity off, no aeration.
200	09.11.98	Add 53.33 mgL^{-1} dye to Rig 2 feed.
202	11.11.98	Add 106.6 mgL^{-1} dye to Rig 2 feed.
204	13.11.98	Add 160 mgL^{-1} dye to Rig 2 feed.
207	16.11.98	Add 213.3 mgL^{-1} dye to Rig 2 feed.
208	17.11.98	5 minutes air at 5 $Lmin^{-1}$ ending at 0500 to Rig 1.
209	18.11.98	Add 266.5 mgL^{-1} dye to Rig 2 feed.
210	19.11.98	Rig 1 diffuser blocked, reactor drained, unblocked and sludge returned.
211	20.11.98	Add 320 mgL^{-1} dye to Rig 2 feed. 10 minutes air at 5 $Lmin^{-1}$ ending at 0500 to Rig 1.
214	23.11.98	Add 373.3 mgL^{-1} dye to Rig 2 feed. 15 minutes air at 5 $Lmin^{-1}$ ending at 0500 to Rig 1.
216	25.11.98	Add 426.6 mgL^{-1} dye to Rig 2 feed. 20 minutes air at 5 $Lmin^{-1}$ ending at 0500 to Rig 1.
218	27.11.98	Add 480 mgL^{-1} dye to Rig 2 feed.

SBRs (Cont.)

Day	Date	Operational change or event
222	01.12.98	Rig 2 lost sludge in effluent, was settled out and returned on 01.12.98 and 02.12.98.
223	02.12.98	30 minutes air at 5 $Lmin^{-1}$ ending at 0500 to Rig 1.
226	05.12.98	Rig 2 effluent pipe broke and lost 4 L of sludge and liquor.
228	07.12.98	Rig 1 not settling and losing sludge in effluent, due to leaking solenoid giving constant gentle aeration.
232	11.12.98	No aeration for either rig.
244	23.12.98	Both reactors shut down.

HABRs

Day	Date	Operational change or event
0	21.04.99	ABR start up. Hydraulic retention time (HRT) 3 days, 8 cells anaerobic only. Add 2 gL^{-1} $NaHCO_3$ to synthetic feed.
5	26.04.99	Add 1.5 gL^{-1} $NaHCO_3$ to synthetic feed. 100 ml sludge transferred from C8 to C1 both reactors.
6	27.04.99	100 ml sludge transferred from C8 to C1 both reactors.
9	30.04.99	Add 1 gL^{-1} $NaHCO_3$ to synthetic feed.
12	03.05.99	50 ml sludge transferred from C8 to C1 both reactors.
19	10.05.99	100 ml sludge transferred from C8 to C1 both reactors.
20	11.05.99	Add 0.75 gL^{-1} $NaHCO_3$ to synthetic feed.
24	15.05.99	Add 0.5 gL^{-1} $NaHCO_3$ to synthetic feed. 100 ml sludge transferred from C8 to C1 both reactors.
26	17.05.99	50 ml sludge transferred from C8 to C2 both reactors. 50 ml sludge transferred from C8 to C3 both reactors.
28	19.05.99	Add no $NaHCO_3$ to synthetic feed.
30	21.05.99	50 ml sludge transferred from C8 to C3 both reactors. 50 ml sludge transferred from C8 to C5 both reactors.
44	04.06.99	HRT set at 2 days, 8 cells all anaerobic.
77	07.07.99	Cell 8 aerobic. No seed sludge added.
112	11.08.99	Added cells 9 & 10, both aerobic, no seed sludge added.
155	23.09.99	Added 250 ml waste activated sludge to each aerobic cell (8, 9 & 10), both reactors.
196	03.11.99	Using lab "soluble starch" in both feeds instead of Kollotex 5. Removed some biomass from Rig B cell 8 to prevent blocking and backing up of liquid in anaerobic cells.
203	10.11.99	Using National starch "plain starch" in both feeds instead of Kollotex 5.
204	11.11.99	Recycle loop installed from C10 to C5, both reactors, flow rate 4.5 $Lday^{-1}$ (ratio 2:1).
229	06.12.99	Both reactors shut down.

Appendix 3: Calibration curves constructed for *Escherichia coli* WP2 and CM871 using the indirect impedance technique

For WP2 see AG37WI. For CM871 see AG37CI.

Appendix 4: Examples of detection times of dyes decolourised by pure cultures and bioreactor samples, obtained using the indirect impedance technique, showing their respective blanks

Dyes decolourised by pure cultures of *Enterococcus faecalis* and *Clostridium butyricum*

Dye	Decolourisation organism	Test *E. coli* strain	Sample TTD[a]	Blank TTD[b]
Orange II	*E. faecalis*	WP2 CM871	03:06-03:42 06:30-08:12	10:00
Sunset Yellow	*E. faecalis*	WP2 CM871	02:12-02:48 03:00-04:06	04:18
C.I. Reactive Black 5	*E. faecalis*	WP2 CM871	02:30-02:42 03:00-03:48	04:06
Hydrolysed C.I. Reactive Black 5	*E. faecalis*	WP2 CM871	02:30-02:48 03:18-03:54	04:48
C.I. Reactive Black 5	*C. butyricum*	WP2 CM871	01:48-01:54 02:18-02:30	>24:00
Hydrolysed C.I. Reactive Black 5	*C. butyricum*	WP2 CM871	01:48-02:00 02:18-02:42	>24:00

N.B. TTD = Time Taken to Detect (h:m). [a]decolourised dye + test *E. coli* strain [b]decolourised dye + sterile 0.85% saline. Sample TTDs are range of triplicate results.

Samples of decolourised effluent from SBRs and HABRs

Reactor type	Test *E. coli* strain	Sample TTD[a]	Blank TTD[b]
SBR	WP2 CM871	02:30-02:42 03:42	09:54
SBR	WP2 CM871	02:24-03:18 02:30-03:24	11:24
SBR	WP2 CM871	02:00-02:18 03:18-03:24	13:30
SBR	WP2 CM871	02:24-02:36 03:12-03:24	10:00
SBR	WP2 CM871	02:36-02:42 03:42-04:06	08:42
ABR	WP2 CM871	02:06-02:24 02:18-02:54	10:18
ABR	WP2 CM871	02:48-02:54 03:42-04:12	11:12
ABR	WP2 CM871	02:24-02:42 03:42-04:18	07:18
ABR	WP2 CM871	02:06-02:12 03:30-03:48	08:12

N.B. TTD = Time Taken to Detect (h:m). [a]bioreactor effluent + test *E. coli* strain [b]bioreactor effluent + sterile 0.85% saline. Sample TTDs are range of triplicate results.