Reductive Decolourisation of Dyes by Thermophilic Anaerobic Granular Sludge

André Bezerra dos Santos

PROMOTOR

prof.dr.ir. C.N. Buisman
hoogleraar in de biologische kringlooptechnologie

CO-PROMOTOREN

dr.ir. J.B. van Lier
onderzoeker bij de sectie milieutechnologie en directeur Lettinga Associates Foundation

dr.ir. F.J. Cervantes
instituto technologico de Sonora, Obregon, Mexico

SAMENSTELLING PROMOTIECOMMISSIE

prof. dr. J.A. Field (University of Arizona)
prof. dr. H.J. Lubberding (UNESCO-IHE, Delft)
dr. R. Mulder (Paques Natural Solution)
prof. dr. A.J.M. Stams (Wageningen Universiteit)

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André Bezerra dos Santos

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To my parents, brothers, Raquel and Lucas.
Without you my life is colourless.

Abstract

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The colour removal achieved under anaerobic conditions is also called reductive decolourisation, which is composed of a biological part, i.e. the reducing equivalents are biologically generated, and a chemical part, i.e. the formed electrons reduce chemically the dyes. The overall objective of this research was to explore different strategies to increase the reduction of dyes in bioreactors by using thermophilic anaerobic granular sludge and redox mediators. Our results clearly show that thermophilic treatment with anaerobic granular sludge does accelerate the reduction of azo dyes compared to the performance achieved under mesophilic conditions. In Chapter 3, batch assays showed increases on the decolourisation rates of azo dyes in sixfold and twofold, in the absence and presence of the redox mediator anthraquinone-2,6-disulphonate (AQDS), respectively, between thermophilic (55ºC) and mesophilic (30ºC) conditions by using the same sludge source. Therefore, most likely the transfer of reducing equivalents at 30ºC was the rate-limiting step. In Chapter 4, thermophilic EGSB (expanded granular sludge blanket) bioreactors were extremely efficient in treating the recalcitrant azo dye Reactive Red 2 (RR2), as well as having a high stability when high loading rates of RR2 were applied (up to 2.7 gRR2 l^{-1} day^{-1}). Long-term experiments revealed that the AQDS-free reactor achieved efficiencies of around 91% in comparison with the efficiencies around 95% for the AQDS-supplemented reactor. In Chapter 5, we demonstrated that the normal rate limiting step, the transfer of reducing equivalents to the azo dye, was accelerated under thermophilic conditions. Both biotic and abiotic mechanisms involved in the biochemistry of reductive decolourisation were enhanced at 55ºC. The faster biological reduction of the redox mediator, AQDS, achieved by sludge incubations at 55°C in comparison with 30°C evidences the biological contribution in enhancing the rate of electron transfer. For instance, about 1 mM of AQDS was completely reduced at 55ºC after 0.7 days of incubation, whereas mesophilic reduction after 0.7 days was just 12.9% of this value. Furthermore, no lag-phase was found at 55ºC. The abiotic chemical reduction of RR2 by sulphide, as expected, followed the Arrhenius equation, and the decolourisation rates were accordingly accelerated by the temperature increase. Furthermore, the AQS-supplemented incubations presented a lower Ea requirement. The calculated Ea values are 27.9 kJ/mol and 22.9 kJ/mol for the AQS-free and AQS-supplemented incubations, respectively. Therefore, the activation energy was decreased 1.2-fold due to the addition of 0.012mM of AQS. In Chapter 6, the significant enhancement of the electron transfer capacity and subsequent increase on the reductive decolourisation of azo dyes, simply by applying high temperature, was demonstrated. For instance, at a hydraulic retention time (HRT) of 2.5 h and in the absence of AQDS, the colour removal was 5.3-fold higher at 55ºC in comparison with the efficiency of bioreactors achieved at 30°C. Furthermore, the catalytic effect of AQDS on the thermophilic reductive decolourisation, i.e. the impact of AQDS on the decolourisation rates, was even

decreased 3.2-fold compared to experiments carried out at 30ºC. Very likely, this indifference to the presence of AQDS was the consequence of the high efficiency reached at 55°C, which masked the impact of AQDS on reductive decolourisation of dyes. The similar degree of COD (chemical oxygen demand) removal achieved in all reactors of about 75% indicated that the reducing equivalents were generated at a similar rate in the mesophilic and thermophilic bioreactors. Apparently, the difference in the decolourisation rates was not related to the difference in the production rates of reducing equivalents. Consequently, we concluded that the higher degree of colour removal was attributed to the impact of temperature and AQDS on electron shuttling. Results described in Chapter 7 with reductive decolourisation of anthraquinone dyes, demonstrated that in comparison with incubations at 30ºC, incubations at 55ºC present distinctly higher decolourisation rates not only with real wastewater, but also with the model compound Reactive Blue 5 (RB5). The k-value of RB5 at 55ºC is enhanced 11.1-fold in the presence of anthraquinone-2-sulphonate (AQS), and sixfold in the absence of AQS, upon comparison with mesophilic incubations at 30ºC. However, the anthraquinone dye Reactive Blue 19 (RB19) exhibited a very strong toxic effect on volatile fatty acids (VFA) degradation and methanogenesis at both 30ºC and 55ºC. Further experiments at both temperatures revealed that RB19 was mainly toxic to methanogens, because the glucose oxidizers including acetogens, propionate-forming, butyrate-forming and ethanol-forming microorganisms are not affected by the dye toxicity. Finally in Chapter 8, we studied the contribution of acidogenic bacteria and methanogenic archaea to azo dye reduction by a thermophilic anaerobic consortium, as well as the competition for reducing equivalents between methanogenesis and azo dye reduction. Our results indicated that acidogenic bacteria and methanogenic archaea play important roles in this reductive process. Experiments with the thermophilic methanogens *Methanothermobacter thermoautotrophicus* ΔH and a *Methanothermobacter*-related strain NJ1 revealed that these strains were unable to reduce the dye in the absence of the redox mediator, riboflavin. This suggested that anaerobic dye reduction is not a universal property among methanogenic archaea and that redox mediators may play an important role for allowing some microbial groups, commonly found in wastewater treatment systems, to participate more effectively in reductive decolourisation of dyes.

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1

Introduction

A huge number of chemicals from textile industries, which have a negative effect on the environment and public health, are released through their wastewaters. Chemicals such as *alkyl phenol ethoxylates* (present in detergents, wetting and levelling agents) are reported to disturb the reproduction of aquatic species; along with *sequestering agents* like *Ethylenediaminetetracetic acid (EDTA) and Diethylenetriaminepentacetic acid* (*DTPA)* are capable of forming very stable complexes with metals, thus affecting their bio availability; *and the dyestuff*, which are recalcitrant by design and not readily amendable to common treatment methods, are examples of hazardous compounds present in textile wastewaters (DEPA, 1997; Hao et al., 2000). Particularly the release of coloured compounds into water bodies is undesirable not only because of their aesthetic appearance and the impact of dyes on photosynthesis of aquatic plants, but also because many of these dyes and their breakdown products are carcinogenic (Weisburger, 2002). Without adequate treatment these dyes can remain in the environment for an extended period of time. For instance, the half-life of hydrolysed Reactive Blue 19 is about 46 years at pH 7 and 25°C (Hao et al., 2000). In addition to the aforementioned problems, the textile industry consumes large amounts of potable water. Mainly in countries where potable water is scarce, this large consumption has become intolerable and wastewater recycling has been recommended.

Textile effluents from the dyebath and rinsing steps represent the most coloured fraction of textile wastewaters. It is estimated that almost 10^9 kg of dyes are produced annually in the world, of which azo dyes represent about 70% by weight, followed by the anthraquinone dyes. The biotechnological approaches to decolourise azo-containing wastewaters are very broad, in which other microorganisms rather than bacteria also have shown this capacity. Under **aerobic conditions**, e.g. in activated sludge systems for wastewater treatment, low azo dye decolourisation is achieved because oxygen is a more effective electron acceptor than the azo dyes (Stolz, 2001). On the other hand, under **anaerobic conditions**, e.g. by using granular or flocculent sludges, azo dyes are generally the sole terminal electron acceptor, and because of this, a better decolourisation is obtained. However, the reduced products of anaerobic treatment (aromatic amines) are in general

anaerobically recalcitrant, which means that the complete biodegradation of azo dyes cannot be accomplished under anaerobic conditions only. Nevertheless, the bioconversion of these aromatic compounds under aerobic conditions is relatively fast. The reduction of the azo dyes is generally regarded as the rate-limiting step in the overall reaction. It has been found that reductive decolourisations could be improved by using **redox mediators**. Many flavin-based vitamins and quinones containing-humus have shown to accelerate azo dye reduction. However, the impact of redox mediators in conjunction with thermophilic conditions has not been investigated yet. In fact, thermophilic decolourisation has only briefly examined in the literature (Willetts and Ashbolt, 2000) even though a large portion of coloured textile wastewaters is discharged at high temperatures. Our work is the follow-up of previous investigations conducted at the Environmental Technology Department of Wageningen University not only with regard to the biotransformation of azo dyes (Chapter 9, item 3.0), but also to the treatment of other xenobiotics like nitroaromatics, BTEX and chlorinated compounds. This **Thesis** focuses on biotechnological approaches to accelerate decolourisations of azo and anthraquinone dyes with the goal of including thermophilic anaerobic treatment as a pre-treatment of coloured wastewaters in closing water systems.

STRUCTURE OF THIS THESIS

Chapter 2 presents a literature review on textile wastewaters, mainly covering the aspects of public health concerns and decolourisation techniques, with an emphasis on the biotechnological approaches. **Chapter 3** describes azo dye reduction by anaerobic granular sludge in which biotic and abiotic contributions are evaluated either in the presence or absence of the redox mediator AQDS. **Chapter 4** is mainly concerned with the reductive decolourisation of azo dyes in bioreactors and the impact of the redox mediator AQDS. **Chapter 5** describes the effect of different redox mediators upon colour removal of azo dyes. The influence of temperature on the biological AQDS reduction and on its chemical regeneration is also assessed. **Chapter 6** investigates the electron transfer capacity and subsequent colour removal in mesophilic and thermophilic anaerobic bioreactors. It also concerns with the importance of the mediator's standard redox potential on the rates of decolourisation. **Chapter 7** deals with the transformation and toxicity of anthraquinone dyes using mesophilic and thermophilic consortia. **Chapter 8** evaluates the contribution of acidogenic bacteria and methanogenic archaea to reductive decolourisation by a thermophilic anaerobic consortium. It also deals with the competition for reducing equivalents between methanogenesis and azo dye reduction. In **Chapter 9** the findings of the previous chapters are discussed in a critical way, and suggestions for further investigations are given.

2

CURRENT TECHNOLOGIES FOR DECOLOURISATION OF TEXTILE WASTEWATERS: PERSPECTIVES FOR ANAEROBIC BIOTECHNOLOGY

Abstract

Dyes are natural and xenobiotic compounds that make the world more beautiful through coloured substances. However, the release of coloured wastewaters represents a serious environmental problem and a public health concern. Colour removal, especially from textile wastewaters, has been a big challenge over the last decades, and up to now there is no single and economically attractive treatment that can effectively decolourise dyes. In the passed years, notable achievements were made in the use of biotechnological applications to textile wastewaters not only for colour removal but also for the complete mineralization of dyes. Different microorganisms such as aerobic and anaerobic bacteria, fungi and actinomycetes have been found to catalyse dye decolourisation. Moreover, promising results were obtained in accelerating dye decolourisation by adding mediating compounds and/or changing process conditions to high temperatures. This paper provides a critical review on the current technologies available for decolourisation of textile wastewaters and it suggests effective and economically attractive alternatives.

🖫 Dos Santos AB, Cervantes FJ and Van Lier JB (2004). Bioresource Technology. Submitted.

1. SOCIAL IMPACT OF TEXTILE INDUSTRY AND ENVIRONMENTAL CONCERNS

1.1. Textile industry in Brazil and Europe

The textile industry represents an important sector of the Brazilian economy. In the past eight years, € 6 billion of investments marked a real revolution in the textile sector, placing Brazil seventh among the world's international markets. Furthermore, an investment of € 11 billion is planned for this sector in the coming seven years. The result is an industry which circulates € 20 billion a year, through 30 thousand companies and 1,4 million workers (ABIT, 2003). In Europe the textile industry is composed of nearly 114 thousands companies, mainly distributed in countries like Italy, Germany, United Kingdom, France and Spain. The European industry moves an average of € 198 billion a year making it the world's leading exporter of textiles and the third largest exporter of clothing (IPPC, 2003). The distribution of textile industry activities (textile and clothing) in EU from the year 2000 is shown in Figure 2.1.

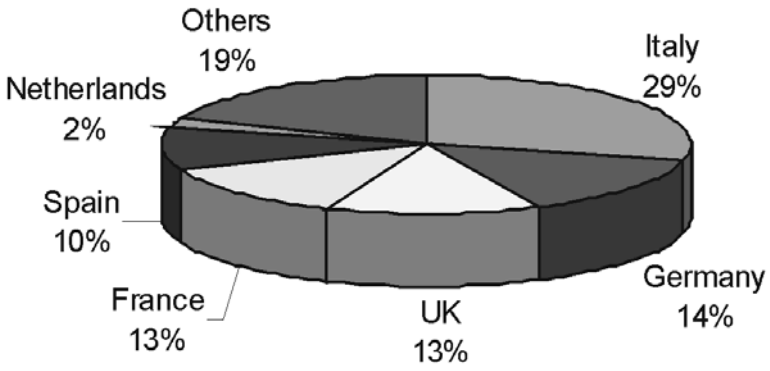

Figure 2.1. Distribution of textile industry activities (textile and clothing) in EU during the year 2000 (IPPC, 2003).

With the increased demand for textile products, the textile industry and its wastewaters have been increasing proportionally, making it one of the main sources of severe pollution problems worldwide. In particular, the release of coloured effluents into the environment is undesirable, not only because of their colour, but also because many dyes from wastewater and their breakdown products are toxic and/or mutagenic to life (Weisburger, 2002). Without adequate treatment these dyes are stable and can remain in the environment for an extended period of time. For instance, the half-life of hydrolysed Reactive Blue 19 (RB19) is about 46 years at pH 7 and 25°C (Hao et al., 2000).

1.2. Textile wastewaters

Textile wastewaters are characterized by extreme fluctuations in many parameters such as Chemical Oxygen Demand (COD), Biochemical Oxygen Demand (BOD), pH, colour, salinity and temperature (Table 2.1). The wastewater composition will depend on the different organic-based compounds, chemicals and dyes used in the industrial dry and wet-processing steps (Talarposhti et al., 2001).

Table 2.1. Characterisation of the cotton wet processing wastewaters (Adapted from Correia et al., 1994; Orhon et al., 2001; Mattioli et al., 2002; Bisschops and Spanjers, 2003).

Process	COD (g/l)	BOD (g/l)	TS (g/l)	TDS (g/l)	pH	Colour (ADMI)	Water usage (l/kg)
Desizing	4.6-5.9	1.7-5.2	16.0-32.0	-	-	-	3-9
Scouring	8.0	0.1-2.9	7.6-17.4	-	10-13	694	26-43
Bleaching	6.7-13.5	0.1-1.7	2.3-14.4	4.8-19.5	8.5-9.6	153	3-124
Mercerising	1.6	0.05-0.1	0.6-1.9	4.3-4.6	5.5-9.5	-	232-308
Dyeing	1.1-4.6	0.01-1.8	0.5-14.1	0.05	5-10	1450-4750	8-300

COD, Chemical Oxygen Demand; BOD, Biochemical Oxygen Demand; TS, Total Solids; TDS, Total Dissolved Solids; ADMI, American Dye Manufacturer Institute.

The most common textile-processing set-up (Figure 2.2) consists of desizing, scouring, bleaching, mercerising and dyeing processes (EPA, 1997; Dos Santos, 2001). **Sizing** is the first preparation step, in which sizing agents such as starch, polyvinyl alcohol (PVA) and carboxymethyl cellulose are added to provide strength to the fibres and minimize breakage. **Desizing** is the employed next to remove sizing materials prior to weaving. **Scouring** then removes impurities from the fibres by using alkali solution (commonly sodium hydroxide) to breakdown natural oils, fats, waxes and surfactants, as well as to emulsify and suspend impurities in the scouring bath. **Bleaching** is the step used to remove unwanted colour from the fibres by using chemicals such as sodium hypochlorite and hydrogen peroxide. **Mercerising** is a continuous chemical process used to increase dye-ability, lustre and fibre appearance. In this step a concentrated alkaline solution is applied and an acid solution washes the fibres before the dyeing step. Finally, **Dyeing** is the process of adding colour to the fibres, which normally requires large volumes of water not only in the dyebath, but also during the **rinsing step**. Depending on the dyeing process, many chemicals like metals, salts, surfactants, organic processing assistants, sulphide and formaldehyde, may be added to improve dye adsorption onto the fibres. Figure 2.2 shows some potential pollutants from cotton processing

operations in which the desizing/scouring and dyebath/rinsing wastewaters are mainly composed of organic pollutants and colour-causing pollutants, respectively (Snowden-Swan, 1995).

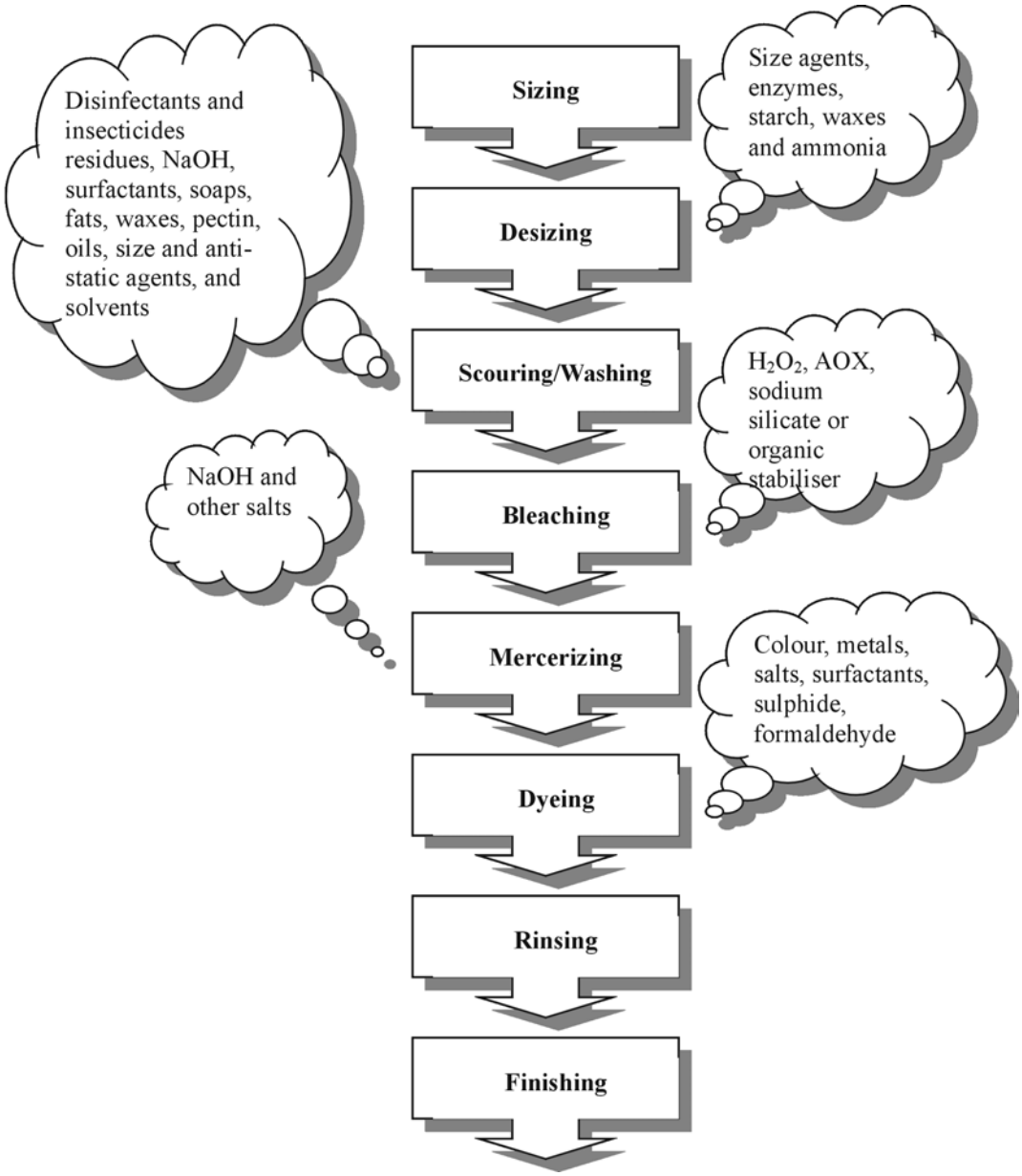

Figure 2.2. Schematic of operations involved in textile cotton industry and the main pollutants from each step (Adapted from EPA, 1997; Mattioli et al., 2002). AOX: Adsorbable Organic Halogens.

2 - DYE CLASSIFICATION AND MECHANISM OF FIXATION

2.1. Dye classification

The history of dyes begins in 2600 BC, according to the earliest written record, with the use of dyestuffs in China. Dyes were originally obtained from animal and vegetable sources. For

example, in the 15th century BC, Phoenicians already used Tyrian purple, which was produced from certain varieties of crushed sea snails, and the well-known plant dye indigo has been used since 3,000 BC (Christie, 2001). Also, Egyptian mummies were discovered wrapped with dyed clothes made of madder plants. In South America, the Incas elaborated fine textures with different colours before being conquered by Spain. The chemical industry started in 1856 when the Englishman William Henry Perkin accidentally synthesised the first dye, 'Mauve' (aniline), a brilliant fuchsia colour, while searching for a cure for malaria. In the following decades a considerable number of new dyes were synthesised (Welham, 2000). Azo dyes are the largest and the most important group of dyes, mainly due to their simple synthesis. The production of azo dyes began in 1858 when the German scientist P. Gries discovered the reaction mechanism diazotisation for the production of azo compounds (Zollinger, 1987).

Dyes are classified according to their application and chemical structure. They are composed of a group of atoms responsible for the dye colour, called chromophores, as well as an electron withdrawing or donating substituents that cause or intensify the colour of the chromophores, called auxochromes (Christie, 2001). The most important chromophores are azo (─N═N─), carbonyl (─C═O), methine (─CH═), nitro (─NO_2) and quinoid groups. The most important auxochromes are amine (─NH_3), carboxyl (─COOH), sulfonate (─SO_3H) and hydroxyl (─OH). The auxochromes can belong to the classes of reactive, acid, direct, basic, mordant, disperse, pigment, vat, anionic and ingrain, sulphur, solvent and disperse dye (Welham, 2000). Figure 2.3 gives an illustration of the chemical structure of several dye molecules.

It is estimated that almost 10^9 kg of dyes are produced annually in the world, of which azo dyes represent about 70% by weight (Zollinger, 1987). This group of dyes is characterised by reactive groups that form covalent bonds with OH─, NH─, or SH─ groups in fibres (cotton, wool, silk, nylon). Azo dyes are mostly used for yellow, orange and red colours (Christie, 2001). To obtain the target colour, normally a mixture of red, yellow and blue dyes is applied in the dyebaths. These three dyes do not necessarily have the same chemical structure. They might contain many different chromophores, in which azo, anthraquinone and phtalocyanine dyes are the most important groups (Hao et al., 2000). Anthraquinone dyes constitute the second most important class of textile dyes, after azo dyes (Baughman and Weber, 1994). Anthraquinone dyes have a wide range of colours in almost the whole visible

spectrum, but they are most commonly used for violet, blue and green colours (Christie, 2001; Fontenot et al., 2003).

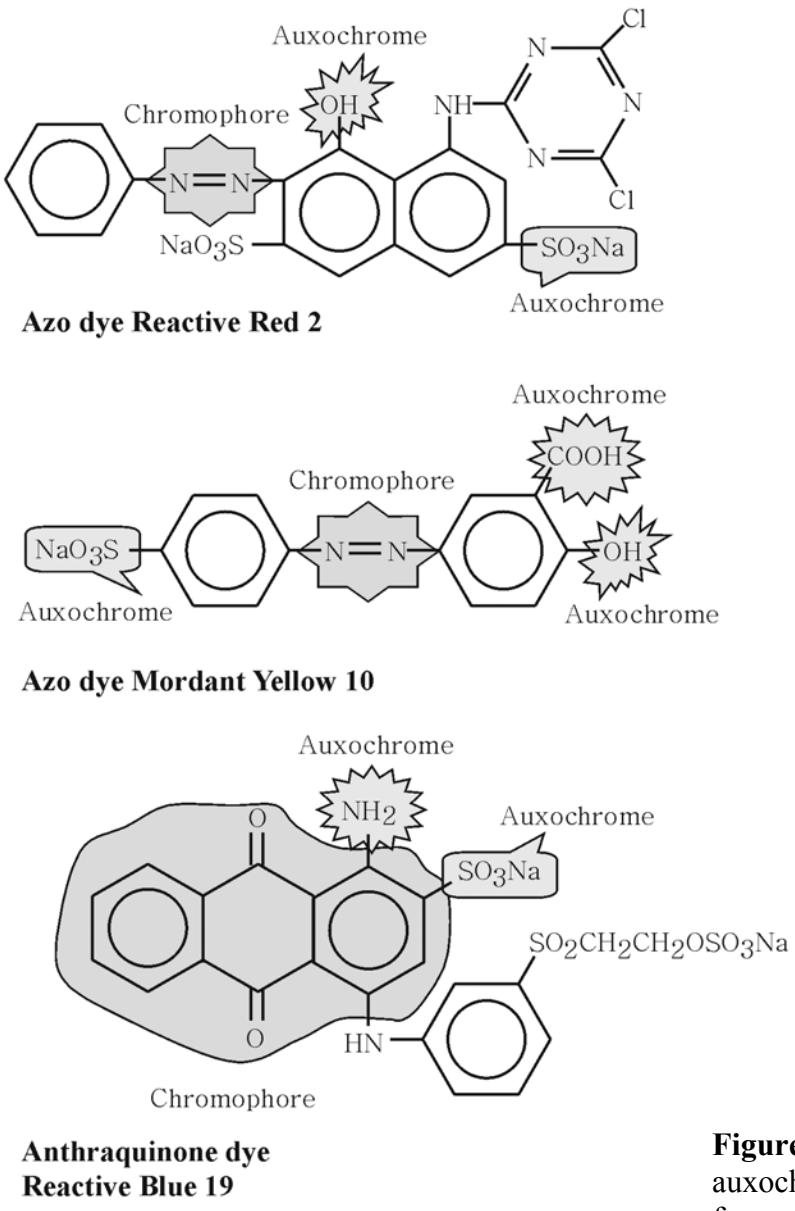

Figure 2.3. Examples of dye-auxochromes and –chromophores for azo and anthraquinone dyes.

2.2. Fixation Mechanisms

Fibres can take up dyes as a result of van der Waals forces, hydrogen bonds and hydrophobic interactions. The uptake of the dye in fibres depends on the dye nature and its chemical constituents. The strongest dye-fibre attachment is a result of a covalent bond with an additional electrostatic interaction where the dye ion and fibre have opposite charges (Welham, 2000).

In alkaline conditions, i.e. pH 9-12 and salt concentration from 40-100 g/l, and at high temperatures (30-70ºC), reactive dyes form a reactive vinyl sulfone (─SO_4─CH═CH_2) group, which forms a bond with the fibres. However, the vinyl sulfone group undergoes hydrolysis, i.e. a spontaneous reaction that occurs in the presence of water, and because the products do not have any affinity with the fibres, they do not form a covalent bond. Therefore, a high amount of dye constituents are discharged in the wastewater (Hao et al., 2000). The fixation efficiency varies with the class of azo dye used, which is around 98% for basic dyes and 50% for reactive dyes (O'Neill et al., 1999). Large amounts of salts such as sodium nitrate, sodium sulphate and sodium chloride are used in the dyebath (Carliell et al., 1998), as well as sodium hydroxide is widely applied to increase the pH to the alkaline range. It is estimated that during the mercerising process the weight of these salts can make up 20% of the fibre weight (EPA, 1997).

3. COLOUR MEASUREMENT IN COLOURED WASTEWATERS

The electromagnetic spectrum can be divided in three different regions: ultraviolet, visible light and infrared (Table 2.2). Although visible light is considered to be between the wavelengths of 350 – 780 nm, the human eye can normally detect radiations between the wavelengths of 380 – 720 nm (Christie, 2001).

Table 2.2. Regions of the electromagnetic spectrum and relationship between wavelength and colour (Christie, 2001).

Electromagnetic Region	Wavelength (nm)	Colour Perception
Ultraviolet	< 350	nd
Visible Light	350 – 400	nd
	400 – 435	Violet
	435 – 480	Blue
	480 – 490	Greenish-blue
	490 – 500	Bluish-green
	500 – 560	Green
	560 – 580	Yellowish-green
	580 – 595	Yellow
	595 – 605	Orange
	605 – 750	Red
	750 – 780	nd
Infrared	> 780	nd

nd is not detected by the eye.

Colour in water can be either a result of natural phenomena like the presence of humic substances, natural metallic ions, e.g. iron and manganese, and/or plankton; or the colour can also result from an artificial phenomenon like the discharge of dyes and pigments. Industrial processes such as textile, chemical and pharmaceutical industries may discharge large amounts of coloured wastewaters into water bodies. In general, colour in water is classified in terms of true colour, i.e. when the sample is turbidity-free, or apparent colour, i.e. when the sample is measured without previous treatment (APHA, 1998). The most common methods to measure the colour of water and wastewater are visual comparison and spectrophotometry, although there is still a lack of a universal method to classify coloured wastewater emissions. By **visual comparison**, colour is quantified by comparing the sample colour with either known concentrations of coloured standards (normally a platinum-cobalt solution), or properly calibrated colour disks. This method is currently used in water treatment plants as a control parameter mainly because of its simplicity, but it is not applicable for highly coloured industrial wastewaters. In the **spectrophotometric method** (Table 2.3), colour-measuring protocols differ between the methodologies, of which the most commonly used are Tristimulus Filter Method, American Dye Manufacturer Institute (ADMI) Tristimulus Filter Method, and Spectra record.

Table 2.3. Spectrophotometric methods of colour determination in water and wastewater (APHA, 1998; Hao et al., 2000; Kao et al., 2001; Bisschops and Spanjers, 2003).

Spectrophotometric Method	Description
Tristimulus	Three tristimulus light filters combined with a specific light source (i.e. tungsten lamp) and a photoelectric cell inside a filter photometer. The output transmittance values are then converted to trichromatic coefficients and colour characteristic values.
ADMI Tristimulus	The ADMI colour value provides a true watercolour measure, which is independent of hue. Depending on the number of wavelengths chosen to calculate the ADMI value, this method can be differentiated in 3 (WL) ADMI, i.e. the transmittance is recorded at 590, 540 and 438nm; or 31 (WL) ADMI, i.e. the transmittance is determined each 10nm in the range of 400-700nm.
Spectra Record	The complete spectrum is recorded in which the entire spectrum, or a part of it, can be used for comparison. A modified method has been suggested in which areas beneath an extinction curve represent the colour intensity, being expressed as space units.

Before measuring colour, the turbidity of the wastewater sample must be removed and the pH must be adjusted to 7.6.

4 - DECOLOURISATION PROCESSES

4.1. Biological colour removal by aerobes

Bacteria

Aromatic compounds are susceptible to biological degradation under both aerobic and anaerobic conditions (Field et al., 1995). Under aerobic conditions, the enzymes mono- and dioxygenase catalyse the incorporation of oxygen from O_2 into the aromatic ring of organic compounds prior to ring fission (Madigan et al., 2003). In most monooxygenases, the electron donor is NADH or NAD(P)H, even though the direct coupling to O_2 is through a flavin that is reduced by the NADH or NAD(P)H donor (Madigan et al., 2003). Although azo dyes are aromatic compounds, their substituents containing mainly nitro and sulfonic groups, are quite recalcitrant to aerobic bacterial degradation (Claus et al., 2002). This fact is probably related either to the electron-withdrawing nature of the azo bond and their resistance to oxygenases attack, or because oxygen is a more effective electron acceptor, therefore having more preference for reducing equivalents than the azo dye (Chung and Cerniglia, 1992; Knackmuss, 1996). However, in the presence of specific oxygen-catalysed enzymes called azo reductases, some aerobic bacteria are able to reduce azo compounds and produce aromatic amines (Stolz, 2001). Examples of aerobic azo reductases were found in *Pseudomonas* species strains K22 and KF46 (Zimmermann et al., 1982; Zimmermann et al., 1984). These enzymes, after purification, characterization and comparison were shown to be flavin-free. The aerobic azo reductases were able to use both NAD(P)H and NADH as cofactors and reductively cleaved not only the carboxylated growth substrates of the bacteria but also the sulfonated structural analogues. Recently, Blümel and Stolz (2003) cloned and characterized the genetic code of the aerobic azo reductase from *Pagmentiphaga kullae* K24. This strain was able to grow with the carboxylated azo compound 1-(4'-carboxyphenylazo)-4-naphtol as a sole source of carbon and energy. Furthermore, the gene encoded a protein with a molecular weight of 20,557 Da, having conserved a putative NAD(P)H-binding site in the amino-terminal region.

Fungi

The capacity of fungi to reduce azo dyes is related to the formation of exoenzymes such as peroxidases and phenoloxidases. Peroxidases are hemoproteins that catalyse reactions in the presence of hydrogen peroxide (Duran et al., 2002). Lignin and manganese peroxidases have a similar reaction mechanism that starts with the enzyme oxidation by H_2O_2 to an oxidized state during their catalytic cycle. Afterwards, in a mechanism involving two successive electron transfers, substrates such as azo dyes reduce the enzyme to its original form (Stolz, 2001).

Eighteen fungal strains able to degrade lignocellulosic material or lignin derivatives were tested with the azo dyes Reactive Orange 96, Reactive Violet 5 and Reactive Black 5. Only the strains of *Bjerkandera adusta, Trametes versicolor* and *Phanerochaete chrysosporium* were able to decolourise all azo dyes (Heinfling et al., 1997). Although lignin peroxidases are able to oxidize both phenolic and nonphenolic aromatic compounds, manganese peroxidases must convert Mn^{+2} to Mn^{+3} in order to oxidize phenolic compounds (Glenn et al., 1986). Phenoloxidases, which can be divided into tyrosinases and laccases, are oxidoreductases that can catalyse the oxidation of phenolic and other aromatic compounds without the use of cofactors (Duran et al., 2002). Laccases are copper-containing enzymes that have a very broad substrate specificity with respect to electron donors, e.g. dyes (Abadulla et al., 2000). However, despite the fact that laccases from *Trametes Versicolor, Polyporus pinisitus* and *Myceliophthora thermophila* were found to decolourise anthraquinone and indigoid-based dyes at high rates, the azo dye Direct Red 29 (Congo Red) was a very poor substrate for laccases (Claus et al., 2002). Chivukula and Renganathan (1995) cited that the azo dye must be electron-rich to be susceptible to oxidation by laccase of *Pyricularia oryzae*. This situation is suitable for the generation of a phenoxy radical, with consequent azo bond cleavage, and the release of molecular nitrogen (Figure 2.4). The addition of redox mediators has been shown to further extend the substrate specificity of laccases with regard to several dye classes, although redox mediators can also be formed from laccase oxidation of phenolic azo dyes (Li et al., 1999; Soares et al., 2001; Claus et al., 2002).

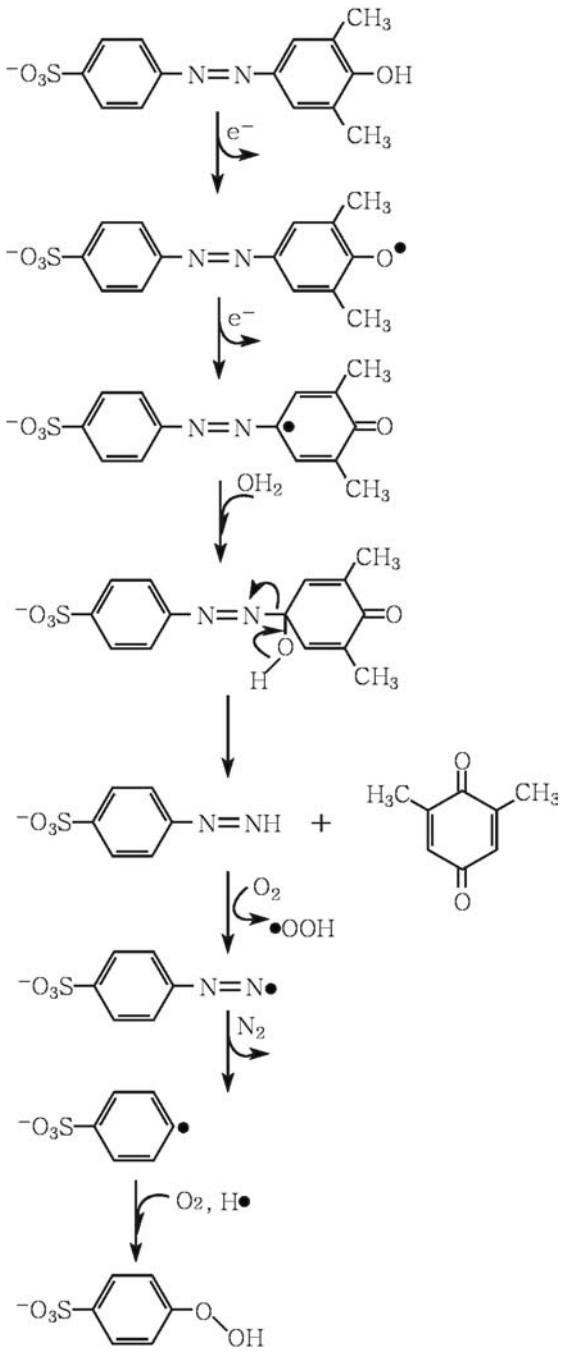

Figure 2.4. Proposed pathway for the peroxidases-catalysed degradation of 4-(4'-sulfophenylazo)-2,6-dimethylphenol by *Pyricularia oryzae* (Chivukula and Renganathan, 1995).

4.2. Biological colour removal by strictly anaerobes or facultative microorganisms incubated under anaerobic conditions

Under anaerobic conditions a low redox potential (< -50 mV) can be achieved, which is necessary for the effective decolourisation of dyes (Beydilli et al., 1998; Bromley-Challenor et al., 2000). Colour removal under anaerobic conditions is also referred as **dye reduction** in which literature mostly covers the biochemistry of azo dye reduction. The azo bond cleavage ─N═N─ involves a transfer of four-electrons (reducing equivalents), which proceeds through two stages at the azo linkage. In each stage two electrons are transferred to the azo dye, which acts as a final electron acceptor:

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

(*Hydrazo intermediate*)

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

(*Reductive cleavage of the azo bond*)

Anaerobic Conditions

The exact mechanism of azo dye reduction, whether occurring intracellularly or extracellularly, is still a subject of investigation, as is the role of biogenic intracellular water-soluble electron carriers such as flavins. Reduced flavins can act as an electron shuttle from nicotinamide adenine dinucleotide phosphate (NADPH)-dependent flavoproteins to azo dye as electron acceptor (Gingell and Walker, 1971). Intracellular azo dye reduction cannot be responsible for the conversion of all types of azo dyes, especially for sulfonated azo dyes, which have limited membrane permeability (Stolz, 2001). Kudlich et al. (1997) demonstrated an increase on colour removal rates of sulfonated azo dyes by cell free-extracts, as well as after addition of toluene, i.e. a membrane-active compound which increases cell lysis, thus showing the limited membrane permeability of this type of dye. The current hypothesis is that azo dye reduction mostly occurs by extracellular or membrane-bond enzymes (Stolz, 2001). Reduced cytoplasmatic cofactors such as reduced flavins do not contribute to the chemical dye reduction due to their inability to cross living cell membranes (Russ et al., 2000). However, cell fractionation experiments demonstrated that a quinone reductase activity located in the cell membranes enhanced the reductive decolourisation of a sulfonated azo compound, and no dye cross-membrane transport was required (Kudlich et al., 1997). Recently, a NADH-dependent lawsone reductase activity located in the cytosolic fraction of *Escherichia coli* also showed the capacity for azo dye reduction (Rau and Stolz, 2003).

Biological and chemical reductive decolourisation

The reductive decolourisation of azo dyes under anaerobic conditions is a combination of both biological and chemical mechanisms. The *biological* contribution can be divided in specialised enzymes called azo reductases, which are present in bacteria that are able to grow using only azo dye as a carbon and energy source. However, up to date there is no clear evidence of anaerobic azo reductase; or non-specific enzymes that catalyse the reduction of a wide range of electron-withdrawing contaminants, including azo dyes (Stolz, 2001). Thus, a co-metabolic reaction is probably the main mechanism of dye reduction (Figure 2.5), in which the reducing equivalents or reduced cofactors like NADH, NAD(P)H, $FMNH_2$ and $FADH_2$ acting as secondary electron donor, channel electrons to cleave the azo bond (Gingell and Walker, 1971). The *chemical* contribution to the reductive decolourisation of azo dyes under anaerobic conditions may involve biogenic reductants like sulfide, cysteine, ascorbate or Fe^{2+} (Yoo, 2002). Figure 2.6 shows the electron flow preference in the presence of different redox couples involved in biological processes. Thus, oxygen is a more effective electron acceptor than azo dyes, which justify the low decolourisation rates (10-30%) under aerobic conditions.

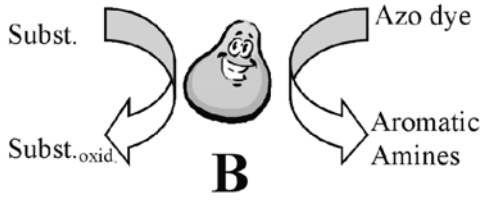

Figure 2.5. Co-metabolic reaction involved in reductive decolourisation of azo dyes. Legend: Subst., substrate or primary electron donor; Subst.$_{oxid}$, products of substrate oxidation.

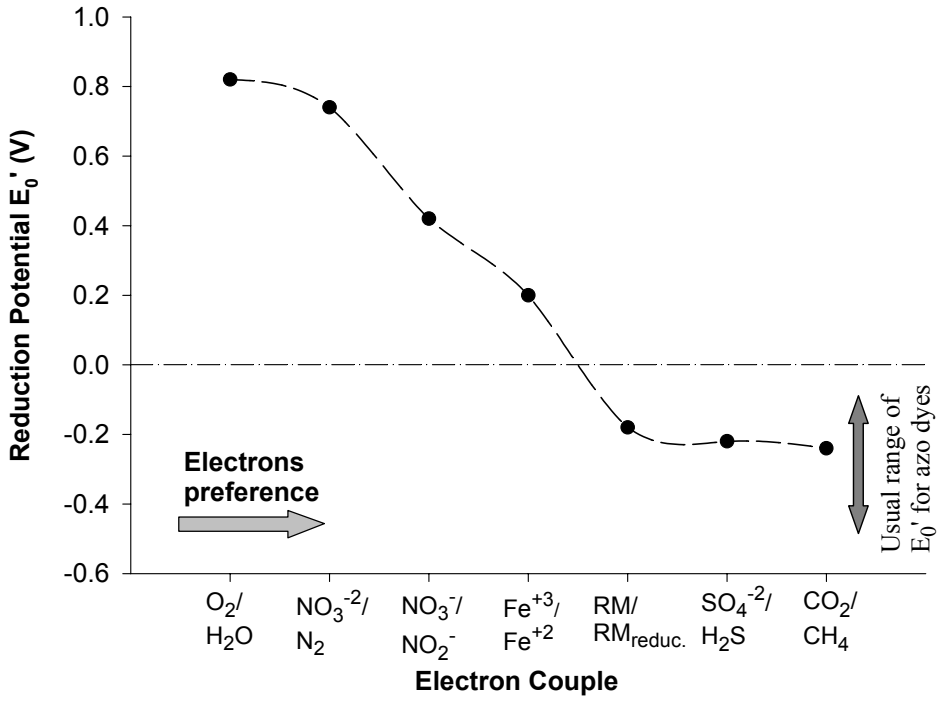

Figure 2.6. Electron flow preference as a function of the different electron couples (Adapted from Dubin and Wright, 1975; Cervantes, 2002; Madigan et al., 2003). RM and RM_{reduc} are the oxidized and the reduced form of the redox mediator, respectively.

Nevertheless, either by using pure cultures or granular sludge under anaerobic conditions, literature reports poor reductive decolourisation with specific dyes (Brown and DeVito, 1993; Van der Zee et al., 2001b). Furthermore, the rates are extremely dependent on the type of dye, in which the azo dyes generally present the highest rates of decolourisation. On the other hand, anthraquinone and phtalocyanine dyes are shown to be rather recalcitrant. Another drawback is that some dyes are quite toxic to the anaerobic microorganisms, which in some cases, may lead to a permanent loss of the methanogenic activity even for low dye concentrations (Brown and Laboureur, 1983; Van der Zee et al., 2001a; Fontenot et al., 2003). For instance, the inhibitory concentration of RB19, exerting 50% reduction in methanogenic activity (IC-value), was 55 mg/l at 30ºC (Dos Santos et al., 2005a). Table 2.4 shows the decolourisation of some dyes by using anaerobic granular sludge under mesophilic conditions. It is important to mention that a sound comparison among investigations is extremely difficult because of the differences in type and concentrations of dyes, sludge source and concentrations, electron donor, the way of calculating the decolourisation rates, etc.

Table 2.4. Colour removal by anaerobic granular sludge under mesophilic conditions.

Type	Name	Decolour. (%)	Decolour. Rates	Comments	Ref.
Anthraquinone	Reactive Blue 19	70	-	About 50 mg/l of dye	1
Anthraquinone	Acid Blue 80	7	-	About 50 mg/l of dye	1
Anthraquinone	Acid Blue 25	67	-	About 50 mg/l of dye	1
Anthraquinone	Basic Blue 22	62	-	About 50 mg/l of dye	1
Anthraquinone	Reactive Blue 4	57	13.4 mg/l/h	About 50 mg/l (0.08 mM) of dye	2
Anthraquinone	Reactive Blue 19	99	14.6 mg/l/h	About 50 mg/l (0.08 mM) of dye	2
Anthraquinone	Reactive Blue 4	73	4.3 mg/l/h	About 50 mg/l (0.08 mM) of dye	3
Anthraquinone	Reactive Blue 19	90	13.0 mg/l/h	About 50 mg/l (0.08 mM) of dye	3
Anthraquinone	Reactive Blue 5	37	-		4
Anthraquinone	Reactive Blue 49	9	-		5
Anthraquinone	Acid Blue 25	68	-		6
Anthraquinone	Disperse Red 159	0	0		7
Anthraquinone	Disperse Blue 56	0	0		8
Azo dye	Acid Orange 7	99	1.49 day^{-1}	About 0.3 mM of dye	9
Azo dye	Acid Red 266	95	0.20 day^{-1}	About 0.3 mM of dye	9
Azo dye	Acid Yellow 137	95	0.35 day^{-1}	About 0.3 mM of dye	9
Azo dye	Acid Yellow 159	97	0.72 day^{-1}	About 0.3 mM of dye	9
Azo dye	Basic Red 23	99	10.00 day^{-1}	About 0.3 mM of dye	9
Azo dye	Direct Black 19	99	3.00 day^{-1}	About 0.3 mM of dye	9

Continue in the next page

Table 2.4. *Continuation*

Type	Name	Decolour. (%)	Decolour. Rates	Comments	Ref.
Azo dye	Direct Blue 53	99	0.24 day^{-1}	About 0.3 mM of dye	9
Azo dye	Direct Blue 71	100	0.61 day^{-1}	About 0.3 mM of dye	9
Azo dye	Direct Red 79	97	16.60 day^{-1}	About 0.3 mM of dye	9
Azo dye	Direct Red 81	99	7.80 day^{-1}	About 0.3 mM of dye	9
Azo dye	Direct Yellow 4	95	1.03 day^{-1}	About 0.3 mM of dye	9
Azo dye	Direct Yellow 12	86	1.17 day^{-1}	About 0.3 mM of dye	9
Azo dye	Direct Yellow 50	99	2.00 day^{-1}	About 0.3 mM of dye	9
Azo dye	Mordant Orange 1	97	1.74 day^{-1}	About 0.3 mM of dye	9
Azo dye	Mordant Yellow 10	95	1.86 day^{-1}	About 0.3 mM of dye	9
Azo dye	Reactive Black 5	99	5.00 day^{-1}	About 0.3 mM of dye	9
Azo dye	Reactive Orange 14	98	0.17 day^{-1}	About 0.3 mM of dye	9
Azo dye	Reactive Orange 16	97	2.10 day^{-1}	About 0.3 mM of dye	9
Azo dye	Reactive Red 2	100	0.31 day^{-1}	About 0.3 mM of dye	9
Azo dye	Reactive Red 4	99	0.45 day^{-1}	About 0.3 mM of dye	9
Azo dye	Reactive Yellow 2	73	0.01 day^{-1}	About 0.3 mM of dye	9
Azo dye	Reactive Red 235	100	4.42 day^{-1}	About 50 mg/l	10
Azo dye	Reactive Blue 235	100	23.5 day^{-1}	About 50 mg/l	10
Azo dye	Reactive Yellow 168	100	23.4 day^{-1}	About 50 mg/l	10
Azo dye	Reactive Red 198	95	11.6 mg/l/h	About 300 mg/l of dye	11
Azo dye	Mordant Blue 13	83	-	About 50 mg/l of dye	1
Azo dye	Mordant Black 9	77	-	About 50 mg/l of dye	1
Azo dye	Basic Red 18	92	-	About 50 mg/l of dye	1
Azo dye	Acid Yellow 151	88	-	About 50 mg/l of dye	1
Azo dye	Direct Red 7	92	-	About 50 mg/l of dye	1
Azo dye	Acid Red 114	62	-	About 50 mg/l of dye	1
Azo dye	Direct Blue 15	83	-	About 50 mg/l of dye	1
Azo dye	Direct Yellow 12	75	-	About 50 mg/l of dye	1
Azo dye	Reactive Black 5	81	-	About 50 mg/l of dye	1
Azo dye	Acid Blue 113	94	-	About 50 mg/l of dye	1
Azo dye	Direct Black 19	51	-	About 50 mg/l of dye	1
Azo dye	Direct Black 22	61	-	About 50 mg/l of dye	1
Methine	Basic Yellow 28	35	-	About 50 mg/l of dye	1
Nitro	Acid Orange 3	62	-	About 50 mg/l of dye	1
Oxazine	Basic Blue 3	62	-	About 50 mg/l of dye	1
Phtalocyanine	Reactive Blue 21	80	8.6 mg/l/h	About 300 mg/l of dye	11
Phtalocyanine	Reactive Blue 21	36	-	About 50 mg/l of dye	1

[1]Brown and Laboureur (1983); [2]Fontenot et al. (2002); [3]Lee and Pavlostathis (2004) [4]Luangdilok and Paswad (2000); [5]Carliell et al. (1994); [6]Brown and Hamburger (1987); [7]Malpei et al. (1998); [8]Delee et al. (1998); [9]Van der Zee et al. (2001b); [10]Willetts (2000); [11] Fontenot et al. (2003).

Reductive decolourisation of azo dyes in the presence of redox mediators

Redox mediators are compounds that accelerate the electron transfer from a primary electron donor to a terminal electron acceptor, which may increase the reaction rates by one to several orders of magnitude (Cervantes, 2002). Redox mediators have shown to be effective not only for reductive decolourisation, but also for the reductive transformation of iron (Lovley et al., 1998), nitroaromatics (Dunnivant et al., 1992), polyhalogenated compounds (O'Loughlin et al., 1999) and radionuclides (Fredrickson et al., 2000). Recently it was found that during the aerobic degradation of naphthalene-2-sulfonate (2NS) by *Sphingomonas xenophaga* strain BN6, quinoid redox mediators were produced, which mediated the reduction of azo dye under anaerobic conditions (Keck et al., 2002). Flavin-based compounds like FAD, FMN and riboflavin, as well as quinone-based compounds like AQS, AQDS and lawsone, have been extensively reported as redox mediators during azo dye reduction (Semdé et al., 1998; Cervantes et al., 2000; Rau et al., 2002a; Field and Brady, 2003). Reductive decolourisation of azo dyes in the presence of redox mediators occurs in two distinct steps, the first step being a non-specific enzymatic mediator reduction, and the second step being a chemical reoxidation of the mediator by the azo dyes (Figure 2.7) (Keck et al., 1997).

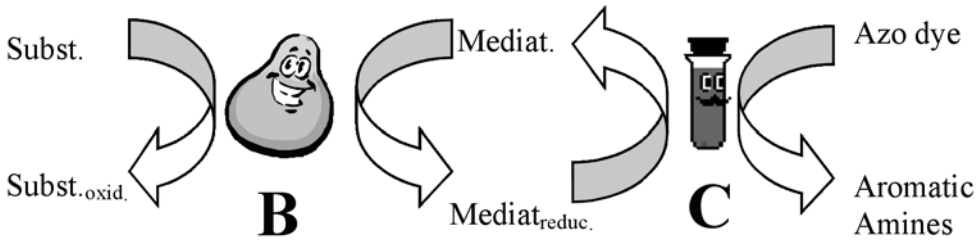

Figure 2.7. Co-metabolic reaction involved in reductive decolourisation of azo dyes in the presence of redox mediators. B and C are the biological and chemical steps, respectively.

Theoretically, feasible redox mediators for biological azo dye reduction must have redox potentials between the half reactions of the azo dye and the primary electron donor (Van der Zee et al., 2003b). Unfortunately, the standard redox potential (E_0') for most azo dyes is unknown, but this information can be obtained by using polarography. In a screening of redox potential values for different azo dyes, it was found that E_0' values are generally between –0.430 and –0.180 V (Dubin and Wright, 1975). Rau et al. (2002) cite that the NAD(P)H cofactor, which has the lowest E_0' value of –0.320 V, seems to set the limits of redox mediators application. The reason for this is that mediators with a more negative E_0' value will not be reduced by the cells, and mediators with E_0' greater than –0.05 V will not

efficiently reduce the azo bond at high rates. Figure 2.8 shows the E_0' values for both quinone-based and non quinone-based redox mediators.

The standard redox potential value (E_0') is a good indication of a compound capacity to function as a redox mediator. However, apparently other factors are of importance as well since different decolourisation rates in the presence of mediators with similar E_0' values have been reported, and similar decolourisation rates with mediators with different E_0' values. For instance, Brown (1981) tested the polymeric nitro dye Poly Y-607 and found that methyl viologen and benzyl viologen increased the decolourisation rates 4.5-fold, even though the E_0' of methyl viologen is much lower than that of benzyl viologen, i.e. –0.440 V and –0.360 V, respectively (Figure 2.8). Walker and Ryan (1971) postulated that decolourisation rates are related to the electron density in the azo bond region. They suggested that colour removal rates would increase by lowering the electron density in the azo linkage. Therefore, the use of redox mediators would not only tend to accelerate the transfer of reducing equivalents to the terminal electron acceptor, i.e. the azo dye, but also to minimize the steric hindrance of the dye molecule (Bragger et al., 1997; Moir et al., 2001). Thus, in estimating the theoretical decolourisation rates by using specific redox mediators, the differences in steric and electro-chemical factors between mediator and azo dye should also be considered.

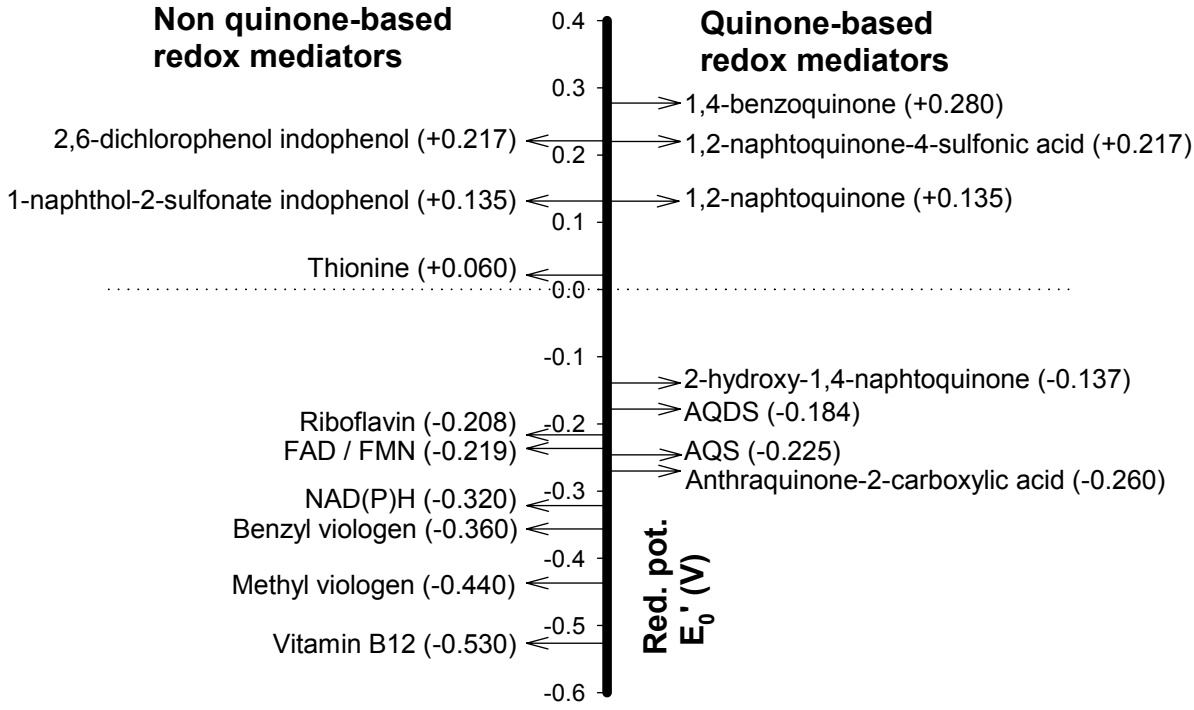

Figure 2.8. E_0' values for both quinone-based and non quinone-based redox mediators (Adapted from Rau et al., 2002a; Fultz and Durst, 1982).

Microbiological aspects of the reductive decolourisation of azo dyes

Pure cultures

The literature extensively reports the use of pure cultures (Table 2.5), either whole cells or specific enzymes, for a better insight of the anaerobic azo dye reduction mechanisms, which are not fully understood yet (Stolz, 2001; Pearce et al., 2003).

Table 2.5. Examples of facultative and strictly anaerobic bacterial cultures, which were able to decolourise azo dyes under anaerobic conditions.

Organism	Dyes	Activity	Decolour. (%)	Comments	Ref.
		μ mol/ml/h			
Clostridium perfringens ATCC 3626	Amaranth	0.74	-	Dye concentration of 0.033 mM.	1
	Methyl Orange	0.62	-		
	Orange II	0.70	-		
	Tartrazine	0.67	-		
		μ mol/ml/h			
Bacteroides fragilis	Amaranth	0.30	66.0	After 6 h of incubation. Dye concentration of 0.1 mM.	2
	Orange II	0.20	37.0		
	Tartrazine	0.08	9.0		
Pseudomonas GM3	Acid Violet 7	-	97.4	After 72 h of incubation. Dye concentration of 100 mg/l.	3
	Reactive Blue 2	-	18.3		
	Acid Green 27	-	75.6		
	Acid Red 183	-	20.1		
	Indigo Carmine	-	69.0		
		μ mol/min/g protein			
Sphingomonas xenophaga BN6	Acid Red 27	0.10	-	Dye concentration of 0.1 mM.	4
	Acid Orange 20	0.10	-		
	Acid Orange 7	0.30	-		
	Acid Red 14	0.20	-		
	Acid Yellow 23	0.10	-		
	Acid Black 1	0.30	-		
		AU x 10^{-2} / mg protein			
Enterococcus faecalis	Methyl Red	1.81	99.4	After 20 h of incubation. Dye concentration of 0.2 mM	5
	Orange II	1.39	95.1		
	Orange G	1.20	64.1		
	Amaranth	1.37	99.5		
Eubacterium biforme	Tartrazine	-	4.0	After 150 minutes of incubation. Dye concentration of 2 mM	6
	Sunset Yellow	-	22.0		
	Methyl Orange	-	79.0		
	Orange II	-	81.0		
	Amaranth	-	19.0		
	Allura Red 40	-	11.0		

[1]Semdé et al., 1998; [2]Bragger et al., 1997; [3]Yu et al., 2001; [4]Rau et al., 2002a; [5]Chen et al., 2004; [6]Chung et al., 1978.

Microbial decolourisation requires an unspecific enzymatic capacity ubiquitously found in a wide diversity of microorganisms (Chung and Stevens, 1993). This has been mainly demonstrated with microorganisms present in the intestine such as *Clostridium, Salmonella, Bacillus, Eubacterium* and *Escherichia coli*, which are able to reduce the dyes ingested through food, drugs and cosmetics (Brown and DeVito, 1993; Rau et al., 2002a; Chen et al., 2004). The understanding of azo dye reduction mechanisms is important not only under a biotechnological approach toward the use of biological processes for decolourisation, but also under a medical approach to have an insight into how the intestinal microflora metabolites the ingested azo dyes (Brown and DeVito, 1993; Semdé et al., 1998). Azo dyes are converted into aromatic amines because of both the presence of microflora and the anaerobic condition found in the human intestine. Aromatic amines present a mutagenic and carcinogenic character much higher than their precursor azo dyes (Weisburger, 2002). Therefore, a lot of effort has been made in the production of compounds, which are resistant to these reductive transformations. Another approach has been investigated in the use of azo polymers that would be insoluble in the upper gastrointestinal track, but susceptible to degradation on the colon, acting as an oral colon-specific drug delivery system (Bragger et al., 1997; Rau et al., 2002b). In this review we will focus on the biotechnological approach of azo dye reduction.

Granular sludge

Even though anaerobic azo dye reduction could be readily achieved with different microorganisms, there is no strain reported so far that is able to decolourise a broad range of azo dyes. Therefore, the use of a specific strain or enzymes on reductive decolourisation does not make much sense in treating textile wastewater, which is composed of many kinds of dyes (Laszlo, 2000). The use of mixed cultures such as anaerobic granular sludge, which is composed of stable microbial pellets with a high activity, is probably a more logic alternative. Different reactor configurations like the widely used upflow anaerobic sludge bed (UASB) system and expanded granular sludge bed (EGSB) system, are used to immobilize high concentrations of biomass (Lettinga et al., 1980; Lettinga, 1995; Van Lier et al., 2001). Indeed, the different microbial consortia present in anaerobic granular sludge can carry out tasks that no individual pure culture can undertake successfully (Nigam et al., 1996; Pearce et al., 2003). However, little is known about the microbiological aspects of the reductive decolourisation of azo dyes with anaerobic consortia commonly found in wastewater treatment plants, although the applicability of the cost-effective high-rate anaerobic reactors

for azo dye reduction has been well demonstrated (Cervantes et al., 2001; Dos Santos et al., 2003; Dos Santos et al., 2004c).

As previously explained, the reductive decolourisation of azo dyes by using methanogenic anaerobic granular sludge is very likely controlled by a co-metabolic reaction in the presence of different electron donors, in which the azo dye is the terminal electron acceptor of the reduced cofactors. Table 2.6 shows the main biochemical reactions involved in the conversion of organic matter in methanogenic environments.

Table 2.6. Biochemical reactions involved in the conversion of organic matter in methanogenic environments

Acetogenic reactions			$\Delta G^{o'}_{25\,^oC}$ (kJ mol^{-1})	$\Delta G^{o'}_{55\,^oC}$ (kJ mol^{-1})
Glucose + 12 H_2O	→	6 HCO_3^- + 12 H_2 + 6H^+	+ 3.2	-51.8
Glucose + 4 H_2O	→	2 $Acetate^-$ + 2 HCO_3^- + 4 H_2 + 4H^+	- 206.3	-232.2
Ethanol + H_2O	→	$Acetate^-$ + 2 H_2 + H^+	+ 9.6	+1.7
$Lactate^-$ + 2 H_2O	→	$Acetate^-$ + HCO_3^- + 2 H_2 + H^+	- 4.2	-12.6
$Acetate^-$ + 4 H_2O	→	2 HCO_3^- + 4 H_2+ H^+	+ 104.2	+ 89.8
$Propionate^-$ + 3 H_2O	→	$Acetate^-$ + HCO_3^- + 3 H_2 + H^+	+ 76.5	+ 64.7
$Propionate^-$ + 2 HCO_3^-	→	$Acetate^-$ + 3 $Formate^-$ + H^+	+ 72.4	+ 61.6
$Butyrate^-$ + 2 H_2O	→	2 $Acetate^-$ + 2 H_2+ H^+	+ 48.3	+ 39.5
$Butyrate^-$ + 2 HCO_3^-	→	2 $Acetate^-$ + 2 $Formate^-$ + H^+	+ 30.6	+ 20.9
Homoacetogenic reactions				
$Acetate^-$ + 4 H_2O	→	2 HCO_3^- + 4 H_2 + H^+	+ 104.6	+ 90.2
2 CO_2 + 4 H_2	→	$Acetate^-$ + 2 H_2O	- 55.0	- 33.5
Methanogenic reactions				
4 H_2 + HCO_3^- + H^+	→	CH_4 + 3 H_2O	- 135.6	- 124.9
4 $Formate^-$ + H_2O + H^+	→	CH_4 + 3HCO_3^-	- 130.4	- 118.9
$Acetate^-$ + H_2O	→	HCO_3^- + CH_4	- 31.0	- 34.7

Energy changes were calculated by using the van 't Hoff equation, standard enthalpy values of compounds (Chang, 1977) and Gibbs free-energy changes at 25ºC (Thauer et al., 1977).

Thus, the reducing equivalents are formed during the conversion of the primary electron donor, i.e. the organic matter, during the different steps of carbon flow under anaerobic conditions (Figure 2.9). The organic matter is initially hydrolysed; namely, enzymes produced by fermentative bacteria hydrolyse complex molecules such as proteins,

polysaccharides, nucleic acid and fats into their corresponding monomers, which are amino acids, sugars and long chain fatty acids. Afterwards, these monomers are fermented to reduced organic compounds like short chain fatty acids, alcohols and lactate in a step called acidogenesis. Subsequently, these acids can either be converted into H_2/CO_2 by hydrogen-forming microorganisms or into acetate by acetate-forming microorganisms. Acetate can also be formed via H_2/CO_2 pathway in a step called homoacetogenesis. Finally, the final product methane/CO_2 can be formed in the methanogenesis step. Methanogens are able to directly use substrates like H_2, acetate, formate and methanol to produce methane.

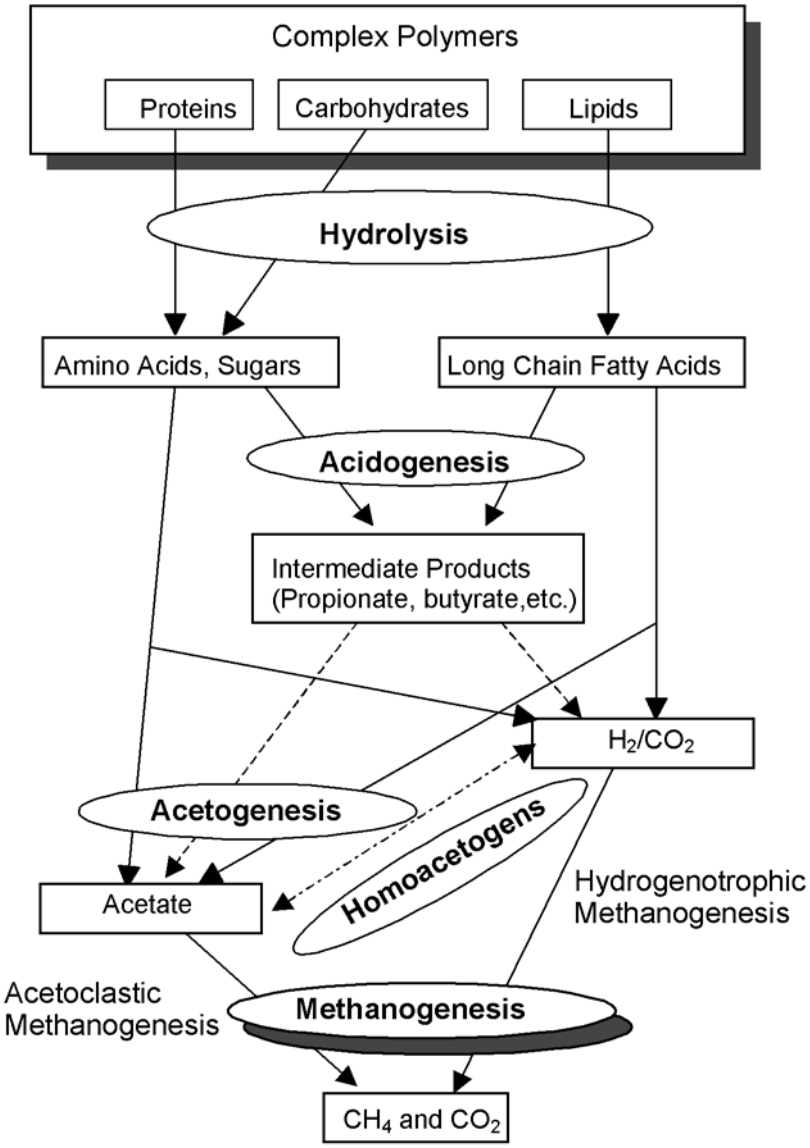

Figure 2.9. Conversion of complex organic matter in methanogenic anaerobic reactors (Adapted from Gujer and Zehnder, 1983).

Reductive decolourisation of azo dyes is extremely dependent on the type of primary electron donor. Acetate and other volatile fatty acids are normally poor electron donors, whereas ethanol, glucose, H_2/CO_2 and formate are more effective electron donors for dye reduction (Tan et al., 1999; Dos Santos et al., 2003). Donlon et al. (1997) reported that interspecies hydrogen resulting from the oxidation of substrates such as butyrate, propionate, and ethanol could provide the medium with reducing equivalents, and thus stimulation of the nitrophenol reduction. In the latter work, the direct methane precursors acetate and methanol did not stimulate the nitrophenol reduction rates. Similar results were also reported during the reductive dechlorination of carbon tetrachloride (CT) by anaerobic granular sludge in which acetate and methanol were marginally utilized to support CT dechlorination (Cervantes et al., 2004).

However, the conversion of the electron donors, e.g. ethanol, glucose, used for both dye reduction and methanogenesis is only maintained if there is equilibrium among the syntrophic group of microorganisms present in the bioreactor (Figure 2.9). The degree of dependence among these microorganisms varies considerably (Schink, 2002). Despite the fact that the last members in the bacterial food chain are dependent on the earlier ones, e.g. interdependence between methanogens and acetogens, they may be extremely important for the first organisms in the consumption chain by removing metabolic products (Schink, 1997). Dos Santos et al. (2005a) observed that the anthraquinone dye RB19 was mainly toxic to the acetate-utilising methanogens, whereas acidogens were not affected by the dye toxicity. Therefore, the feasibility of coupling, in the same bioreactor, dye reduction and complete electron donor conversion into CH_4 must be carefully attempted.

4.3. Non-biological colour removal

Physical-chemical methods

In physical-chemical methods coagulant agents like ferric salts or aluminium polychloride are used to form flocs with the dyes, which are then separated by filtration or sedimentation. Polyelectrolyte can also be dosed during the flocculation phase to improve the flocs settleability (Vandevivere et al., 1998). The coagulation-flocculation method is one of the most widely used processes in textile wastewater treatment plants in many countries such as Germany and France. It can be used either as a pre-treatment, post-treatment, or even as a main treatment system (Gähr et al., 1994; Marmagne and Coste, 1996). Marmagne and Coste (1996) reported that coagulation-flocculation methods were successfully applied for colour

removal of sulphur and disperse dyes, whereas acid, direct, reactive and vat dyes presented very low coagulation-flocculation capacity. On top of the problem of low colour removal efficiency with some dyes, physical-chemical methods demand large chemicals inputs, and produce high volumes of polluted sludge, which then must be treated (Robinson et al., 2001).

Chemical Methods

Chemical oxidation typically involves the use of an oxidising agent such as ozone (O_3), hydrogen peroxide (H_2O_2) and permanganate (MnO_4) to change the chemical composition of a compound or a group of compounds, e.g. dyes (Metcalf and Eddy, 2003). Among these oxidants, ozone is the most widely used because of its high reactivity with many dyes, usually providing good colour removal efficiencies (Alaton et al., 2002). In a process called selective oxidation, ozone can be designed in such a way that only ─N═N─ bond scission occurs, and biodegradable compounds remain non-oxidised (Boncz, 2002). However, disperse dyes and those insoluble in water represent a drawback for the process, as well as the high cost of ozone (Hassan and Hawkyard, 2002a). The usual low efficiency of both colour and COD removals of conventional chemical oxidation techniques have been overcome by the development of the so-called advanced oxidation processes (AOP). In this process, oxidizing agents such as O_3 and H_2O_2 are used with catalysts (Fe, Mn and TiO_2), either in the presence or absence of an irradiation source (Alaton et al., 2002). Consequently, an improvement in the generation and use of the free hydroxyl radical ($HO^{\bullet}$) is obtained, which may represent a rate increase of one to several orders of magnitude compared with normal oxidants in the absence of a catalyst (Ince and Tezcanli, 1999). Table 2.7 gives an indication of the oxidative power of the hydroxyl radical based on the electrochemical oxidation potential (EOP) capacity compared with other oxidants.

Table 2.7. Oxidation capacity of different oxidants in terms of electrochemical oxidation potential (EOP) (Adapted from Metcalf and Eddy, 2003).

Oxidizing agent	EOP (V)
Fluorine	3.06
Hydroxyl radical	2.80
Oxygen (atomic)	2.42
Ozone	2.08
Hydrogen peroxide	1.78
Hypochlorite	1.49
Chlorine	1.36
Chlorine dioxide	1.27
Oxygen (molecular)	1.23

At present, many different combinations of these AOP have been investigated for colour removal, all of which are capable of producing the free hydroxyl radical ($HO^{\bullet}$). The first example is a reaction called the **Fenton reaction**, in which hydrogen peroxide is added in an acid solution (pH 2-3) containing Fe^{+2} ions:

$$Fe^{+2} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^{-} \quad \text{(I)}$$

In comparison with ozonation, this method is relatively cheap and also presents high COD removal and decolourisation efficiencies (Van der Zee, 2002). The main process drawbacks are the high sludge generation due to the flocculation of reagents and dye molecules (Robinson et al., 2001), as well as the need for decreasing the bulk pH to acidic conditions. Hassan and Hawkyard (2002) reported that a pre-ozonation of coloured wastewaters prior to Fenton reaction not only considerably accelerated the overall colour removal rates, but also decreased the sludge generation.

In **H_2O_2/UV** process $HO^{\bullet}$ radicals are formed when water-containing H_2O_2 is exposed to UV light, normally in the range of 200-280 nm (Metcalf and Eddy, 2003). The H_2O_2 photolysis follows the reaction:

$$H_2O_2 + \text{UV (or } h\nu, \lambda \approx 200\text{-}280 \text{ nm}) \rightarrow HO^{\bullet} + HO^{\bullet} \quad \text{(II)}$$

This process is the most widely used AOP technology for the treatment of hazardous and refractory pollutants present in wastewaters, mainly because no sludge is formed and a high COD removal in a short retention time is achieved (Safarzadeh et al., 1997). Additionally, it has been successfully applied for colour removal. For instance, more than 95% decolourisation was achieved in treating reactive, basic, acid and direct dyes at pH 5, whereas disperse and vat dyes were only partially decolourised (Yang et al., 1998). A comparative study between ozone and H_2O_2/UV was carried out in treating a concentrated reactive dyebath from a textile factory. The H_2O_2/UV system presented decolourisation rates close to those rates obtained with ozone but with a lower cost (Alaton et al., 2002). In some cases however, the H_2O_2/UV process presents low COD and colour removal efficiency due to inefficient use of UV light (mainly for highly coloured wastewaters) (Moraes et al., 2000), or

because of the low molar extinction coefficient of H_2O_2 (specific oxidation capacity), requiring high dosages of the latter.

The UV-based methods in the presence of a catalyst, e.g. a semiconductive material such as TiO_2, have also shown to distinctly enhance colour removal (So et al., 2002; Grzechulska and Morawski, 2002). Thus, different combinations such as Ozone/TiO_2, Ozone/TiO_2/H_2O_2 and TiO_2/H_2O_2 have been investigated, but they are enormously influenced by the type of dye, dye concentration and pH (Galindo et al., 2000). Recently, the utilization of solar technologies instead of UV-based methods has been attracting attention (Wang, 2000).

Physical Methods

Filtration methods such as ultrafiltration, nanofiltration and reverse osmosis have been used for water reuse and chemical recovery. In the textile industry these filtration methods can be used for both filtering and recycling not only pigment-rich streams, but also mercerising and bleaching wastewaters. The specific temperature and chemical composition of the wastewater determine the type and porosity of the filter to be applied (Porter, 1997). The main drawbacks of membrane technology are the high investment costs, the potential membrane fouling, and the production of a concentrated dyebath which needs to be treated (Robinson et al., 2001). The recovery of concentrates from membranes, e.g. recovery of the sodium hydroxide used in the mercerising step or sizing agents such as polyvinyl alcohol (PVA), can attenuate the treatment costs (Porter, 1997). Water reuse from dyebath effluents has been successfully achieved by using reverse osmosis. However, a coagulation and micro-filtration pre-treatment was necessary to avoid membrane fouling (Vandevivere et al., 1998).

Adsorption methods for colour removal are based on the high affinity of many dyes for adsorbent materials. Decolourisation by adsorption is influenced by some physical-chemical factors like dye-adsorbent interactions, adsorbent surface area, particle size, temperature, pH and contact time (Mattioli et al., 2002). The main criteria for the selection of an adsorbent should be based on characteristics such as high affinity and capacity for target compounds and the possibility of adsorbent regeneration (Karcher et al., 2001a). Activated carbon (AC) is the most common adsorbent and can be very effective with many dyes (Walker and Weatherley, 1997). However, its efficiency is directly dependent upon the type of carbon material used and the wastewater characteristics, i.e. types of dyes present in the stream. Additionally, AC is relatively expensive and has to be regenerated offsite with losses of about 10% in the thermal

regeneration process. In order to decrease the adsorbent losses during regeneration, new adsorbent materials have been tested for their ability for on-site regeneration. Karcher et al. (2001) studied alternative materials such as zeolites, polymeric resins, ion exchangers and granulated ferric hydroxide. It was found that zeolites and microporous resins were unsuitable due to their low sorption capacity. Although the ion exchangers provided good sorption capacity, regeneration was sometimes difficult. A number of low-cost adsorbent materials like peat, bentonite clay and fly ash, have been investigated on colour removal (Ramakrishna and Viraraghavan, 1997). However, the efficiency of these materials varied with the dye class. For instance, fly ash presented high sorption affinity for acid dyes, whereas peat and bentonite presented high affinity for basic dyes. Table 2.8 shows the advantages and drawbacks of some non-biological decolourisation processes applied to textile wastewaters.

Table 2.8. Advantages and drawbacks of some non-biological decolourisation processes applied to textile wastewaters (after Robinson et al., 2001).

Physical/Chemical Methods	**Method Description**	**Advantages**	**Disadvantages**
Fenton reagents	Oxidation reaction using mainly H_2O_2-Fe(II)	Effective decolourisation of both soluble and insoluble dyes	Sludge generation
Ozonation	Oxidation reaction using ozone gas	Application in gaseous state: no alteration of volume	Short half-life (20 min)
Photochemical	Oxidation reaction using mainly H_2O_2-UV	No sludge production	Formation of by-products
NaOCl	Oxidation reaction using Cl^+ to attack the amino group	Initiation and acceleration of azo-bond cleavage	Release of aromatic amines
Electrochemical destruction	Oxidation reaction using electricity	Breakdown compounds are non-hazardous	High cost of electricity
Activated carbon	Dye removal by adsorption	Good removal of a wide variety of dyes	Very expensive
Membrane filtration	Physical separation	Removal of all dye types	Concentrated sludge production
Ion exchange	Ion exchange resin	Regeneration: no adsorbent loss	Not effective for all dyes
Electrokinetic coagulation	Addition of ferrous sulphate and ferric chloride	Economically feasible	High sludge production

5. CONCLUSIONS

All the decolourisation methods described in this review have advantages and drawbacks, and their selection will depend on the wastewater characteristics like class and concentration of dye, pH, salinity and toxic compounds. Compared to individual bacterial cells or specific enzymes, anaerobic colour removal by granular sludge as a pre-treatment for coloured wastewaters from dyebath and rinsing steps seems to be a very attractive technology. Moreover, the use of redox mediators and/or thermophilic treatment to accelerate decolourisation rates in bioreactors is very promising, while ways to immobilize redox mediators in bioreactors or their recovery when continuously dosed, still represents a challenge.

Efficient post-treatment methods, e.g. aerobic biological post-treatment, ozone and fenton reagents, for the complete mineralization of the aromatic amines, which are formed in the anaerobic step, must be utilized. Therefore, the treatment of textile wastewater either to guarantee the emission requirements or to close the water cycle should be composed of a sequence of treatments, and each scenario should be analysed individually.

Acknowledgements

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3

Azo dye reduction by thermophilic anaerobic granular sludge, and the impact of the redox mediator AQDS on the reductive biochemical transformation

Abstract

Azo dye reduction by thermophilic anaerobic granular sludge was investigated, of which the biotic and abiotic contributions were distinguished. The impact of the redox mediator anthraquinone-2,6-disulfonate (AQDS) on colour removal and co-substrate conversion was investigated as well. Metabolic activities of the thermophilic inoculum induced a fast azo dye reduction and indicated a biotic predominance in the process. The addition of co-substrate enhanced the decolourisation rates 1.7-fold compared with the bottles free of co-substrate. Addition of AQDS together with co-substrate enhanced the k value 1.5-fold, compared with batch assays containing co-substrate but lacking AQDS. During a comparative study between sludge samples incubated under mesophilic (30ºC) and thermophilic (55ºC) conditions, the decolourisation rate at 55ºC reached values up to sixfold higher than at 30°C. Biological treatment at 55ºC showed a fast initial generation of reducing compounds via electron donor conversion, with AQDS increasing the azo dye reduction rate in all incubations tested. Nevertheless, high concentrations of AQDS showed severe inhibition of thermophilic acetate and propionate conversion as well as methane production rates.

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1. Introduction

Almost 10^9 kg of dyes are produced annually in the world, of which azo dyes, characterised by an azo-bond (R_1–N=N–R_2), represent about 70% by weight (Zollinger, 1987). Reactive azo dyes, i.e. dyes with reactive groups that form covalent bonds with OH–, NH– or SH– groups are extensively used in the textile industry, despite the fact that they have a low degree of fixation into the fibres (efficiency 10–50%). Since textile industry wastewaters are generally discharged at high temperatures (40–70°C), thermophilic anaerobic treatment could serve as an interesting option for azo dye reduction, especially when closing process water cycles are considered. Anaerobic microorganisms maintain low the redox potential conditions (< -50 mV) and generate the reducing equivalents required for reductive decolourisation (Beydilli et al., 1998; Bromley-Challenor et al., 2000). The exact mechanism, whether occurring intracellularly or extracellularly, is still a subject of investigation, as is the role of biogenic water-soluble electron carriers, e.g. flavins. Reduced flavins can act as an electron shuttle from nicotinamide adenine dinucleotide phosphate (NADPH)-dependent flavoproteins to azo dye as electron acceptor (Gingell and Walker, 1971). Intracellular azo dye reduction cannot be responsible for the conversion of all types of azo dyes, especially for sulfonated azo dyes, which have limited membrane permeability (Stolz, 2001). An increase on colour removal rates of sulfonated azo dyes by cell free-extracts as well as after addition of toluene, i.e. a membrane-active compound which increases cell lysis, demonstrate the limited membrane permeability of the dye (Kudlich et al., 1997). The current hypothesis is that azo dye reduction mostly occurs by extracellular or membrane-bond enzymes (Stolz, 2001). Reduced cytoplasmatic cofactors such as reduced flavins do not contribute to the chemical reductive decolourisation due to their inability to cross living cell membranes (Russ et al., 2000). However, cell fractionation experiments demonstrated that a quinone reductase activity located in the cell membranes enhanced the reductive decolourisation of a sulfonated azo compound, and no dye cross-membrane transport was required (Kudlich et al., 1997). Recently, a NADH-dependent lawsone reductase activity located in the cytosolic fraction of *Escherichia coli* also showed the capacity for azo dye reduction (Rau and Stolz, 2003). Quinones are the electron accepting moieties of humic substances. Such compounds have been shown to play an important role not only as final electron acceptors for many recalcitrant organic compounds, but also facilitating electron transfer from an electron donor to an electron acceptor, e.g. azo dyes (Cervantes et al., 2000; Field et al., 2000). The first step is the non-specific enzymatic reduction of quinone to hydroquinone, and the second step is the chemical reoxidation of hydroquinone by azo dyes (Keck et al., 1997). The chemical

regeneration of hydroquinone is sometimes the rate-limiting step, e.g. using lawsone as redox mediator at 30°C during reduction of the azo dye Amaranth (Rau et al., 2002a). In general, the chemical reaction follows the Arrhenius equation, indicating that an increase in temperature will proportionally increase the collision frequency of the reactants, thus improving the kinetics of the reaction. Therefore, the use of redox mediators to accelerate reductive decolourisation of azo dyes under thermophilic conditions might be advantageous, not only for the expected faster enzymatic reduction of quinone to hydroquinone compared to mesophilic conditions, but also for the faster regeneration of hydroquinone by the chemical reaction with azo dyes. The main objective of this study is to investigate in batch assays the feasibility of applying thermophilic anaerobic treatment on reductive decolourisation. Therefore, the importance of biotic and abiotic activities is distinguished at 55°C. Additionally, the initial generation of reducing compounds after co-substrate oxidation is assessed by comparing sulphide-free incubations with those supplemented with sulphide as the reducing agent. Finally, the impact of the redox mediator anthraquinone-2,6-disulfonate (AQDS) azo dye reduction and co-substrate conversion is evaluated.

2. Materials and methods

2.1. Chemicals

Reactive Red 2 (RR2), a sulfonated reactive azo dye, was selected as model compound for this study (Procion Red MX-5B, ~50% purity) (Aldrich, Gillingham, UK). RR2 was used without additional purification. Prior to utilisation, RR2 was hydrolysed by increasing the pH to 11 with NaOH, heating at 80°C for 1 h, and by decreasing the pH to 7 with HCl (after Beydilli et al., 1998). This procedure aims to simulate the hydrolysed dye structure found in industrial textile wastewaters. Figure 3.1 shows the chemical structure before and after hydrolysis.

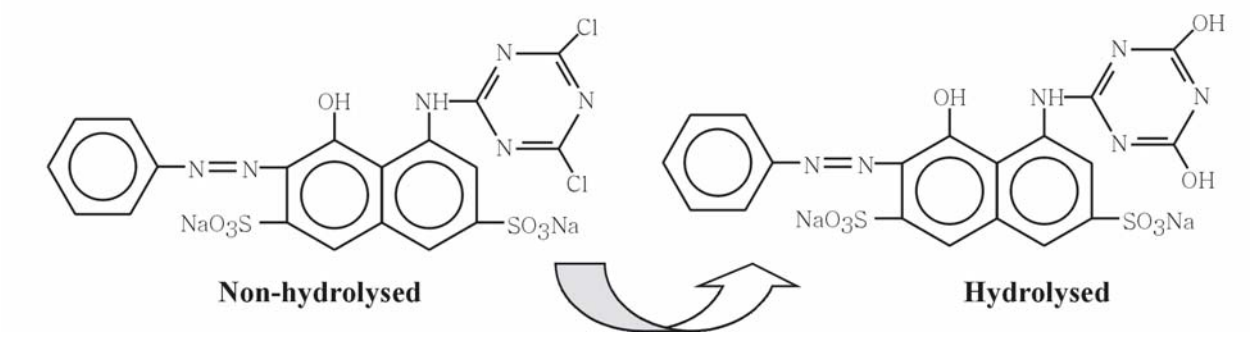

Figure 3.1. Chemical structure of the reactive azo dye Reactive Red 2 (RR2) used as model compound, before and after hydrolysis.

AQDS (Aldrich, Gillingham, UK) was used as redox mediator model compound, without additional purification (Figure 3.2).

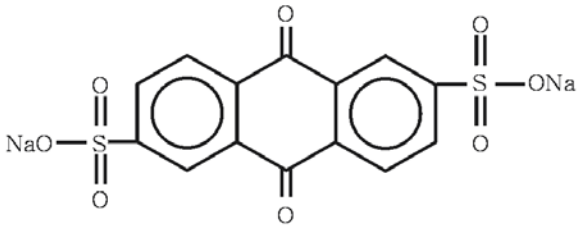

Figure 3.2. Chemical structure of AQDS.

2.2. Batch and continuous experiments

Seed inoculum and basal medium for decolourisation assays

Granular anaerobic sludge was collected from a full-scale mesophilic upflow anaerobic sludge blanket (UASB) reactor treating paper mill wastewater (Eerbeek, The Netherlands). The mesophilic sludge was acclimated for 3 months at 55ºC in an expanded granular sludge bed (EGSB) reactor (5.6 l) operating at a hydraulic retention time (HRT) of about 6 h and an organic loading rate (OLR) of 2.5 kgCOD m^{-3} day^{-1}. The chemical oxygen demand (COD) consisted of a mixture of glucose and volatile fatty acids (VFA) at a COD ratio of 1:3. The neutralized VFA solution contained acetate, propionate and butyrate at a COD ratio of 1:1:1. The glucose–VFA mixture simulates the organic compounds normally present in textile wastewaters. The influent stock solution was free of dye and AQDS during the whole period.

For batch tests at 30°C the same mesophilic granular sludge was acclimated in an EGSB reactor (30°C) following the above-mentioned protocol until reaching stable efficiency performance.

The basal medium consisted of (mg/l): NH_4Cl (280), K_2HPO_4 (250), $MgSO_4 \cdot 7H_2O$ (100) and $CaCl_2 \cdot 2H_2O$ (10) and 1 ml/l of trace elements containing (mg/l): H_3BO_3 (50), $FeCl_2 \cdot 4H_2O$ (2000), $ZnCl_2$ (50), $MnCl_2 \cdot 4H_2O$ (500), $CuCl_2 \cdot 2H_2O$ (38), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (50), $AlCl_3 \cdot 6H_2O$ (90), $CoCl_2 \cdot 6H_2O$ (2000), $NiCl_2 \cdot 6H_2O$ (92), $Na_2SeO_3 \cdot 5H_2O$ (162), EDTA (1000) and HCl 36% (1). The medium was buffered with 5.0 and 6.2 g/l sodium bicarbonate, for the temperatures of 30ºC and 55ºC, respectively, to keep the pH around 7.1. Resazurin was not included in the trace elements solution due to its mediating properties.

Batch experiments

Activity tests

In the activity tests 1.3 ± 0.1 gVSS/l of the previously described stabilized sludge were added to 117 ml serum bottles with 50 ml basal medium. The bottles were then sealed with butyl

rubber stoppers and aluminium crimp caps. Anaerobic conditions were established by flushing the headspace with N_2/CO_2 (70%:30%) and 2 gCOD/l co-substrate (mixture of glucose and VFA at a COD ratio of 1:3) was supplemented as electron donor and carbon source. RR2 (0.3 mM), AQDS (variable) and sulphide (variable) were also added. Sludge-free and autoclaved sludge were used as a control for abiotic colour removal. The incubations were conducted under non-static conditions by applying 50 shakes per minute. Sterile controls were autoclaved once at 122ºC for 240 min and again following a 5 days incubation period, after which sterile co-substrate, mediator, dye and sulphide stock solutions were added. The pH and the amount of VSS were determined after completion of the experiment.

Biotic and abiotic azo dye reduction at 55ºC

A first-order reaction with respect to the dye concentration was used, whereas the first-order rate constant "k" was determined using the equation 1:

$$A_t = A_0 \, e^{-kt} \quad (1)$$

A_t is the absorbance at time "t", A_0 is the initial absorbance at t=0, "k" is the first-order rate constant (day^{-1}) and "t" is the time elapsed (days). Time was plotted against $\ln(A_t/A_0)$ and the k-value was estimated by the slope of a linear regression.

Autoclaved sludge was used as a control of abiotic decolourisation by reducing compounds and adsorption in the sludge, and sludge-free controls were used to monitor the stability of the azo dye at 55ºC. The effects of co-substrate and AQDS (0.012 mM) were also evaluated for living and autoclaved sludges. To assess the reduction capacity in terms of the redox potential (ORP), a comparative study was conducted between sludge samples stabilized under mesophilic (30ºC) and thermophilic (55ºC) conditions. The ORP values of the serum bottles with living sludge were measured in an anaerobic chamber under a N_2/H_2 (95%:5%) atmosphere, after an incubation time of 48 h. The ORP values were reported in terms of the reference electrode (+207 mV at 25ºC). The serum bottles contained RR2 and co-substrate, either in the presence or absence of AQDS (0.012 mM).

Effect of VSS concentration on the azo dye reduction at 55ºC

The impact of AQDS (0.012 mM) on colour removal at 55ºC for different VSS concentrations was assessed. The VSS concentrations tested were 1.3, 3.0, 6.0 and 12.0 g/l. A mixture of glucose and VFA at a COD ratio of 1:3 (2 gCOD/l) was used as co-substrate, i.e. the electron donor.

Initial generation of reducing equivalents at 55ºC

The biological generation of reducing equivalents from co-substrate conversion at 55ºC was investigated. For this purpose, different concentrations of sulphide as reducing compound were added to living sludge, autoclaved sludge and sludge-free media. The k-values were determined in a sulphide-free medium, sulphide at sub-stoichiometric concentration (≤ 2 mM) and excess of sulphide (> 2 mM). AQDS (0.012 mM) was used to evaluate the impact of the external mediator on azo dye reduction. The concentrations of sulphide were measured at the start and completion of the experiment.

Effects of AQDS dosage on the azo dye reduction and co-substrate conversion

To assess the catalytic potential of redox mediators on colour removal at 55ºC, different AQDS concentrations were tested in batch assays. The effect of AQDS on co-substrate conversion and methanogenesis, after 2 days of incubation, was also observed. For this purpose, an AQDS gradient of 0, 0.006, 0.012, 0.024, 0.120, 0.600, 1.2, 5.0 and 10.0 mM, was tested with both living and autoclaved sludge. A mixture of glucose and VFA at a COD ratio of 1:3 (2 gCOD/l) was used as co-substrate or electron donor. A sludge-free control was used to monitor the azo dye stability at 55ºC.

3. Analysis

Colour removal was determined photometrically (Spectronics 60, Milton-Roy Analytical Products Division, Belgium), reading the absorbance at the maximum absorbance wavelength of RR2, i.e. 539 nm. Samples were diluted in phosphate buffer (10.86 g/l of $NaH_2PO_4 \cdot 2H_2O$ and 5.98 g/l of $Na_2HPO_4 \cdot 2H_2O$), and then centrifuged for 3 minutes at 10000 rpm. After hydrolysis 1.72 mM of RR2 was equivalent to 57.2 AU/cm, yielding a molar extinction coefficient of 33.3 AU cm^{-1} per mM.

VFA, methanol and ethanol were measured with a Hewlett Packard 5890 gas chromatograph (Palo Alto, USA), equipped with a 2 m x 2 mm glass column packed with Supelcoport (100-120 mesh) coated with 10% Fluorad FC 431. Temperatures used for column, injection port and flame ionisation were 130ºC, 200ºC and 280ºC, respectively. For alcohols determinations the temperature of column was switched to 70ºC.

Methane production was monitored adding 100 µL volume of sample in a gas chromatograph model 438/S (Packard-Becker, Delft, The Netherlands), equipped with a steel column (2 m x 2 mm) packed with Porapak Q (80/100 mesh, Milipore Corp., Bedford, M.A.). The

temperature of column, injection port and flame ionisation detector were 60ºC, 200ºC and 220ºC, respectively, with nitrogen as carrier gases (20 ml/min).

Sucrose, fructose, glucose, lactate and formate were measured on a High Pressure Liquid Chromatograph (HPLC) equipped with an Ion-300 column and a refractive index detector according to Van Lier et al. (1997).

Sulphide was measured photometrically as described by Trüper and Schlegel (1964).

The ORP was measured using a Sentix ORP 0-100ºC combination electrode (platinum – silver/silver chloride) (WTW, Weilheim, Germany), using a KCl solution (3 M) as the electrolyte.

VSS were analysed according to APHA standard methods (1998).

4. Results

Biotic and abiotic azo dye reduction at 55ºC

An anaerobic granular sludge originated from a mesophilic full-scale UASB reactor treating paper mill wastewater was tested for its capacity to reduce an azo dye, RR2, under thermophilic conditions. The sludge was previously stabilized at 55°C in a laboratory-scale EGSB reactor prior the assays. Azo dye reduction by thermophilic living sludge in a sulphide-free medium followed a first-order reaction with respect to the dye concentration. For bottles supplemented with co-substrate, the k-value enhanced 1.7-fold compared to the endogenous control, i.e. the incubation free of external electron donor (Figure 3.3). Addition of AQDS together with co-substrate enhanced the k-value 1.5-fold compared to bioassays supplemented with co-substrate and lacking AQDS. Addition of AQDS had no effect on the k-values observed in endogenous controls.

During a time elapsed of 6 days, negligible (< 1%) colour removal occurred in sludge-free controls in the presence of AQDS. However, controls conducted with autoclaved sludge in AQDS-supplemented assays achieved about 10% of colour removal during the same period (results not shown), and a complete decolourisation after prolonged periods (> 90 days).

The anaerobic granular sludge under study was also stabilized at 30°C in a laboratory-scale EGSB reactor in a parallel experiment, which followed the same protocol described for the thermophilic (55°C) EGSB reactor. During a comparative study between sludge samples incubated under mesophilic (30ºC) and thermophilic (55ºC) conditions, in the presence of the azo dye RR2 (0.3 mM), the decolourisation rate at 55ºC ($k = 0.93\ day^{-1}$) was about sixfold higher than at 30ºC ($k = 0.15\ day^{-1}$) in the absence of AQDS. Meanwhile, enhanced decolourisation rates were obtained in the presence of AQDS (0.012 mM) both at 30°C (k =

0.77 day^{-1}) and 55°C (k = 1.52 day^{-1}), which represents increments of fivefold and 1.6-fold, respectively, in comparison with the controls lacking AQDS. Furthermore, comparing the rate of decolourisation achieved in the AQDS-supplemented bottles, it can be deduced that there is a twofold higher rate for the thermophilic (55°C) controls compared to mesophilic (30ºC) assays. In the same experiment, a variation of the redox potential values at 30ºC and 55ºC was verified, after 48 h of incubation, in which AQDS (0.012 mM) stimulated more negative values. For AQDS-free assays the redox values were –148 mV at 30ºC and –204 mV at 55ºC. For the assays supplemented with AQDS the redox values were –158 mV at 30ºC and –214 mV at 55ºC. Therefore, AQDS lowered redox potential values by around –10 mV at both 55ºC and 30ºC.

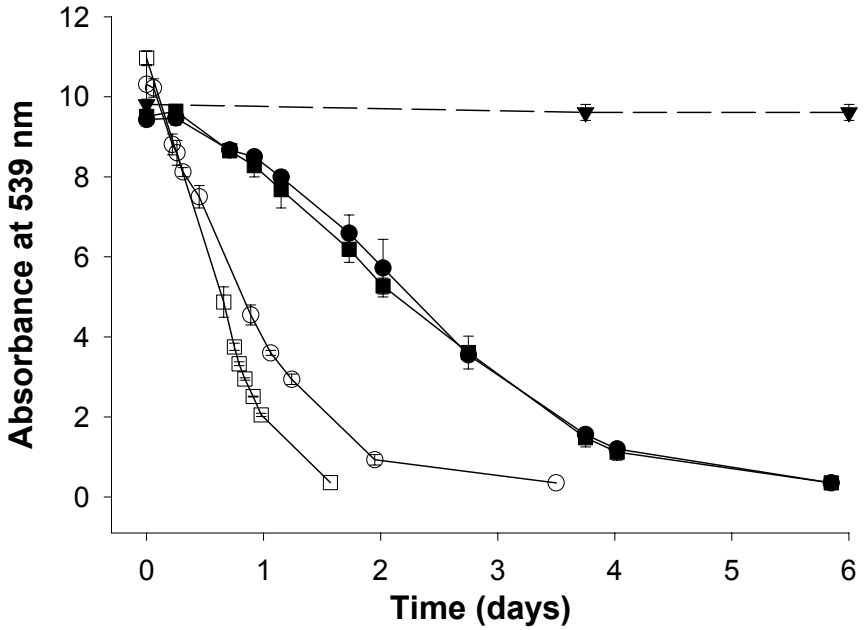

Figure 3.3. Colour removal of RR2 (0.3 mM) in the presence and absence of co-substrate (2 gCOD/l) and AQDS (0.012 mM). Symbols for the bottles contained: dye (●), dye+substrate (○), dye+AQDS (■), dye+substrate+AQDS (□), sludge-free controls (▼). Endogenous controls (co-substrate free) were used to assess the improvement of colour removal by addition of external co-substrate and AQDS. Sludge-free incubations were used to assess the stability of the basal medium at 55ºC and contained RR2, AQDS and co-substrate. The results are means of triplicate bottles and the bars indicate the standard deviation.

Effect of VSS concentration on azo dye reduction at 55ºC

The increase in VSS concentration enhanced the k-value for both AQDS-supplemented and AQDS-free incubations (Figure 3.4). A pseudo-exponential approximation curve fitted well in both cases, in which AQDS stimulated the rate of decolourisation in all cases. Nevertheless, the ratio k_{AQDS} / $k_{AQDS\text{-}free}$ was reduced with the increase in VSS concentration (Figure 3.4). A

pseudo-exponential approximation curve could approximately represent the fall in the ratio k_{AQDS} / $k_{AQDS\text{-}free}$.

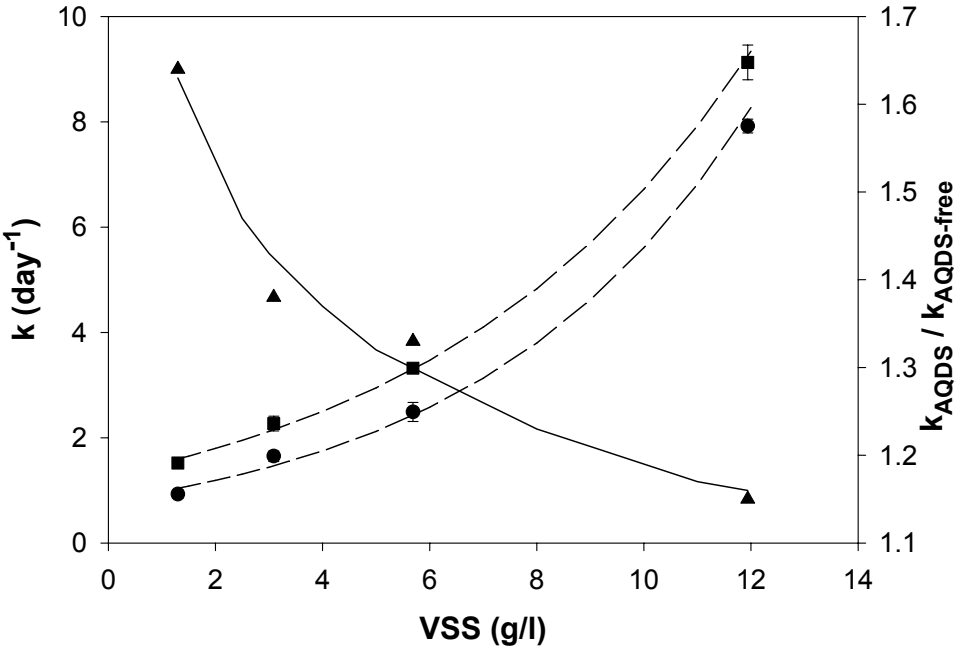

Figure 3.4. First-order rate constant (k, left Y-axis) for different VSS concentrations, in azo dye reduction of RR2 (0.3 mM), either in the presence (■) or absence (●) of AQDS (0.012 mM). The ratio k_{AQDS} / $k_{AQDS\text{-}free}$ decrease (right Y-axis) was also plotted against VSS concentration (▲). The dashed line represents an exponential approximation and the continuous line a pseudo-exponential fall. Co-substrate (2 gCOD/l) was present in all bottles. The results are means of triplicate bottles and the bars indicate the standard deviation.

Initial generation of reducing equivalents at 55ºC

Sulphate is a common pollutant in textile wastewaters, and it can readily be reduced to sulphide by sulphate reducing bacteria. The biogenic sulphide may play an important role as a reducer of azo dyes (**Chapter 2**), thus contributing to the decolourising processes. Therefore, an experiment was conducted to assess the impact of different sulphide concentrations on the reduction of RR2. In a sulphide-free medium, no dye reduction was observed in the absence of sludge, whereas batch assays with living as well as autoclaved sludge achieved total colour removal. AQDS enhanced the reduction of the azo dye RR2 in all cases. In serum bottles with living sludge, no difference in the decolourisation rates for a sulphide gradient up to 0.5 mM was observed (Table 3.1). Nevertheless, for sulphide concentrations above 0.5 mM, there were increases in the decolourisation rates, which were dependent on the sulphide concentration. For instance, bottles with living sludge and 2 mM of sulphide showed a k-value increase of 1.8-fold in the presence of AQDS and 2.0-fold in the absence of AQDS, in comparison with the sulphide-free bottles. For sulphide concentrations above 0.5 mM, a complete colour removal was achieved in the assays free of sludge (Table 3.1). When

sulphide concentrations were ≥ 1 mM, the same decolourisation rates were obtained for both autoclaved sludge and sludge-free bottles. For living sludge bottles with sulphide concentrations up to 2 mM, there was a biological predominance in the process (Figure 3.5). Namely, the k-values obtained with living sludge assays were significantly higher compared to those obtained with autoclaved and sludge-free assays.

Table 3.1. First-order rate constant (k) values (day^{-1}) for batch assays with living sludge, autoclaved sludge and sludge-free control, during azo dye reduction in the presence of sulphide.

Sulphide	Living Sludge		Autoclaved Sludge		Sludge-free Control	
(mM)	+AQDS	-AQDS	+AQDS	-AQDS	+AQDS	-AQDS
0	1.5	0.9	[a]	[a]	[b]	[b]
0.5	1.5	0.9	[a]	[a]	[b]	[b]
1	2.1	1.2	0.8	0.6	0.6	0.4
2	2.7	1.9	1.6	1.4	1.7	1.4
5	6.2	3.9	5.5	3.8	5.4	3.7
10	9.9	7.1	9.8	7.2	10.0	7.2

Co-substrate (2 gCOD/l as a mixture of glucose and VFA, ratio 1:3) was present in all bottles.
[a] Complete colour removal after prolonged periods (>90 days) of incubation.
[b] Incomplete colour removal even after prolonged period of incubation.
The results are means of triplicate bottles and the SD was lower than 10% in all cases.

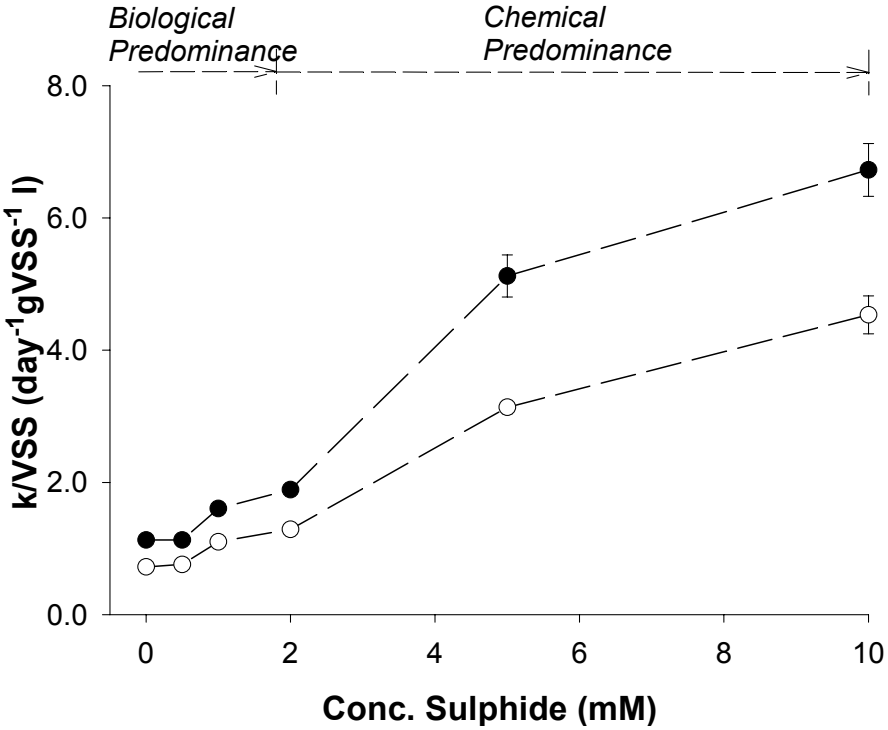

Figure 3.5. First-order rate constant (k) value for azo dye reduction of RR2 (0.3 mM), normalized by the VSS content of living sludge, in the presence of sulphide (variable) and living sludge. Symbols for AQDS-supplemented (●) and AQDS-free (○) bottles. AQDS (0.012 mM) was added and co-substrate (2 gCOD/l) was present in all bottles. The results are means of triplicate incubations and the bars indicate the standard deviation.

Therefore, the reducing equivalents generated via electron donor conversion by living sludge and endogenous respiration were the predominant mechanisms for colour removal. With excess of sulphide (> 2 mM) the k-values for living sludge, autoclaved sludge and sludge-free bottles were almost the same, indicating the prevalence of chemical azo dye reduction in which the reducing equivalents generated via electron donor conversion and endogenous respiration were masked by the reducing equivalents provided by sulphide (Figure 3.5).

Effects of AQDS concentration on the azo dye reduction and co-substrate conversion

Figure 3.6 presents the normalized k-values as a function of the supplemented AQDS concentration. A linear correlation was found for AQDS concentrations up to 0.024 mM. The k-value increased 1.9-fold for the bottles with 0.024 mM of AQDS compared to those lacking this mediator (Figure 3.6). For AQDS concentrations exceeding 1.2 mM, only a small increase on the rate of decolourisation was observed, e.g. 10 mM of AQDS enhanced the k-value 1.1-fold compared to 1.2 mM of AQDS. At incubations with AQDS concentrations of 10 mM, the k-value was 5.9-fold higher than in AQDS-free bottles.

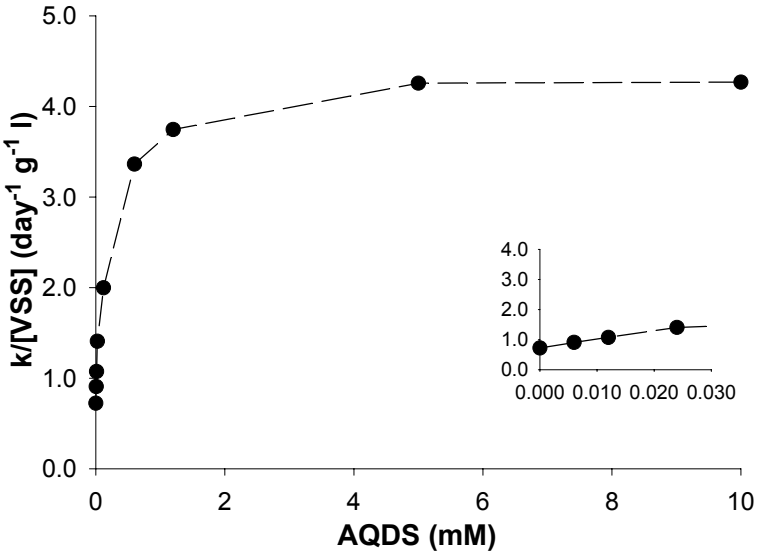

Figure 3.6. First-order rate constant (k) values for azo dye reduction normalized by the VSS content. Different concentrations of AQDS were added to the bottles. Co-substrate (2 gCOD/l) was present in all bottles. The results are means of triplicate incubations.

The use of high concentrations of AQDS however, showed severe inhibition of thermophilic acetate conversion, since acetate accumulated (Figure 3.7A) and methanogenesis concomitantly decreased (Figure 3.7B). Propionate oxidation was also affected by the AQDS

concentration increase, in which the propionate conversion almost ceased at AQDS concentrations exceeding 1.2 mM. On the other hand, glucose fermentation was not affected by high AQDS concentrations as demonstrated by the negligible detection of this substrate at the end of the experiment. After 2 days of experiment, the final co-substrate (glucose-VFA mixture) mass balance presented about a 20% deficit. The glucose fermentation products pyruvate, formate, lactate and alcohols were not detected. Thus, such a deficit was attributed mainly to the high maintenance energy requirements of the inoculum, as well as production of soluble microbial products and non-identified products. AQDS at low concentrations (< 0.024 mM) had no influence in generating reducing compounds and further conversion to methane, via external electron donor conversion (Figure 3.7B). AQDS was just accelerating the shuttle of electrons to the dye (Figure 3.6). For instance, the addition of co-substrate and AQDS (0.024 mM) increased the k-value 1.9-fold compared to the AQDS-free control with co-substrate (Figure 3.6); however, no difference in methane production rate was observed under these conditions (Figure 3.7B).

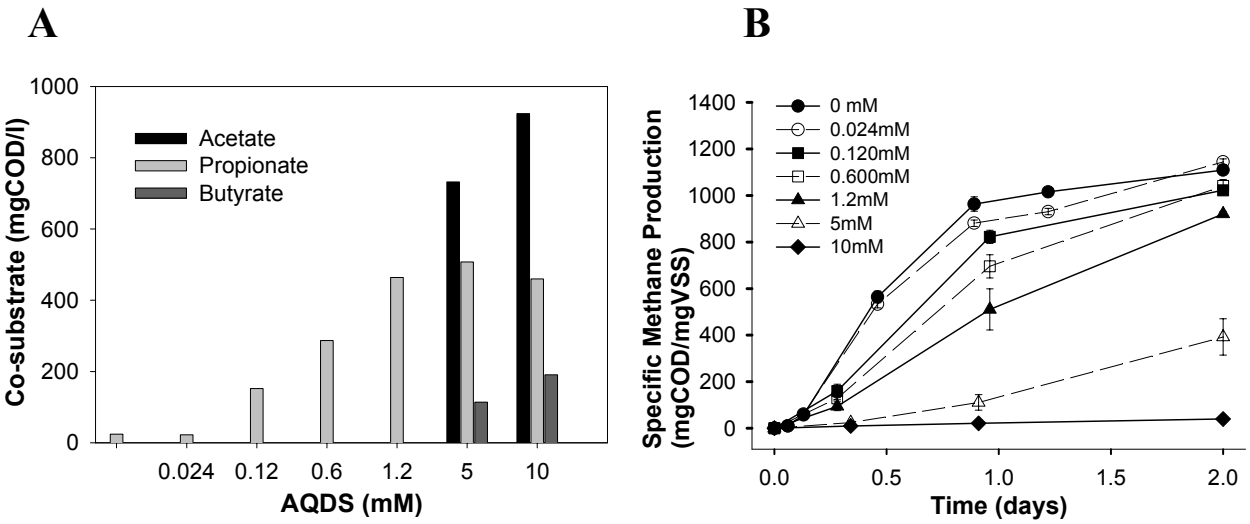

Figure 3.7. (A) Increase on VFA concentrations after 2 days of experiment showing the inhibitory effect of high concentrations of AQDS on acetate and propionate conversion. (B) Concomitant decrease of methanogenesis for high AQDS concentrations. Co-substrate (2 gCOD/l) was present in all bottles. The results are means of triplicate incubations and the bars indicate the standard deviation.

5. Discussion

The aim of this paper was to investigate the feasibility of applying thermophilic anaerobic treatment for reductive decolourisation of coloured wastewaters. Metabolic activities of the thermophilic inoculum used in this study induced a fast reduction of RR2, indicating a biotic predominance in the process. However, addition of high concentrations of sulphide (≥ 5 mM)

revealed the potential contribution of this reducing agent to chemical decolourisation of azo dyes. The supplemented co-substrate, acting as a primary electron donor, is important not only for improving the colour removal rates but also for supplying the high maintenance requirements of the thermophilic microorganisms. Biological treatment at 55ºC shows a fast initial generation of reducing compounds via electron donor conversion, which we verified by comparing the k-values of batch assays free of sulphide with incubations containing 2 mM of sulphide in the presence of granular sludge. The pseudo-exponential increase at 55ºC on the decolourisation rates by increasing VSS concentration is another example of the fast initial generation and/or transfer of reducing compounds via electron donor conversion at 55ºC. In addition, the more negative redox potential values obtained at 55ºC compared to those at 30ºC confirm a better reduction capacity at 55ºC. As a result of the afore-mentioned factors, sixfold and twofold increases on the decolourisation rates were verified in the absence and presence of AQDS, respectively, between thermophilic (55ºC) and mesophilic (30ºC) conditions using the same sludge source. Therefore, most likely the transfer of reducing equivalents at 30ºC was the rate-limiting step.

AQDS (0.012 mM) in the presence of co-substrate increased the reductive decolourisation of RR2. However, there is no effect on the observed k-values of the endogenous controls. This suggests that the electron donor conversion coupled to electron transfer through AQDS was required for the enhanced decolourisation. Moreover, the impact of AQDS as a redox mediator at high VSS concentrations was less evident than at low VSS concentrations. Based on these results, it was suggested that intracellular components with mediating properties were released to the medium by the addition of biomass. In general, compared to mesophilic microorganisms, thermophilic microorganisms present higher metabolic rates (2-3 times), maintenance energy requirements and sludge turnover rates (Van Lier et al., 1993b). Due to the expected higher sludge turnover rates of thermophilic microorganisms compared to mesophilic microorganisms, it is expected that the concentration of reducing and mediating compounds in the reactor bulk would be higher. Keck et al. (2002) reported that during aerobic degradation of naphthalene-2-sulfonate (2NS) at 30ºC for *Sphingomonas xenophaga* strain BN6, redox mediators were produced that increased the efficiency of the strain to reduce azo dyes anaerobically. Furthermore, Van der Zee et al. (2003a) showed that riboflavin, present in flavin coenzymes, had a superior capacity as a redox mediator compared to AQDS during anaerobic reduction of Acid Orange 7 at 30ºC in the presence of sulphide. This reducing/mediating effect seems to be more evident at 55ºC than at 30ºC based on the higher k-values obtained under thermophilic conditions. Probably such an effect is due to the higher sludge turnover rate and cell lysis at 55ºC than at 30ºC, in

which reducing/mediating compounds, previously inactive due to inability to cross the cell membrane, can actively participate in the azo dye reduction. In addition, cell lysis and sludge disruption can provide the medium with reducing agents, e.g. sulphide, iron and other reduced cofactors which may reduce azo dyes chemically (Yoo et al., 2000).

The negligible increase on colour removal at high AQDS concentrations is in accordance with previous studies using redox mediators to accelerate azo dye reduction (Keck et al., 1997; Kudlich et al., 1997; Rau et al., 2002a). As with high AQDS concentrations there was an inhibitory effect on acetate and propionate conversion (Figure 3.7A), and methanogenesis (Figure 3.7B), the enzymatic generation of hydroquinone probably was the rate-limiting step. Apparently, the consortium used in the current experiment had a poor AQDS reducing capacity with acetate and propionate as electron donors. This is in accordance with the fact that up to date literature has reported only a few microorganisms that can couple the conversion of acetate or propionate to quinone reduction under thermophilic or hyperthermophilic conditions (Kashefi et al., 2002). On the other hand, acetate-utilizing and propionate-oxidizing quinone-reducing microorganisms are more abundant under mesophilic conditions (Field et al., 2000). Hydrogen-oxidizing rather than acetate-utilizing bacteria seem to be more actively involved in quinone-respiration under thermophilic or hyperthermophilic conditions (Field et al., 2000; Lovley et al., 2000). Considering the increasing role of H_2 at high temperatures (Van Lier et al., 1993a; Stams, 1994), thermophilic anaerobic treatment seems to be advantageous for hydroquinone generation when redox mediators are involved. Saturation kinetics could also explain the negligible increase on colour removal for high AQDS concentrations. Field and Brady (2003) used riboflavin as a redox mediator at 30ºC for the reduction of Mordant Yellow 10. They reported that there was a small variation in the k-values for riboflavin concentrations higher than 0.055 mM, which they attributed to saturation kinetics of the enzymatic reduction of riboflavin. Nevertheless, in the present experiment at 55ºC, the generation of hydroquinone rather than saturation kinetics of AQDS seems to explain more readily the constant decolourisation rates for the high concentrations of AQDS tested. The later is in accordance with Rau et al. (2002a) who demonstrated at 30ºC that the reduction of anthraquinone-2-sulfonate to the hydroquinone form by *Sphingomonas xenophaga* strain BN6 was the rate-limiting step in the reduction of the azo dye Amaranth.

Acknowledgements

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4

Effect of redox mediator, AQDS, on the decolourisation of a reactive azo dye containing triazine group in a thermophilic anaerobic EGSB reactor

Abstract

The feasibility of thermophilic (55°C) anaerobic treatment applied to colour removal of a triazine contained reactive azo dye was investigated in two 0.53 l expanded granular sludge blanket (EGSB) reactors in parallel at a hydraulic retention time (HRT) of 10 h. Generally, this group of azo dyes shows the lowest decolourisation rates during mesophilic anaerobic treatment. The impact of the redox mediator addition on colour removal rates was also evaluated. Reactive Red 2 (RR2) and anthraquinone-2,6-disulfonate (AQDS) were selected as model compounds for azo dye and redox mediator, respectively. The reactors achieved excellent colour removal efficiencies with a high stability, even when high loading rates of RR2 were applied (2.7 gRR2 l^{-1} per day). Although AQDS addition at catalytic concentrations improved the decolourisation rates, the impact of AQDS on colour removal was less apparent than expected. Results show that the AQDS-free reactor R2 achieved excellent colour removal rates with efficiencies around 91%, compared with the efficiencies around 95% for the AQDS-supplemented reactor R1. Batch experiments confirmed that the decolourisation rates were co-substrate dependent, in which the volatile fatty acids (VFA) mixture was the least efficient electron donor. The highest decolourisation rate was achieved in the presence of either hydrogen or formate, although the presence of glucose had a significant effect.

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1. Introduction

Almost one million tons of dyes are annually produced in the world (Cao et al., 1999), of which azo dyes, characterised by an azo-bond (R_1–N═N–R_2), represent about 70% by weight (Zollinger, 1987). Reactive azo dyes, i.e. dyes with reactive groups that form covalent bonds with OH–, NH–, or SH– groups, are extensively used in the textile industry, despite the fact that they have a low degree of fixation into the fibres (efficiency 10–50%). Reactive azo dyes are highly water-soluble. Therefore, wastewater treatment processes such as activated sludge systems or by using coagulation methods, may not be efficient to treat them (Hao et al., 2000).

Biological treatment applied to azo dye removal has been extensively researched. Under aerobic conditions low colour removal efficiencies are achieved (10–30%), because oxygen is a more effective electron acceptor, therefore having a greater preference for reducing equivalents than azo dyes (Stolz, 2001). In contrast, anaerobic treatment generally gives good decolourisation efficiencies (Tan et al., 2000; Van der Zee et al., 2001a; Cervantes et al., 2001). Since textile industry wastewaters are generally discharged at high temperatures (40–70°C), thermophilic anaerobic treatment could serve as an interesting option, especially when closing process water cycles is considered.

Some reactive azo dyes containing triazine as reactive group presented the lowest decolourisation rates during mesophilic anaerobic treatment (Van der Zee et al., 2001b). The required high HRT in the reactors could be lowered by using quinone compounds to accelerate the electron transfer from the microorganisms to the dye (Van der Zee et al., 2001a; Cervantes et al., 2001). Quinones are the electron accepting moieties of humic substances. Such compounds have been shown to play an important role not only as final electron acceptor for many recalcitrant organic compounds, but also in facilitating electron transfer from an electron donor to an electron acceptor, e.g. azo dyes (Cervantes et al., 2000; Field et al., 2000). The first step is the non-specific enzymatic reduction of quinone to hydroquinone, and the second step is the chemical reoxidation of hydroquinone by the azo dye (Keck et al., 1997). The redox potential prevailing in the medium is an important factor determining the rate of electron transfer when redox mediators are involved (Rau et al., 2002a). Either the mediator reduction or its regeneration can be the rate-limiting steps. Therefore, compared to mesophilic anaerobic treatment, thermophilic anaerobic treatment seems to be advantageous not only for the expected faster enzymatic reduction of the mediator, but also for its chemical regeneration.

The main objective of this paper is to evaluate the reductive decolourisation of an azo dye containing triazine as reactive group in a continuous flow EGSB reactor at 55°C. The

impact of AQDS as a redox mediator on the decolourisation rates is also studied. Co-substrate and temperature dependency are researched by using standardised activity tests.

2. Materials and methods

2.1. Chemicals

Reactive Red 2 (RR2) was selected as model compound in this study (Procion Red MX-5B, ~50% of purity) (Aldrich, Gillingham, UK). RR2 was used without additional purification. Prior to utilisation, RR2 was hydrolysed as described in Chapter 3 (Figure 3.1).

Anthraquinone-2,6-disulfonate (AQDS) (Aldrich) was used as redox mediator model compound, without additional purification. The molecular structure of AQDS is shown in Chapter 3 (Figure 3.2).

2.2. Batch and continuous experiments

Seed inoculum and basal medium for decolourisation assays

Granular anaerobic sludge was collected from a full-scale mesophilic upflow anaerobic sludge blanket (UASB) reactor treating paper mill wastewater (Eerbeek, The Netherlands). The mesophilic sludge was converted into a thermophilic sludge as described in Chapter 3. The glucose–VFA mixture simulates the organic compounds normally present in textile wastewaters. The influent stock solution was free of dye and AQDS during the whole period.

The basal medium consisted of (mg/l): NH_4Cl (280), K_2HPO_4 (250), $MgSO_4 \cdot 7H_2O$ (100) and $CaCl_2 \cdot 2H_2O$ (10) and 1 ml/l of trace elements containing (mg/l): H_3BO_3 (50), $FeCl_2 \cdot 4H_2O$ (2000), $ZnCl_2$ (50), $MnCl_2 \cdot 4H_2O$ (500), $CuCl_2 \cdot 2H_2O$ (38), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (50), $AlCl_3 \cdot 6H_2O$ (90), $CoCl_2 \cdot 6H_2O$ (2000), $NiCl_2 \cdot 6H_2O$ (92), $Na_2SeO_3 \cdot 5H_2O$ (162), EDTA (1000) and HCl 36% (1). The medium was buffered with 6.21 g/l sodium bicarbonate to keep the pH around 7.1. Resazurin was not included in the trace elements solution due to its mediating properties.

Batch experiments

Activity tests

In the activity tests 1.3 ± 0.1 gVSS/l of the previously described stabilized sludge were added to 117 ml serum bottles with 50 ml basal medium. The bottles were then sealed with butyl rubber stoppers and aluminium crimp caps. Anaerobic conditions were established by flushing

the headspace with N_2/CO_2 (70%:30%), and 2 gCOD/l co-substrate (variable) and RR2 (0.3 mM) were added. To some of the bottles AQDS (variable) was supplemented to control the impact of an external redox mediator. Sludge-free and autoclaved sludge were used as a control for abiotic colour removal. The incubations were conducted under non-static conditions by applying 50 shakes per minute. Sterile controls were autoclaved once at 122ºC for 240 min and again following a 5 days incubation period, after which sterile co-substrate, mediator and dye stock solutions were added. The pH and the amount of VSS were determined after completion of the experiment.

Effect of different co-substrates

VFA mixtures of acetate:propionate:butyrate (1:1:1) on a COD basis, glucose–VFA mixtures (1:3) on a COD basis, glucose, and H_2/CO_2 (80%:20%) were used as co-substrates (2 gCOD/l), during azo dye reduction at 55°C either in the presence or absence of AQDS (12 μM). When H_2/CO_2 was used as an electron donor, the shaker rotation was increased from 50 to 100 shakes per minute to facilitate the hydrogen transfer to the liquid phase. The incubation with endogenous substrate was used to control the effect of electron donor addition.

Effect of temperature on thermophilic range

The incubation temperatures (°C) were 45, 55, 60, 65 and 75, for the serum bottles with both living and autoclaved sludge. The co-substrates used were VFA mixture of acetate:propionate:butyrate (1:1:1), and glucose–VFA mixture (1:3), either in the presence or absence of AQDS (120 μM).

Continuous Experiment

Stock solution

The 10 l influent stock solution was composed of glucose–VFA mixture at a COD ratio of 1:3 as co-substrate, azo dye and demineralised water. The stock solution of reactor R1 also contained AQDS. Different concentrations of co-substrate, RR2 and AQDS were added to the medium (Table 4.1). To prevent acidification of the reactor medium, the medium was buffered with sodium bicarbonate, i.e. 1 g $NaHCO_3$ for 1 g COD–glucose. The VFA stock solution at a final concentration of 150 gCOD/l was neutralised with NaOH. The influent

stock solution had a final pH adjustment to 7 by adding some drops of NaOH or HCl. The influent stock solutions and basal media were stored at 4°C.

Reactor

Two 0.53 l EGSB reactors were operated in parallel, in which reactor R1 was AQDS-supplemented and reactor R2 was AQDS-free, i.e. control. The reactors were seeded to a final concentration of 30 gVSS/l. The reactors were operated at an HRT of 10 h and an up-flow velocity of 4 m/h was imposed by recirculating the flow with peristaltic pumps (505S, Watson–Marlow, Cornwall, UK). The reactors were operated at 55°C using a thermostat bath. Biogas was collected in a gas–solid–liquid separator, and afterwards washed in a NaOH (10% w/v) solution with timol blue as indicator. Methane was measured in a Mariotte Flask at 30°C and 1 atm. Figure 4.1 shows a schematic set-up of the continuous flow experiment.

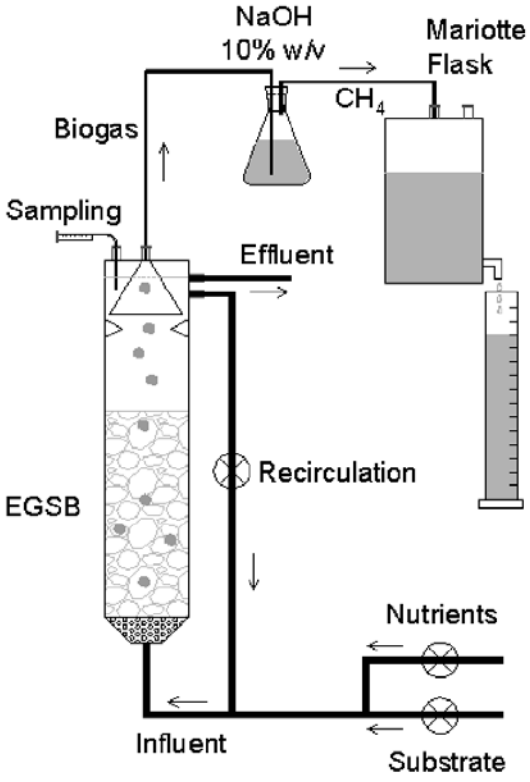

Figure 4.1. Experimental set-up of the EGSB reactor system.

2.3. Analysis

Colour removal was determined photometrically (Spectronics 60, Milton-Roy Analytical Products Division, Belgium) as described in Chapter 3, reading the absorbance at the maximum absorbance wavelength of RR2, i.e. 539 nm. After hydrolysis 1.72 mM of RR2 was equivalent to 57.2 AU/cm, yielding a molar extinction coefficient of 33.3 AU cm^{-1} mM^{-1}. The daily methane production was measured by using a Mariotte Flask. The conversion factor used was 2.58 gCOD/l CH_4 at 30°C and 1 atm. VFA, methane, methanol, ethanol, sucrose, fructose, glucose, lactate, formate and VSS were measured as described in Chapter 3. Soluble COD was analysed photometrically on a Spectronics 20 Genesis model 4001/4 (Spectronics Instruments, NY) using a micro method according to APHA (1998). Samples were centrifuged for 3 min at 13,000 rpm and absorbance was read at a wavelength of 600 nm.

The redox potential (ORP) was measured using a Sentix ORP 0–100°C combination electrode (platinum—silver/silver chloride) (WTW, Weilheim, Germany), using as electrolyte a KCl solution (3 M). ORP values were corrected to the redox values of reference electrode (+207 mV at 25°C).

3. Results

Batch experiments

Effect of different co-substrates

The addition of co-substrate accelerated the rate of colour removal at 55°C, even though the endogenous control showed reasonable rate (Figure 4.2). During the continuous flow experiment, the impact of co-substrate on the reductive decolourisation was more pronounced, probably owing the fact that the reducing equivalents from endogenous respiration were limited. Results also show different rates for the different electron donors tested, even though a complete decolourisation was achieved in all cases.

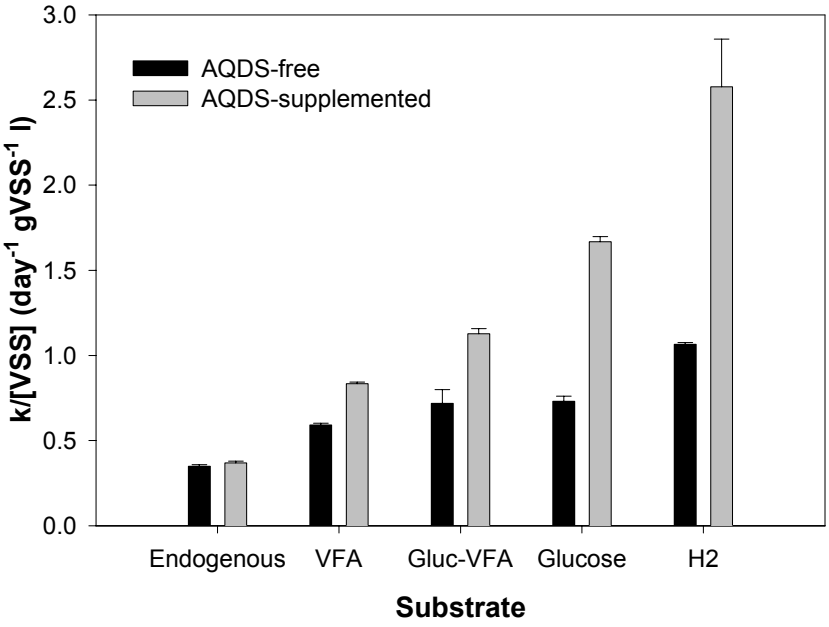

Figure 4.2. First-order rate constant (k) value for colour removal of RR2 (0.3 mM) at 55ºC normalized by the VSS content, in the presence of different co-substrates (2 gCOD/l). AQDS (0.012 mM) was added to some of the bottles. The results are means of triplicate incubations with the standard deviation being indicated by vertical bars.

The highest rate of colour removal was achieved when hydrogen was used as co-substrate for both AQDS-free and AQDS-supplemented medium, whereas the VFA mixture was the least efficient electron donor. Interestingly, in the presence of AQDS, the reductive

decolourisation increased by increasing the glucose concentration, i.e. comparing the incubations when glucose–VFA mixture or glucose solely was used as co-substrate, whereas the rates for both co-substrates were comparable in the absence of AQDS. The rates of colour removal with formate as the electron donor (results not shown) achieved values close to those when hydrogen was tested. This suggests that both interspecies formate and interspecies hydrogen could be involved upon electron transfer in the system. During the course of experiment, the decolourisation was lower than 10% in the autoclaved sludge controls and lower than 1% in the sludge-free controls.

Effect of temperature

The temperature dependency was assessed in the range of 45–75°C, showing a maximum rate of colour removal at 60°C (Figure 4.3). The temperature response at 60°C was similar for both VFA and glucose–VFA mixture, enhancing 1.1-fold the first-order rate constant "k" (day^{-1}), in comparison with incubations at 55 and 65°C. The lowest k values were found for the bioassays at 75°C.

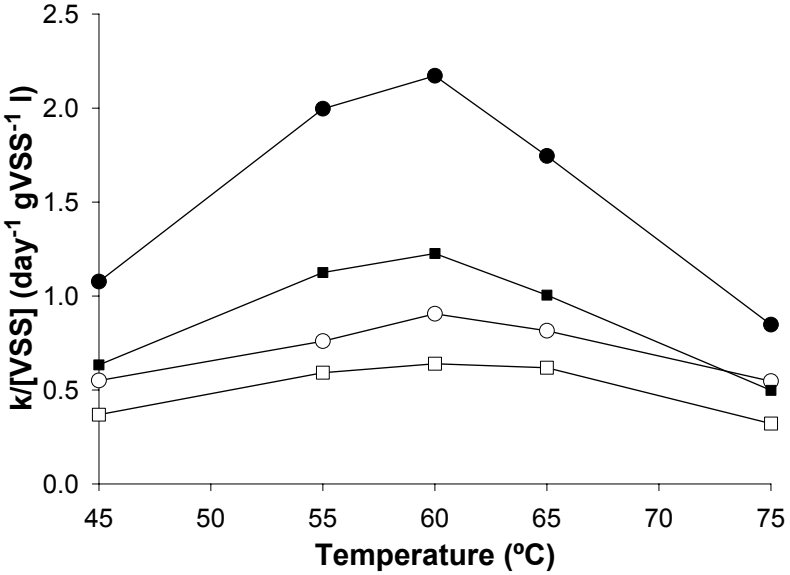

Figure 4.3. First-order rate constant (k) value for colour removal of RR2 (0.3 mM) at different incubation temperatures, normalized by the VSS content: ○ glucose-VFA AQDS-free, ● glucose-VFA AQDS-supplemented, □ VFA AQDS-free, ■ VFA AQDS-supplemented. The co-substrate concentration was 2 gCOD/l. AQDS (0.12 mM) was supplemented to some of the bottles. The results are means of triplicate bottles, in which the standard deviation was lower than 10% in all cases.

Results again show the stimulatory effect of glucose on the decolourisation rates. For instance, at the optimum temperature of 60°C and 120 μM-AQDS, the k value was 1.3-fold higher using a glucose–VFA mixture as electron donor compared to the vials supplemented

with solely VFA mixture. AQDS stimulated reductive decolourisation in all cases and its effect on the decolourisation rates varied with the electron donor and temperature tested. For instance, for the AQDS-supplemented bottles at 60°C with glucose–VFA mixture as the electron donor, the k value enhanced 2.4-fold compared to those lacking AQDS. Doing the same comparison but considering the VFA mixture, the k value increased 1.9-fold. During the course of the experiment, the decolourisation was lower than 10% in the autoclaved sludge controls and lower than 1% in the sludge-free controls.

Continuous experiment

The operating data of the continuous flow EGSB experiments are presented in Table 4.1.

Colour removal

Both reactors were started and stabilised with a dye-free and AQDS-free medium (period I). In period II, RR2 (0.10 g/l) was introduced in both reactors media at sub-stoichiometric concentration, i.e. in a low concentration to prevent some toxicity during the start-up, and AQDS (6 μM) was supplemented to reactor R1. Results reveal a similar performance of reactors R1 (Figure 4.4A) and R2 (Figure 4.4B), probably due to the initial adsorption of RR2 in the sludge bed. In period III, the RR2 concentration was increased to 0.17 g/l to exhaust the adsorption capacity of the sludge bed, which was likely reached around 30 days, i.e. 25 days after RR2 was introduced in the medium. At the same period the colour removal in R2 started to drop. At the beginning of period IV, the efficiency of R2 has dropped to 87 ± 0.7%, and was kept around this value until day 54. For the reactor R1 however, the efficiency values were the same as in period III. In period V, the loading rate of co-substrate and RR2 was doubled, which resulted in an increase in colour removal efficiency for both reactors. In this period, the ratio co-substrate/dye was not changed. In the following periods co-substrate/azo dye ratio was decreased from 7.4 to 3.6 gCOD/gRR2 (period VI) and 1.8 gCOD/gRR2 (period VII), limiting the availability of reducing equivalents. It was observed that the reduced ratios affected the colour removal efficiency in R2. In period VIII, a further decrease in the ratio co-substrate/azo dye to 0.9 gCOD/gRR2 was imposed. In contrast to R1, reactor R2 responded with a significant drop in the decolourisation to 86 ± 0.5%. In period IX, both reactors were refrained from co-substrate, which resulted in a sharp decrease in the colour removal efficiency values in both reactors to about 30% (Figures 4.4A and 4.4B). Once the feeding with co-substrate was resumed to the loading rate of 2.5 gCOD l^{-1} day^{-1} (period X), the reactor R1 reached the same colour removal efficiency observed during period VIII and in reactor R2 the efficiency values increased to higher values to those of period VIII (Figure 4.4B).

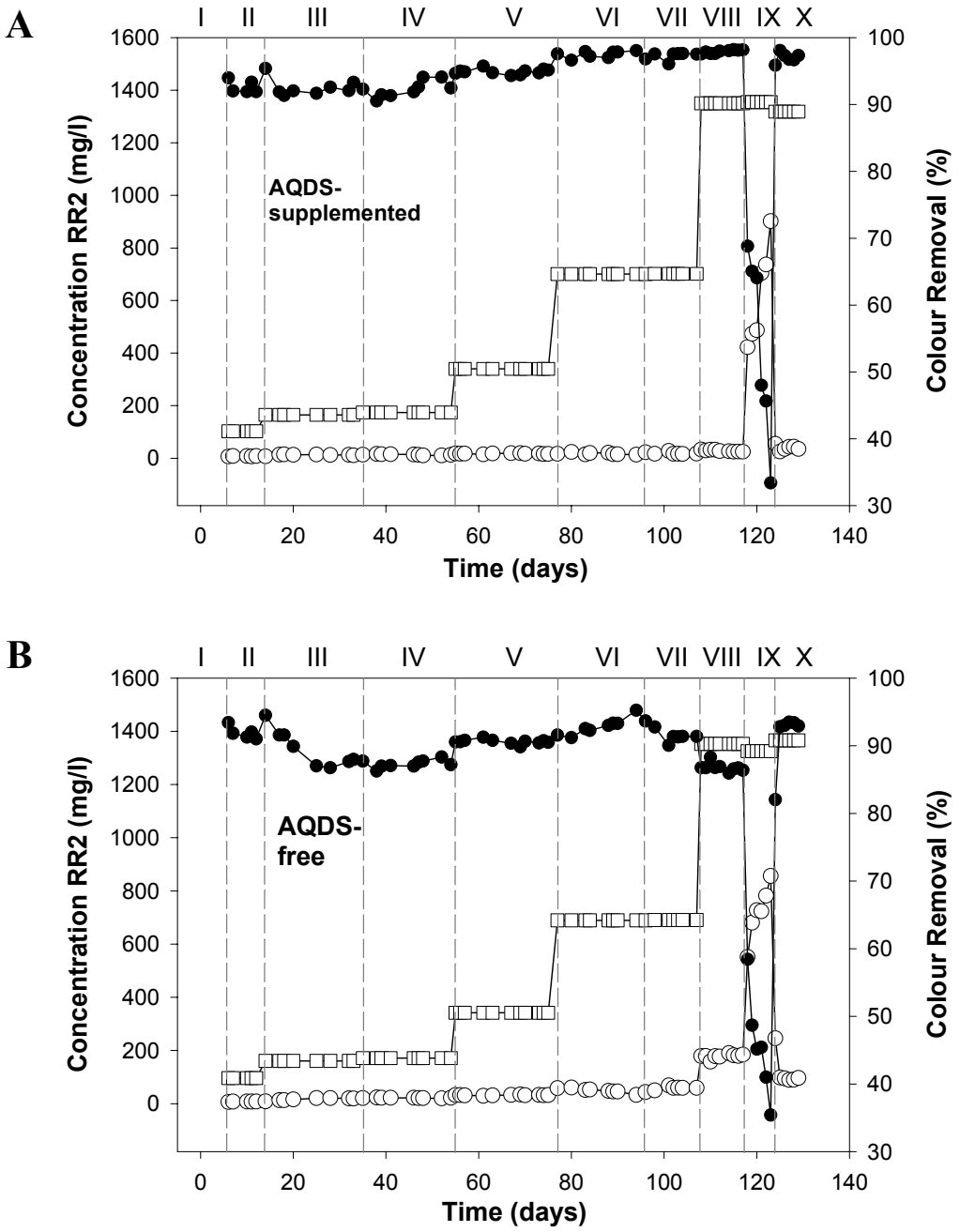

Figure 4.4. Performance of the continuous flow EGSB reactors at 55ºC indicating the concentration of RR2 on the left Y axis and the colour removal on the right Y axis, (A) reactor R1 AQDS-supplemented and (B) reactor R2 AQDS-free (control). Symbols for: influent RR2 concentration (□), effluent RR2 concentration (○), colour removal efficiency (●). - - - - - corresponds to the different periods of the experiment (Table 4.1).

Addition of AQDS resulted in a different colour fixation in the sludge bed. For the AQDS-supplemented reactor R1, almost no red colour was attached to the granules, which contrasted the strongly red-coloured sludge bed of the AQDS-free reactor R2. The latter sludge was apparently reversibly coloured since all colour easily disappeared after washing the granules.

Co-substrate removal and methane production

The system efficiency for co-substrate removal was constantly evaluated in terms of soluble COD. In addition, the products formed from glucose fermentation and VFA conversion, were also monitored, but in a lower frequency. The products of glucose pyruvate, formate, lactate and alcohols were not detected in the effluent, but only traces of VFA, i.e. acetate (< 5 mgCOD/l) and butyrate (< 19 mgCOD/l). Propionate accumulation was not observed. The COD influent was composed of co-substrate and RR2, while the COD effluent contained traces of co-substrate, RR2 not removed and aromatic amines. The decrease in COD removal (Figure 4.5) can be attributed to the increase in RR2 and aromatic amines (mainly aniline) concentrations in the effluent. Interestingly, the efficiency of the thermophilic bioreactors in terms of colour removal and complete electron donor conversion to methane was never affected by the applied dye-loading rate.

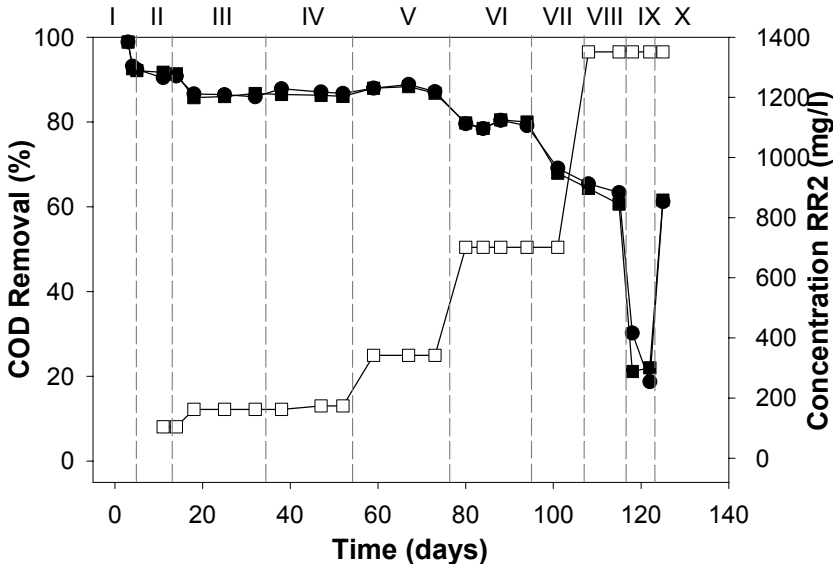

Figure 4.5. Performance of the continuous flow EGSB reactors at 55ºC in terms of COD removal. Symbols for: COD-efficiency of R1 (■), COD-efficiency of R2 (●), RR2 concentrations (□). Dashed lines correspond to the different periods of the experiment (Table 4.1).

Aromatic amines are known to be extremely toxic to methanogens even in small concentrations. Nonetheless, during the continuous flow experiments even when a high loading rate of RR2 was applied, the methanogenesis remained stable. The aromatic amine aniline, which was detected as product of azo-bond cleavage (results not shown), was probably not converted since it is known that aniline is a rather stable compound anaerobically (Razo-Flores et al., 1997).

Table 4.1. Operational data of the continuous flow EGSB experiments at 55ºC. R1 and R2 are the reactor AQDS-supplemented and AQDS-free, respectively.

Operational Parameters of the Reactors										
Period	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**
End of period (days)	**5**	**14**	**35**	**54**	**76**	**96**	**107**	**117**	**123**	**129**
Co-substrate (gCOD/l)	1.25	1.25	1.25	1.25	2.50	2.50	1.25	1.25	-	1.25
RR2 (g/l)	-	0.10	0.17	0.17	0.34	0.70	0.70	1.35	1.35	1.35
OLR sub. (gCOD l^{-1} day^{-1})	2.5	2.5	2.5	2.5	5.0	5.0	2.5	2.5	-	2.5
OLR RR2 (g l^{-1} day^{-1})	-	0.2	0.3	0.3	0.7	1.4	1.4	2.7	2.7	2.7
Co-sub./azo dye (gCOD/gRR2)	-	12.5	7.4	7.4	7.4	3.6	1.8	0.9	-	0.9
AQDS (μM) R1	-	6	6	24	24	24	24	24	24	24
Performance of the Reactors										
COD removal R1(%)	96 (4.4)	92 (0.4)	86 (0.5)	86 (0.2)	88 (0.8)	80 (0.8)	60 (11.8)	63 (2.6)	21 (0.9)	62 (0.0)
COD removal R2 (%)	96 (4.0)	91 (1.0)	86 (0.4)	87 (0.6)	88 (0.8)	79 (0.8)	62 (10.1)	64 (2.5)	29 (10.2)	61 (0.0)
Colour removal R1 (%)	-	93 (1.0)	93 (1.3)	92 (1.2)	93 (0.4)	97 (0.5)	97 (0.6)	98 (0.2)	54 (13.9)	97 (0.8)
Colour removal R2 (%)	-	92 (1.2)	90 (1.2)	87 (0.7)	91 (0.3)	93 (1.2)	91 (0.9)	87 (0.6)	46 (7.7)	91 (4.5)
Methane R1 (gCOD/l)	1.0 (0.0)	0.8 (0.1)	0.9 (0.1)	0.9 (0.1)	1.7 (0.1)	1.7 (0.1)	0.9 (0.1)	0.9 (0.1)	0.2 (0.0)	0.8 (0.1)
Methane R2 (gCOD/l)	1.0 (0.0)	0.7 (0.1)	0.8 (0.1)	0.8 (0.1)	1.7 (0.1)	1.7 (0.1)	0.8 (0.1)	1.0 (0.1)	0.1 (0.0)	0.7 (0.1)
ORP R1 (mV)	-248	-	-	-	-	-	-	-215	-53	-210
ORP R2 (mV)	-248	-	-	-	-	-	-	-205	-53	-203

OLR is the abbreviation of organic loading rate. Co-substrate, Reactive Red 2 (RR2) and anthraquinone-2,6-disulfonate (AQDS) concentrations in the influent. 1 gRR2/l is about 1.1 gCOD/l. Redox potential (ORP) values were corrected to the redox values of reference electrode (+207 mV at 25ºC). The standard deviation is shown in parenthesis.

4. Discussion

The results of the continuous flow experiments clearly show the potentials of thermophilic anaerobic treatment on the reductive decolourisation of an azo dye containing triazine as the reactive group. The results also show that the rates of colour removal improve by using catalytic concentrations of the redox mediator AQDS. The reducing equivalents generated after co-substrate conversion biologically reduce AQDS to the hydroquinone form, AH_2QDS, with consequent chemical reoxidation of hydroquinone by the azo dye (Field et al., 2000). So far, there are only a few publications on anaerobic azo dye reduction at high temperatures. Furthermore, differences in type and loading rates of azo dye, co-substrate and redox mediator, as well as the temperature and sludge concentration in the reactor hamper a sound comparison with previously reported results under mesophilic conditions.

The EGSB reactors achieved excellent colour removal efficiencies with a high stability even when high loading rates of azo dye were applied (2.7 gRR2 l^{-1} day^{-1}). However, the impact of AQDS on this reductive decolourisation was less apparent than expected, based on the results achieved under mesophilic conditions. Long-term experiments show that the AQDS-free reactor R2 achieved excellent colour removal rates with efficiencies around 91%, in comparison with the efficiencies around 95% (Table 4.1) for the AQDS-supplemented reactor R1. This was in contrast to the findings of Cervantes et al. (2001), who reported a considerable impact of AQDS on the anaerobic colour removal at 30°C, using Acid Orange 7 as azo dye model compound. When AQDS was absent, the efficiency values were around 86%, but increased to about 99% after 30 μM of AQDS was introduced in the system. The stability of the system in our experiment, in terms of colour removal and complete co-substrate conversion to methane, was also in contrast to the results of Van der Zee et al. (2001a). They reported a decrease in the decolourisation efficiencies to around 25% after 40 days of feeding with non-hydrolysed RR2 in the absence of AQDS, during anaerobic treatment at 30°C. Furthermore, the co-substrate (VFA) removal efficiencies dropped to around 10%. Nevertheless, the system capacity for colour removal and co-substrate conversion was recovered after the reactor was supplemented with 19 μM of AQDS. In our experiments, no toxicity effects were observed, which might be attributed to the following differences with the above-cited work. Firstly, Van der Zee et al. (2001a) used non-hydrolysed RR2 in their experiments (Figure 3.1), whereas we used hydrolysed RR2. The toxicity effect of the former RR2 could be more pronounced as indicated by the results of Chung and Stevens (1993) who reported microbial respiration inhibition due to the presence

of chloride or bromide as functional groups. The azo dye concentrations in and around the biofilm could be lower under thermophilic conditions, owing to the fact that at 55°C the decolourisation rates are higher compared to those at 30°C. As a result, a much lower fraction of the sludge is exposed to the toxic concentrations of RR2. Azo dyes are known to be more toxic to methanogens than their cleavage products, i.e. aromatic amines (Donlon et al., 1997).

The decolourisation rate showed to be co-substrate dependent, in which the VFA mixture was the least efficient electron donor. The highest decolourisation rate was achieved in the presence of hydrogen. Also glucose has a significant impact on the colour removal rates, probably because of the concentrations of H_2 (or formate) that are likely formed in glucose fermentation, i.e. maximum of 4 mol H_2 per mol glucose. The findings are similar to Donlon et al. (1996) who reported that interspecies hydrogen resulting from the oxidation of substrates such as butyrate, propionate, and ethanol, could provide the medium with reducing equivalents, and stimulation of the nitrophenol reduction. In the latter work, the direct methane precursors acetate and methanol did not stimulate the nitrophenol reduction rates.

AQDS has a clear effect on the reductive decolourisation of RR2 either when glucose–VFA mixture or glucose solely is used as an electron donor, whereas the rates for both co-substrates were similar in the absence of AQDS. This is an indication that AQDS was facilitating the electron transfer, most probably interspecies hydrogen or interspecies formate that is generated via glucose fermentation, to the reductive cleavage of the azo bond. The redox couple AQDS/AH_2QDS (reduction potential E_0' equal to -0.18 V) is thermodynamically more favourable than the redox couple CO_2/CH_4 (E_0' equal to -0.24 V) indicating the preference of AQDS as an electron acceptor (Cervantes et al., 2000; Cervantes, 2002). Recently, a NADH-dependent lawsone reductase activity located in the cytosolic fraction of *Escherichia coli* also showed the capacity for azo dye reduction (Rau and Stolz, 2003). Hydrogen-oxidising rather than acetate-utilising microorganisms seem to be more actively involved in quinone-respiration under thermophilic and hyperthermophilic conditions (Field et al., 2000). Considering the increasing role of H_2 (or formate) at high temperatures (Van Lier et al., 1993a; Stams, 1994) and their effectiveness as electron donor for dye reduction, thermophilic anaerobic treatment seems to be advantageous either on the direct reductive decolourisation or on the hydroquinone generation, which is sometimes the rate-limiting step, when quinone-based redox mediators are used to accelerate azo dye reduction (Rau et al., 2002a).

The highest decolourisation rate at 60°C agrees with the generally observed optimum temperature for thermophilic methanogenic consortia (Van Lier et al., 1996), and the resulting

highest production of reducing equivalents. During continuous flow experiment however, it was decided to operate the reactors at 55°C following the recommendations of Ahring (1995) and Van Lier et al. (1993b) who reported a dramatic decrease in the VFA oxidising populations (particularly C_3 oxidisers) of temperatures higher than 55°C.

The promising results achieved in this study, suggest good prospects for the application of thermophilic anaerobic treatment for treating reactive azo dyes, in which high efficiency values on colour removal were obtained either in the absence or presence of AQDS at catalytic concentrations.

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5

Effect of different redox mediators during thermophilic azo dye reduction by anaerobic granular sludge and comparative study between mesophilic (30ºC) and thermophilic (55ºC) treatments for decolourisation of textile wastewaters

Abstract

The impact of different redox mediators on colour removal of azo dye model compounds and textile wastewater by thermophilic anaerobic granular sludge (55ºC) was investigated in batch assays. Additionally, a comparative study between mesophilic (30ºC) and thermophilic (55ºC) colour removal was performed with textile wastewater, either in the presence or absence of a redox mediator. The present work clearly evidences the advantage of colour removal at 55ºC compared to 30ºC when dealing with azo coloured wastewaters. The impact of the redox mediators anthraquinone-2,6-disulfonate (AQDS), anthraquinone-2-sulfonate (AQS) and riboflavin was evident with all dyes, increasing decolourisation rates up to 8-fold compared to the mediator-free incubations. The generation of the hydroquinone form AH_2QDS, i.e. the reduced form of AQDS, was extremely accelerated at 55ºC compared to 30ºC. Furthermore, no lag-phase was observed at 55ºC. Based on the present results we postulate that the production/transfer of reducing equivalents was the rate-limiting step, which was accelerated by the temperature increase. It is conclusively stated that 55ºC is a more effective temperature for azo dye reduction than 30ºC, which is a result of the improvement of both biotic and abiotic mechanisms involved in this reductive transformation.

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1. Introduction

Dyes make the world more beautiful through coloured substances, but on the other hand they represent a serious pollution problem for the environment. Almost one million tons of dyes are annually produced in the world, of which azo dyes, characterized by an azo-bond (R_1–N=N–R_2), represent about 70% by weight (Hao et al., 2000). Azo dyes are the most common synthetic colourants released to the environment via textile, pharmaceutical and chemical industries (Chung et al., 1992). The discharge of azo dyes in water bodies is problematic not only for aesthetic reasons, but also because azo dyes and their cleavage products (aromatic amines) are carcinogenic (Brown and DeVito, 1993; Weisburger, 2002).

The biological treatment of azo dye wastewaters has been extensively researched. Under aerobic conditions low colour removal efficiencies are achieved because oxygen is a more effective electron acceptor, therefore having more preference for electrons than azo dyes (Stolz, 2001). In contrast, anaerobic treatment generally gives good colour removal efficiencies (Van der Zee et al., 2001b; Cervantes et al., 2001; Dos Santos et al., 2004c). The anaerobic microorganisms not only generate the electrons to cleave the azo bond, but also maintain the low redox potential (< -50 mV) which is required for the transfer of reducing equivalents to the dye molecule (Beydilli et al., 1998; Bromley-Challenor et al., 2000). Although a complete mineralization cannot generally be reached anaerobically, the reductive transformation increases the susceptibility of the aromatic molecule to oxygenases attack (Field et al., 1995). Therefore, for most of the azo dyes, a sequenced anaerobic/aerobic treatment is suggested not only for the reduction of the azo dye but also for the mineralization of its cleavage products (Tan et al., 2000).

The transfer of reducing equivalents from a primary electron donor (co-substrate) to a terminal electron acceptor (azo dye) generally acts as the rate-limiting step in anaerobic azo dye reduction (Van der Zee et al., 2003b). However, the addition of redox mediators has been shown to accelerate this electron transfer (Kudlich et al., 1997; Keck et al., 2002), and higher decolourisation rates can be achieved in bioreactors operated with a low hydraulic retention time (HRT) (Cervantes et al., 2001; Dos Santos et al., 2003).

Flavin-based compounds, such as flavin adenine dinucleotide (FAD), flavin adenine mononucleotide (FMN) and riboflavin as well as quinone-based compounds such as AQS, AQDS and lawsone have been extensively reported as redox mediators (Semdé et al., 1998; Cervantes et al., 2000; Rau et al., 2002a). In this case, non-specific enzymes biologically reduce the mediator, as the direct electron acceptor of the primary electron donor. Secondly,

the electrons are chemically transferred to the azo dye, the terminal electron acceptor, with consequent mediator regeneration (Figure 5.1).

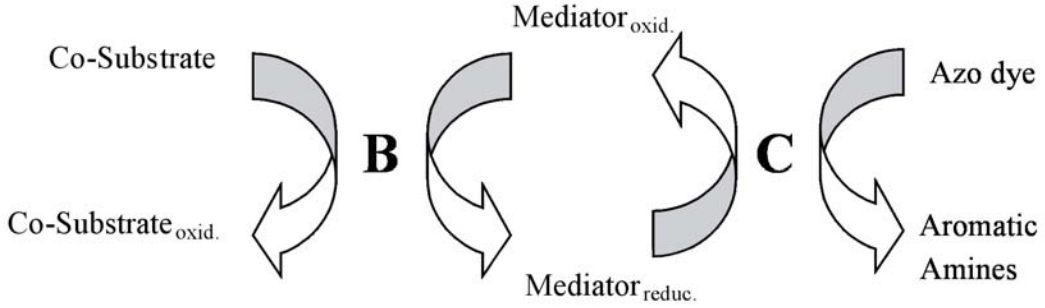

Figure 5.1. Schematic of the biological and chemical steps involved in the azo dye reduction in the presence of redox mediators. Letters B and C are representing the biological enzymatic reaction and the pure chemical reaction, respectively.

The effect of redox mediators on decolourisation rates has generally been investigated with azo model compounds, and their effectiveness in enhancing the decolourisation of textile wastewaters is still unclear due to the wide range of redox potentials among azo dyes (-180 to –430 mV) (Rau and Stolz, 2003). Moreover, a comparative study between mesophilic and thermophilic treatments of textile wastewater in the presence of a redox mediator has never been conducted.

In the current investigation, the impact of different redox mediators upon colour removal of azo dye model compounds and textile wastewater is assessed in batch assays containing thermophilic anaerobic granular sludge (55ºC). Additionally, a comparative study between mesophilic and thermophilic decolourisation is performed using textile wastewater, either in the presence or absence of a redox mediator.

2. Materials and Methods

2.1. Chemicals

Reactive Red 2 (RR2), Acid Orange 7 (AO7) and Mordant Yellow 10 (MY10) were selected as azo dye model compounds (Figure 5.2). Anthraquinone-2,6-disulfonate (AQDS), Anthraquinone-2-sulfonate (AQS), Riboflavin (Vitamin B2) and Cyanocobalamin (Vitamin B12) were selected as redox mediator model compounds (Figure 5.3).

Chemicals were purchased from Aldrich (Gillingham, UK), Sigma (Bornem, Belgium) or Acros (Geel, Belgium) and used without additional purification.

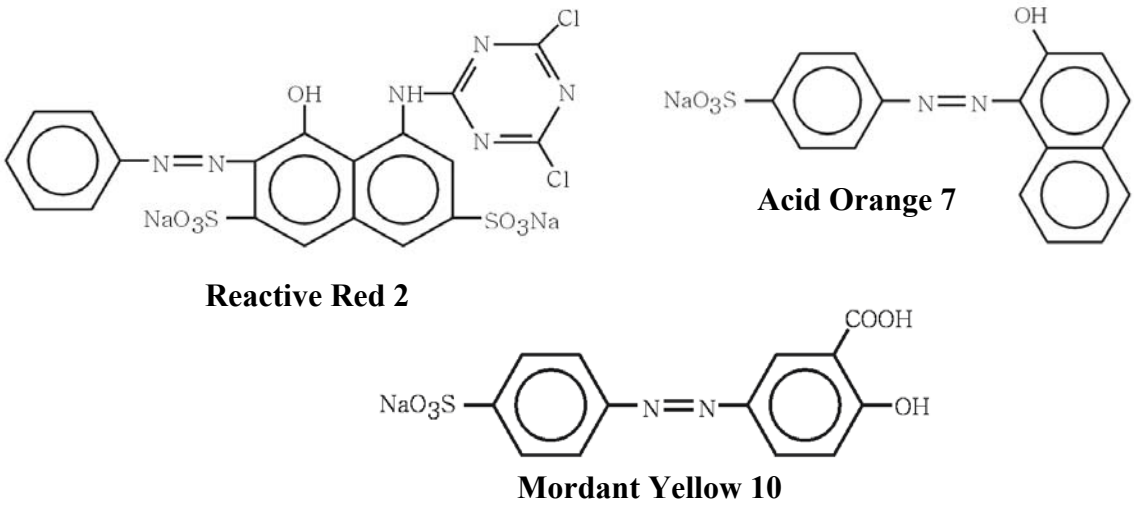

Figure 5.2. Chemical structure of the azo dyes Reactive Red 2 (RR2), Acid Orange 7 (AO7) and Mordant Yellow 10 (MY10), used as model compounds.

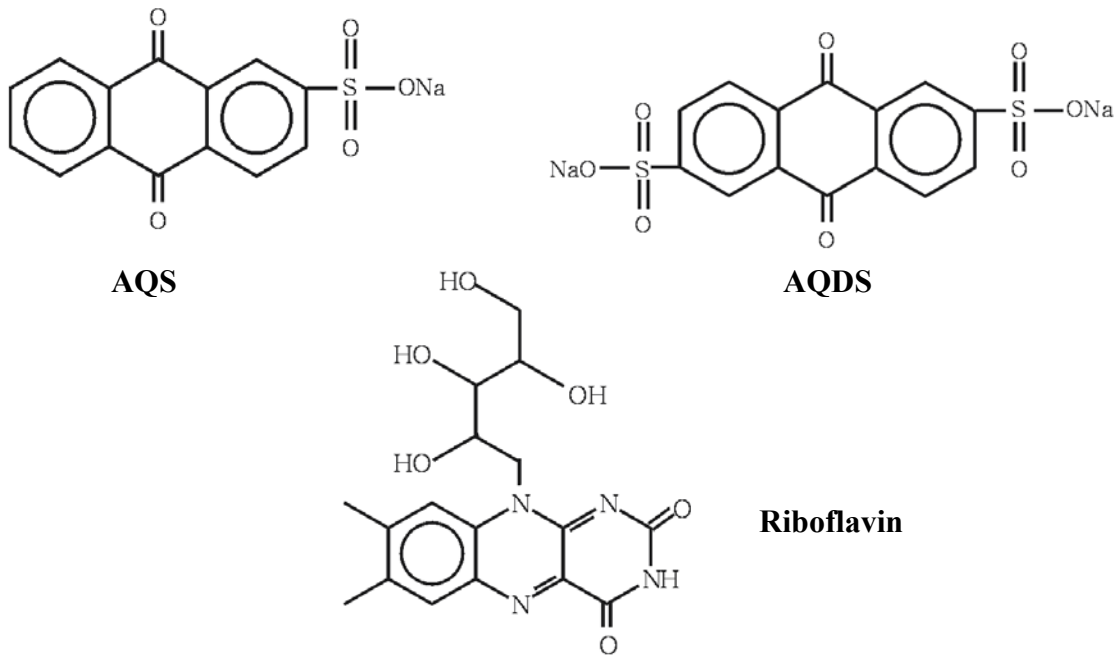

Figure 5.3. Chemical structure of the external redox mediators antraquinone-2-sulfonate (AQS), antraquinone-2,6-disulfonate (AQDS) and riboflavin, used as model compounds.

2.2. Seed inoculum and basal medium for decolourisation assays

Seed inoculum

Anaerobic granular sludge was collected from a full-scale mesophilic upflow anaerobic sludge blanket (UASB) reactor treating paper mill wastewater (Eerbeek, The Netherlands). The mesophilic sludge was converted into a thermophilic sludge as described in Chapter 3.

For batch tests at 30ºC the same mesophilic granular sludge was first acclimated to stable performance in an EGSB reactor (30ºC) as described in Chapter 3.

Basal medium for activity tests

A previously described basal medium (Chapter 3) was used for tests with model compounds. The basal medium for the AQDS reducing capacity test contained (mg/l): NH_4Cl (100), K_2HPO_4 (50), $MgSO_4 \cdot 7H_2O$ (100) and $CaCl_2 \cdot 2H_2O$ (5), 1 ml/l trace elements and 1 ml/l vitamins solution. The medium was buffered with 6.21 g/l sodium bicarbonate at around pH 7.1. The vitamin solution contained (mg/l): biotin (20), *p*-aminobenzoate (50), pantothenate (50), folic acid dihydrate (20), lipoic acid (50), pyridoxine (100) and nicotinamide (50).

When a wastewater derived from cotton processing textile factory was investigated, the wastewater was used undiluted and without the addition of nutrients or trace elements. The pH was adjusted to 7 with NaOH or HCl.

Activity Test

In the activity tests 1.3 ± 0.1 gVSS/l of the previously described stabilized sludge were added to 117-ml serum bottles with 50 ml basal medium and sealed with butyl rubber stoppers. Anaerobic conditions were established by flushing the headspace with N_2/CO_2 (70%:30%) and 2 gCOD/l (glucose:VFA mixture at a COD ratio of 1:3) co-substrate was added as electron donor and carbon source. After a pre-incubation time of 2 days, the azo dyes (variable) and redox mediators (variable) were added. Sterile controls were autoclaved once at 122ºC for 240 min and again following a 5 days incubation period, after which sterile co-substrate, mediator and dye stock solutions were added. The pH and the amount of VSS were determined upon completion of the experiment.

Effect of different redox mediators on thermophilic azo dye reduction

Model compounds

The impact of the redox mediators AQDS, AQS, riboflavin and cyanocobalamin (vitamin B12) on the thermophilic anaerobic reductive transformation of three azo dyes (0.3 mM) was investigated at 55ºC. Co-substrate (2 gCOD/l) and redox mediator (0.012 mM) were added to some bottles. Autoclaved sludge was used as a control for dye adsorption to the sludge and abiotic azo dye reduction by reducing agents. A sludge-free control was used to monitor the azo dye stability at 55ºC.

Decolourisation of acid dye wastewater

The three most efficient redox mediators obtained from the previous study were investigated at 55ºC with acid dye wastewater from a silk yarn-processing factory in Italy. The mediator concentration was increased from 0.012 mM to 0.024 mM to assess its impact on the decolourisation rates. An excess co-substrate concentration of 2 gCOD/l (glucose:VFA mixture at a COD ratio of 1:3) was used to avoid any lack of primary electron donor. The previously stabilized thermophilic sludge was taken from a 4ºC storage and added with the wastewater. Abiotic colour removal was assessed with both autoclaved sludge and sludge-free bottles.

Comparative study between mesophilic and thermophilic anaerobic treatments

Decolourisation of reactive dye wastewater

A comparative study between mesophilic (30ºC) and thermophilic (55ºC) conditions on anaerobic azo dye reduction was conducted with a reactive dyeing wastewater. The light brown wastewater was taken from a Belgian cotton processing textile factory. The best mediating compound from the previous screening was used at a concentration of 0.5 mM, either in presence or absence of 2 gCOD/l of co-substrate, i.e. glucose:VFA mixture on a COD ratio of 1:3. Both previously stabilized mesophilic and thermophilic sludges were taken from 4ºC storage and added with the wastewaters.

AQDS as electron acceptor in mesophilic and thermophilic incubations with granular sludge

To compare the capacity of microbial communities to use AQDS as a terminal electron acceptor, an AQDS reducing capacity test (AH_2QDS) was performed. AQDS (1 mM) was added to basal medium incubated at both 30ºC and 55ºC, in which 2 gCOD/l of a glucose:VFA mixture at a COD ratio of 1:3 was either present or absent. Sludge-free and autoclaved sludge bottles were used as controls for abiotic AQDS reduction. Both previously stabilized mesophilic and thermophilic sludges were taken from a 4ºC storage. The pH of the medium was 7 in all bottles.

Activation energy (Ea) determination during chemical decolourisation

The activation energy requirements of the model compound Reactive Red 2 (RR2, 0.3 mM) were determined in sludge-free assays by way of chemical reaction between a reducing compound and the dye. Sulphide (4.5 mM) was selected as a reducing agent and added with

RR2 and AQS (variable) at temperatures of 30, 45 and 55ºC. AQS (0.012 mM) was added to some of the bottles. The concentration of sulphide was measured initially and upon completion of the experiment.

2.3. Analysis

For azo dye model compounds, colour removal was determined photometrically (Spectronics 60, Milton-Roy Analytical Products Division, Belgium) as described in Chapter 3. The absorbance was read at the maximum absorbance wavelength, i.e. RR2 at 539 nm, AO7 at 484 nm and MY10 at 355 nm. The extinction coefficients used (AU cm^{-1} mM^{-1}) were 33.3, 20.0 and 17.3 for RR2, AO7 and MY10, respectively.

Wastewater decolourisation was determined in a 1 cm quartz cuvette by scanning the VIS spectra (Perkin-Elmer UV/VIS Lambda 12, Rodgau-Jügesheim, Germany) and comparing the wavelengths of two absorbance peaks.

AH_2QDS was determined anaerobically in a Type B Coy anaerobic chamber (Coy Laboratory Products Inc., USA) under a N_2/H_2 (96%:4%) atmosphere according to Cervantes et al. (2000). An extinction coefficient of 2.08 AU cm^{-1} mM^{-1} was obtained at 450 nm by chemical reduction of AQDS in a hydrogen atmosphere, in the presence of a hydrogenation catalyst according to Kudlich et al. (1999).

Sulphide was determined photometrically by using the Dr. Lange cuvette method and VSS were analysed according to APHA standard methods (1998).

3. Results

Effect of different redox mediators during thermophilic colour removal at 55ºC

Model compounds

Colour removal with living sludge in the presence of the azo dyes RR2 and AO7 followed a first-order reaction with respect to the dye concentration. However, for MY10 a zero-order reaction fit the colour data more accurately. Thus, the zero-order rate constant "k_0" (mM day^{-1}) and the first-order rate constant "k_1" (day^{-1}) were determined based on the dye concentration depletion.

The impact of the redox mediators AQDS, AQS and riboflavin at a catalytic concentration of 0.012 mM was evident with all three dyes, which increases the decolourisation rates up to 8-fold (Table 5.1) compared to the mediator-free bottles. Mediator addition did not affect the reaction-order. Riboflavin was by far the best mediator with the

reactive azo dye RR2, increasing the k_1-value 3-fold compared to the bottles supplemented with AQS (Table 5.1). However, the same tendency was not observed with AO7 and MY10, for which AQS had a higher catalytic capacity than riboflavin.

Table 5.1. First-order constant (k_1) and zero-order constant (k_0) on colour removal of azo dye model compounds RR2, AO7 and MY10 by thermophilic (55ºC) anaerobic granular sludge, either in the presence or absence of the redox mediators AQS, AQDS, riboflavin and vitamin B12.

Incubation	**k_1 / VSS (day^{-1} g^{-1} l)**		**k_0 / VSS ($day^{-1}g^{-1}$ mM l)**
	RR2	**AO7**	**MY10**
Mediator-free	0.72	4.20	0.44
AQDS	1.07	5.38	0.64
Vitamin B12	0.87	3.83	0.36
Riboflavin	5.83	6.79	0.64
AQS	1.92	7.30	0.66

2 gCOD/l co-substrate (glucose:VFA, at a COD ratio of 1:3). The concentration of azo dye and mediators are 0.3 mM and 0.012 mM, respectively. The first-order constant k_1 was calculated assuming a first-order reaction for RR2 and AO7. The zero-order constant k_0 was calculated assuming a zero-order reaction for MY10. The k-values were normalized by the amount of VSS present in the bottle (1.3 gVSS/l). The results are means of triplicate bottles.

Comparing the decolourisation rates between AQS- and AQDS-supplemented bottles, it is observed that the mediating capacity of AQS is much higher than AQDS, i.e. with RR2 1.8-fold and AO7 1.4-fold. Surprisingly, with MY10 all mediators catalysed the colour removal at similar rates (Table 5.1). Vitamin B12 was found to be a very poor redox mediator, having no effect on the decolourisation rates of AO7 and MY10 (Table 5.1). Based on this finding vitamin B12 was excluded during the investigations with textile wastewater.

Acid dyeing wastewater

All redox mediators accelerated the decolourisation rates of the acid dyeing wastewater, which can be observed from the spectra plotted in Figure 5.4A. The colour removal followed a first-order reaction both in the presence and absence of mediators (Figure 5.4B). However, the increase in decolourisation rate was not so evident as in the case of azo dye model compounds, regardless of the higher concentration of the mediator. For the mediator-

supplemented bottles the k_1-value increased 1.3-fold for AQS, 1.2-fold for riboflavin and 1.1-fold for AQDS compared to the bottles lacking mediators (Figure 5.4B).

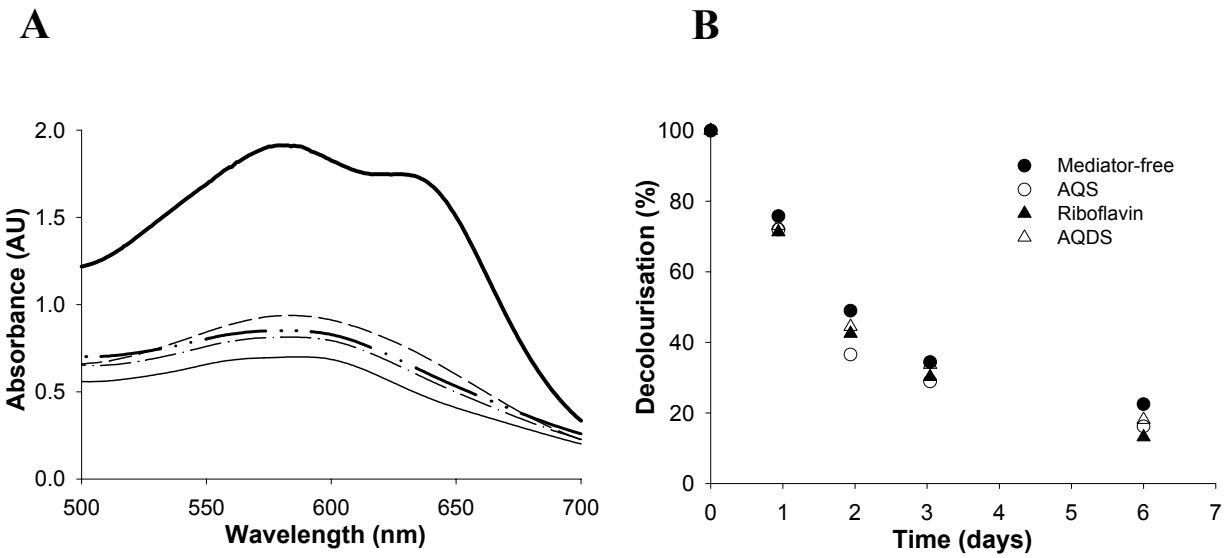

Figure 5.4. (A) Wastewater spectra after 22.5 h of incubation (T_1) with granular sludge at 55°C in the presence of the redox mediators AQS (continuous line on the bottom), riboflavin (dashed line with single point) and AQDS (dashed line with double point), and the mediator-free bottles (dashed line). T_0 was the initial wastewater spectrum (continuous line on the top). (B) Decolourisation of the wastewater measured at 580 nm. The results are means of duplicate bottles. The standard deviation was lower than 5% in all cases.

Comparative study between mesophilic and thermophilic anaerobic treatments

Decolourisation of reactive dyeing wastewater

Batch assays showed that decolourisations under thermophilic conditions were distinctly faster than under mesophilic conditions (Figures 5.5A and 5.5B). Moreover, the relative impact of the external redox mediator AQS (0.5 mM) on colour removal was considerably decreased under thermophilic conditions. Colour removal was accelerated by addition of co-substrate either in the presence or absence of mediator. Incubations supplemented with mediator in the absence of co-substrate showed no difference in rates (Figures 5.5A and 5.5B). Thus, in the case of separate treatments of dyeing- and rinsing-step wastewaters the addition of co-substrate should be considered.

Negligible (< 4%) decolourisation occurred in sludge-free controls in the presence of AQS during the incubation time of 2.7 days.

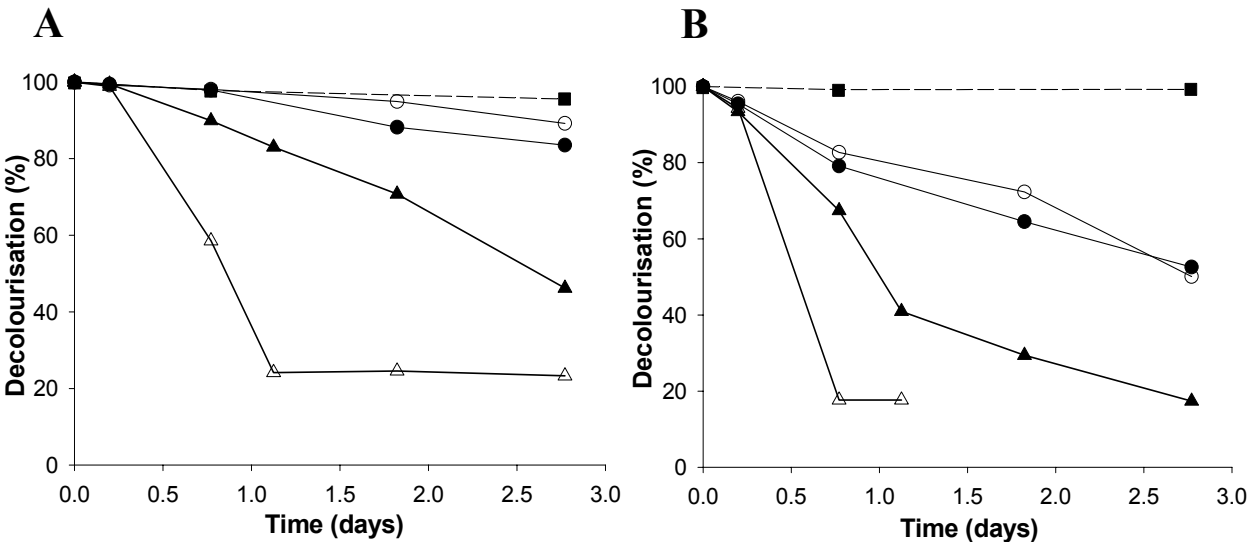

Figure 5.5. Decolourisation of the textile wastewater at 500 nm by mesophilic (A) and thermophilic (B) granular sludge incubated with wastewater (●), wastewater/AQS (○), wastewater/co-substrate (▲), wastewater/co-substrate/AQS (△). Sludge-free bottles controlled the abiotic colour removal (■). AQS (0.5 mM) was added to some assays and 2 gCOD/l co-substrate. The results are means of duplicate bottles. The standard deviation was lower than 5% in all cases.

AQDS as electron acceptor for microbial incubations at 30ºC and 55ºC with granular sludge

Anaerobic granular sludge incubated under mesophilic and thermophilic conditions was capable to couple the reduction of AQDS as a final electron acceptor with the primary electron donor oxidation (co-substrate). The generation of AH_2QDS, the reduced form of AQDS, was greatly accelerated at 55ºC (Figure 5.6) compared to 30ºC. Furthermore, no lag-phase was observed under thermophilic conditions. For instance, about 1 mM AQDS was completely reduced at 55ºC after 0.7 days of incubation, whereas mesophilic reduction after 0.7 days represented just 12.9% of this value (Figure 5.6). AH_2QDS formation in endogenous controls, autoclaved sludge and sludge-free bottles was negligible during the 2 days experiment at both 30ºC and 55ºC.

Ea determination during chemical decolourisation

The chemical decolourisation of RR2 by sulphide followed a first-order reaction with respect to the dye concentration. Thus, the first-order rate constant "k_1" (day^{-1}) was determined in each temperature tested. In order to calculate Ea values ln (k_1) versus 1000/T was plotted, and the slope Ea/R was obtained by the linear regression. This ratio was multiplied by the universal gas constant R (8.314 J K^{-1} mol^{-1}) to obtain the Ea value. In Figure 5.7 is shown that the Arrhenius equation could describe the chemical decolourisation of RR2 by sulphide at different temperatures. The slopes of the AQS-free and AQS-supplemented plots were indeed

different (Figure 5.7). The calculated Ea values were 27.9 kJ/mol and 22.9 kJ/mol for the AQS-free and AQS-supplemented bottles, respectively. Therefore, the activation energy was decreased 1.2-fold due to the addition of 0.012 mM of AQS.

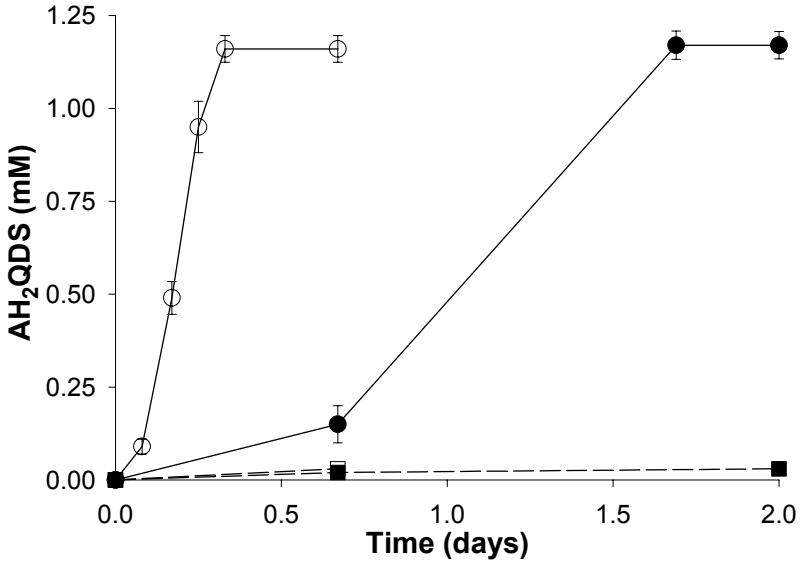

Figure 5.6. AH_2QDS formation (1 mM) for living sludge at 30ºC (●) and 55ºC (○). No AH_2QDS formation was found in the endogenous controls at 30ºC (■) and 55ºC (□). Measurements were conducted under anaerobic conditions, in which the samples were diluted in a bicarbonate buffer (60 mM, pH 6.8 ± 0.1), and the absorbance was read at 450 nm. The results are means of triplicate bottles.

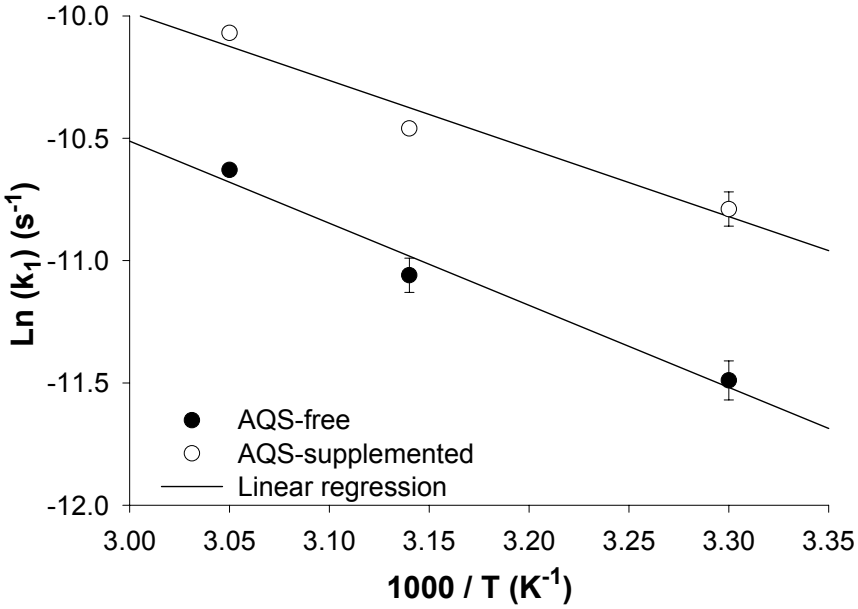

Figure 5.7. Arrhenius plot and linear regression for the chemical decolourisation of RR2 (0.3mM) by sulphide (4.5 mM) in sludge-free assays. AQS (0.012 mM) was added to some of the bottles. The value of the ratio Ea/R was obtained by the slope of the linear regression, in which the parameter R was the universal gas constant (8.314 J K^{-1} per mol). The results are means of duplicate bottles with the vertical bars representing the standard deviation.

4. Discussion

The present work clearly demonstrates the advantage of thermophilic treatment at 55ºC over mesophilic treatment at 30ºC for the decolourisation of azo coloured wastewaters. The normal rate limiting step, the transfer of reducing equivalents, was accelerated under thermophilic conditions. Both biotic and abiotic mechanisms may contribute to enhance the observed decolourisation under thermophilic conditions. The faster biological reduction of the redox mediator, AQDS, achieved by sludge incubations at 55°C in comparison with 30°C, evidenced the biological contribution in enhancing the rate of electron transfer. On the other hand, AQS-supplemented bottles presented a lower Ea requirement during the chemical reduction of RR2 by sulphide. Therefore, at 55ºC, the external mediator dosage can be decreased to achieve the colour removal requirements.

The improvement on the decolourisation rates at 55ºC due to the addition of low molecular weight redox mediators at catalytic concentration was clear with all tested azo dye model compounds (Table 5.1). Riboflavin and AQS had similar mediating properties, which is most likely related to the more or less equal redox potentials (E_0') of riboflavin and AQS (–208 mV and –225 mV, respectively) (Sober et al., 1970). However, riboflavin was a far better mediator than AQS with regard to the azo dye RR2. Such a result indicates that other factors, such as electro-chemical interactions between azo dye and redox mediator, also determine the decolourisation rates in the system. This is in contrast with Chung et al. (1978) who tested different redox mediators in the reduction of tartrazine by *Bacteroides thetaiotaomicron*. The use of methyl viologen and benzyl viologen increased the decolourisation rates 4.5-fold. However, the E_0' of methyl viologen and benzyl viologen are –440 mV and –360 mV, respectively (Brown, 1981), although this high redox difference was not shown to affect decolourisation rates. Walker and Ryan (1971) postulated that decolourisation rates are related to the electron density in the region of the azo bond. They suggested a colour removal increase by lowering the electron density in the azo link. Therefore, the use of redox mediators would not only tend to accelerate the reducing equivalents transfer to the terminal electron acceptor azo dye, but also to minimize the steric hindrance of the dye molecule (Bragger et al., 1997; Moir et al., 2001). Vitamin B12 showed to be a very poor redox mediator with the azo dye model compounds. This is probably due to its highly negative redox potential E_0' of –530 mV (Chiu and Reinhard, 1996), which in fact requires a higher

reducing capacity than was available in the present anaerobic incubation. This is in accordance with Chiu and Reinhard (1996) who verified that vitamin B12 had little effect on carbon tetrachloride reduction in the absence of the external reductant cysteine. Therefore, the redox potential of the mediator E_0' is an indication of its catalytic capacity and must be in the range of –440 mV (Brown, 1981) to –50 mV (Rau and Stolz, 2003). Other information should be derived from the difference in steric and electro-chemical factors between mediator and azo dye.

In the acid dyeing wastewater experiment, an increase in decolourisation rates due to the addition of redox mediators at catalytic concentrations (0.024 mM) was not so evident. This difference was probably due to the high dye COD concentration (1.4 g/l) and the properties of the dyes present in the wastewater. The dyebath contained three dyes, the final hue being a combination of red, yellow and blue. As the dyebath colour was dark blue, the blue dye was most likely present in a greater concentration than the other two dyes. The structure of the dyes in the wastewater was not known, but many blue dyes have an anthraquinone structure. An experiment with anthraquinone dye Reactive Blue 5 showed that because this type of dye is electronically very stable, the chemical electron transfer from the reduced form of the mediator to the dye is less effective, and thus the rate-limiting step (Dos Santos et al., 2004b).

Thermophilic anaerobic treatment showed a higher decolourisation rate compared to mesophilic anaerobic treatment (Figure 5.5) with the reactive dyeing wastewater. The impact of temperature on colour removal also corroborated Willetts and Ashbolt (2000), who reported faster decolourisation rates at 55ºC compared to 35ºC while treating the azo dye Reactive Red 235 in UASB bioreactors free of external redox mediator. Furthermore, Laszlo (2000) reported a higher decolourisation of the azo dyes Orange II and Remazol Red F3B at 43ºC than at 28ºC by anaerobically incubating the facultative organism *Burkholderia cepacia* NRRL B-14803. Based on these reports and our present results we postulate that the transfer of reducing equivalents to the azo dye is the rate-limiting step, which is accelerated by the temperature increase.

Thus, it is conclusively stated that 55ºC is a more effective temperature than 30ºC for colour removal, which brings good prospects on the application of thermophilic treatment for decolourisation processes.

Acknowledgements

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6

Enhancing the electron transfer capacity and subsequent colour removal in bioreactors by applying thermophilic anaerobic treatment and redox mediators

Abstract

The effect of temperature, HRT and the redox mediator AQDS, on electron transfer and subsequent colour removal from textile wastewater was assessed in mesophilic (30ºC) and thermophilic (55ºC) anaerobic bioreactors. The results clearly show that compared to mesophilic anaerobic treatment, thermophilic treatment at 55ºC is an effective approach for increasing the electron transfer capacity in bioreactors, and thus improving the colour removal efficiencies. Furthermore, similar efficiency values on colour removal were found under thermophilic conditions between the AQDS-free and AQDS-supplemented reactors, whereas a significant difference (up to 3.6-fold) on decolourisation rates occurred under mesophilic conditions. For instance, at an HRT of 2.5 h and in the absence of AQDS, the colour removal was 5.3-fold higher at 55ºC compared to 30ºC. The impact of a mix of mediators with different redox potentials on the decolourisation rate was investigated with both industrial textile wastewater and the model azo compound RR2. Colour removal of RR2 in the presence of AQS (E_0' of -225 mV), was 3.8-fold and 2.3-fold higher at 30ºC and 55ºC, respectively, than the values found in the absence of AQS. Furthermore, when the mediators 1,4-benzoquinone (BQ) (E_0' of +280 mV) and AQS were incubated together, there was no improvement on the decolourisation rates compared to the bottles solely supplemented with AQS, even though a broader range of the electron transfer mediation capacity could be covered in the solution with dyes. Results imply that the use of mixed redox mediators with positive and negative E_0' under anaerobic conditions is not an efficient approach to improve colour removal in textile wastewaters.

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1. Introduction

With the increased demand for textile products, the textile industry and its wastewaters have been increasing proportionally, making it one of the main sources of severe environmental problems worldwide (Vandevivere et al., 1998). Particularly the release of coloured compounds into water bodies is undesirable not only because of their aesthetic appearance and the impact of dyes on photosynthesis of aquatic plants, but also because many of these dyes and their breakdown products are carcinogenic (Weisburger, 2002). Without adequate treatment these dyes can remain in the environment for a long time. For instance, the half-life of the hydrolyzed Reactive Blue 19 is about 46 years at pH 7 and 25°C (Hao et al., 2000). Dyes are recalcitrant by design and not readily amendable to common treatment methods, imposing a challenge for closed water systems. Extensive research in the field of biological azo dye decolourisation has shown promising results, but much of this work has been done with single model compounds (Tan et al., 2000; Cervantes et al., 2001). However, industrial textile wastewater presents the additional complexity of dealing with unknown quantities and varieties of many kinds of dyes (Bisschops and Spanjers, 2003), as well as low BOD/COD ratios (Pagga and Brown, 1986), which may affect the efficiency of the biological decolourisation.

Many microorganisms and inorganic electron donors are able to anaerobically reduce azo dyes. The reductive cleavage generally produces the anaerobically recalcitrant aromatic amines (Chung et al., 1995). However, many of these amines are readily mineralized aerobically (Field et al., 1995), so an anaerobic-aerobic treatment strategy has been repeatedly proposed (O'Neill et al., 2000a; Tan et al., 2000).

Possibly, due to unsuitable redox potentials, complex structure or steric hindrance of the molecules, the reductive cleavage reaction generally represents one of the rate-limiting step in the overall mineralization of an azo dye (Dos Santos et al., 2004a). It has been suggested that redox mediators could increase the rate of dye decolourisation as an electron shuttle from the primary electron donor to the azo dye (Rau and Stolz, 2003). Theoretically, feasible redox mediators for biological azo dye reduction must have redox potentials between the half reactions of the azo dye and the primary electron donor (Van der Zee et al., 2003b). Unfortunately the standard redox potential (E_0') for most azo dyes is unknown. In a screening of redox potential values for different azo dyes, it was found that E_0' values are normally between –430 and –180 mV (Dubin and Wright, 1975). Rau et al. (2002) cites that the NAD(P)H cofactor, which has the lowest E_0' value of –320 mV, seems to set the lower limits of redox mediators application. The reason is that mediators with a more negative E_0' value

will not be reduced by the cells and mediators with E_0' higher than –50 mV will probably not efficiently reduce the azo bond at high rates. Although this observation was confirmed with a model compound (Rau et al., 2002a), it has yet to be shown in the case of complex textile wastewater. Furthermore, the colour removal achieved in the presence of a mix of mediators with different redox potentials has not yet been described.

Quinones, the electron accepting moieties of humus substances, have been shown to effectively accelerate electron transfers in biological azo dye reduction (Cervantes et al., 2001; Dos Santos et al., 2003), but their impact in conjunction with thermophilic conditions has not been well investigated (Dos Santos et al., 2004c; Dos Santos et al., 2004a). In fact, thermophilic textile wastewater decolourisation has only been briefly examined in the literature (Willetts and Ashbolt, 2000) notwithstanding that a large portion of textile wastewaters, particularly those coming from dyebath, are discharged at high temperatures. In the present study the electron transfer capacity and subsequent colour removal in thermophilic anaerobic bioreactors was compared to mesophilic reactors. The impact on the decolourisation rates of a quinone-based redox mediator is also evaluated at both 30ºC and 55ºC. Additionally, the importance of the mediator's standard redox potential with respect to its impact on decolourisation is evaluated in batch assays.

2. Materials and Methods

2.1. Wastewater characterization

Industrial wastewater was collected from a textile factory in Ronse, Belgium. The water was obtained from the rinsing step, and a concentrated solution was obtained from the dyebath. The water was mainly composed of reactive azo dyes, sodium hydroxide, urea, sodium silicate, wetting and antifoam agents. Table 6.1 gives the characterization of the water used in the current experiment.

2.2. Chemicals

Reactive Red 2 (RR2) was selected as azo model compound for this study as described in Chapter 3.

Anthraquinone-2,6-disulfonate (AQDS), anthraquinone-2-sulfonate (AQS) and 1,4-benzoquinone (BQ) were used as redox mediator model compounds without additional purification. All chemicals used were of analytical grade and purchased from Sigma-Aldrich (Gillingham, UK).

Table 6.1. Characterization of the wastewater used in the current experiment.

Parameter	Unit	Concentration	
		Rinsing step	Dyebath
Colour	AU (475 nm)	1.24	245.90
pH		10.2	11.6
Conductivity	mS/cm	2.7	29.9
Soluble COD	mgO_2/l	747	32600
Nitrate	mg/l	-	-
Nitrite	mg/l	-	-
Sulphate	mg/l	990	12600
Sulphide	mg/l	-	[a]
Acetate	mg/l	36	475
Propionate	mg/l	-	77
Butyrate	mg/l	-	255

[a]Sulphide concentration in the dyebath was not determined due to the strong colour interference on the measurement.

2.3. Batch and continuous experiments

2.3.1. Seed inoculum and basal medium

Anaerobic granular sludge was collected from a full-scale mesophilic upflow anaerobic sludge blanket (UASB) reactor treating paper mill wastewater (Eerbeek, The Netherlands). The mesophilic sludge was acclimated for 2 months at 55ºC in an expanded granular sludge bed (EGSB) reactor (0.53 l) operating at a hydraulic retention time (HRT) of about 10 h and an organic loading rate (OLR) of 5.0 kgCOD (chemical oxygen demand) m^{-3} day^{-1}. The COD consisted of a mixture of glucose and volatile fatty acids (VFA) at a COD ratio of 1:3. The neutralized VFA solution contained acetate, propionate and butyrate at a COD ratio of 1:1:1. The basal medium was prepared as described in Chapter 4.

For the two 0.53 l mesophilic (30ºC) EGSB reactors, granular sludge was derived from the same source as previously described and was acclimated until reaching steady-state conditions.

2.3.2. Continuous Experiment

Reactor

Two mesophilic 0.53 l EGSB reactors, R1 and R2, were investigated both with AQDS-free and AQDS-supplemented medium, respectively. They were seeded to a final concentration of 30 gVSS/l. The reactors were operated at HRT of 10, 5 and 2.5 h, and an up-flow velocity of 4 m/h was imposed by recirculating the flow with peristaltic pumps (505S, Watson-Marlow, Cornwall, UK). They were placed in a temperature-controlled room at 30ºC. Two thermophilic 0.53 l EGSB reactors, R3 and R4, were investigated both with AQDS-free and

AQDS-supplemented medium, respectively. The operational parameters sludge concentration, HRT and up-flow velocity were as those described for the mesophilic reactors. The reactors were maintained at 55ºC using a thermostat bath. Biogas was collected in a gas-solid-liquid separator, and afterwards washed in a NaOH (10% w:v) solution with timol blue as indicator. Methane was measured in a wet test gas meter. The redox potential (ORP) was monitored on-line in all reactors.

Stock solution

The 10 l influent stock solution was composed of textile wastewater, a glucose-VFA mixture at a COD ratio of 1:3 as co-substrate and demineralized water. The stock solution of reactors R2 and R4 also contained AQDS. Different concentrations of rinsing step wastewater, dyebath and AQDS were added to the medium (Table 6.2). The 300 gCOD/l VFA stock solution was neutralized with NaOH. To keep the pH around 7.5 in the reactors, the pH of the influent stock solution was adjusted to 5.5 with HCl. The influent stock solutions and basal media were stored at 4ºC.

2.3.3. Batch Experiments

Activity tests

In the activity tests 1.3 ± 0.1 gVSS/l of the previously described stabilized sludge were added to 117-ml serum bottles with 50 ml basal medium. The bottles were then sealed with butyl rubber stoppers and aluminium crimp caps. Anaerobic conditions were established by flushing the headspace with N_2/CO_2 (70%:30%) after which 1.5 gCOD/l co-substrate (glucose), dyes or wastewater (variable) and redox mediators (variable) were added. Sterile controls were autoclaved once at 122ºC for 240 min and again following a 5-days incubation period, after which sterile co-substrate, mediator and dye were added. The pH and the amount of VSS were determined after completion of the experiment.

Impact of pre-exposure biomass to continuous AQDS dosage in bioreactors

An AQDS reducing capacity test was performed to evaluate the possible enrichment of AQDS-respiring microorganisms between AQDS-free and -supplemented reactors during the continuous flow experiment time. AQDS (1 mM) was added to the biomass containing-basal medium at both 30ºC and 55ºC. Glucose (1.5 gCOD/l) was added to some of the bottles. Sludge-free and autoclaved sludge bottles were used as controls for abiotic AQDS reduction.

Both mesophilic and thermophilic active sludges were taken from the operating bioreactors. The medium was buffered with 5.0 and 6.2 g/l sodium bicarbonate, for the temperatures of 30ºC and 55ºC, respectively, to keep the pH around 7.1. The basal medium for the AQDS reducing capacity test is described in Chapter 5.

Effect of different ratios of dyebath and rinsing water on colour removal and COD removal in the same bioreactor

As previously mentioned, the industrial wastewater was collected from the rinsing and dyeing steps. The concentrated dyebath was from a semi-continuous system, which discharged this heavily coloured water to the treatment plant. However, in case of combined treatment of dyeing- and rinsing-step with anaerobic reactors, the ratio of dyebath and rinsing waters should be considered. Therefore, we assessed in batch experiments the effect of increasing the ratio of concentrated dyebath to rinsing water in terms of colour removal and methane production. The ratios of concentrated dyebath and rinsing water used (% v:v) were 0:100, 0.10:99.9, 0.50:99.5 and 2.0:98.0. A co-substrate concentration of 1.5 gCOD/l as glucose was the primary electron donor. AQS (0.050 mM) was added to some of the bottles. The mesophilic acclimated sludge was incubated with the wastewater at a concentration of 1.3 ± 0.1 gVSS/l. Abiotic colour removal was assessed with both autoclaved sludge and sludge-free bottles.

Effect of the redox mediator standard redox potential (E_0') in the decolourisation rates

To assess the decolourisation rate-limiting step, i.e. the enzymatic mediator reduction versus the chemical electron transfer from the mediator to the dye, textile wastewater containing many dyes with different redox potentials was used in mesophilic and thermophilic incubations. Quinone-based redox mediators with different E_0' values AQS (–225 mV) and BQ (+280 mV) were selected as model compounds. The mediators were tested both individually at concentrations of 0.050 mM or together at a ratio of 1:1, to give the same final concentration of 0.050 mM. In addition to the investigation with industrial wastewater, the model compound RR2 (0.3mM) was also tested. A co-substrate concentration of 1.5 gCOD/l as glucose was the primary electron donor. Both mesophilic and thermophilic acclimated sludges were added with the wastewater at the same VSS concentration. Abiotic colour removal was assessed with both autoclaved sludge and sludge-free bottles.

2.4. Analysis

For the azo dye model compound RR2 colour removal is determined photometrically as described in Chapter 3. Wastewater decolourisation was determined as described in Chapter 5. Soluble COD was analysed photometrically by the Dr. Lange cuvette method. Samples were centrifuged for 3 minutes at 13000 rpm prior to analysis. Methane, VFA, methanol, ethanol, sucrose, fructose, glucose, lactate, formate and VSS determinations were described in Chapter 3. The redox potential (ORP) was measured as described in Chapter 4.

3. Results

Effect of temperature, HRT and redox mediator on electron transfer and colour removal with mesophilic and thermophilic anaerobic bioreactors

Investigation with wastewater

The operational data of the continuous flow EGSB reactors experiments are presented in Table 6.2. With textile wastewater (Periods II-V) all mesophilic (Figure 6.1A) and thermophilic (Figure 6.1B) reactors achieved similar efficiency values of around 85%, and colour removal was independent of the concentrated dyebath and rinsing water ratio, i.e. the dye concentration in the influent. Furthermore, the impact of the redox mediator AQDS (0.025 mM) for the acceleration of decolourisation rates was not evident (Figures 6.1A and 6.1B). Very likely, the wastewater was composed of a larger fraction of easily reducible dyes and a smaller fraction of recalcitrant dyes. Therefore, the effects of AQDS and temperature were likely masked because of the wastewater composition, as their effect can mainly be observed with those dyes recalcitrant to reductive transformations. This is probably the reason why the colour removal efficiency in all reactors was similar, even though more negative values of redox potentials profiles were measured at 55ºC, which represent a better capacity of the terminal electron acceptor azo dye to be reduced (Figure 6.2).

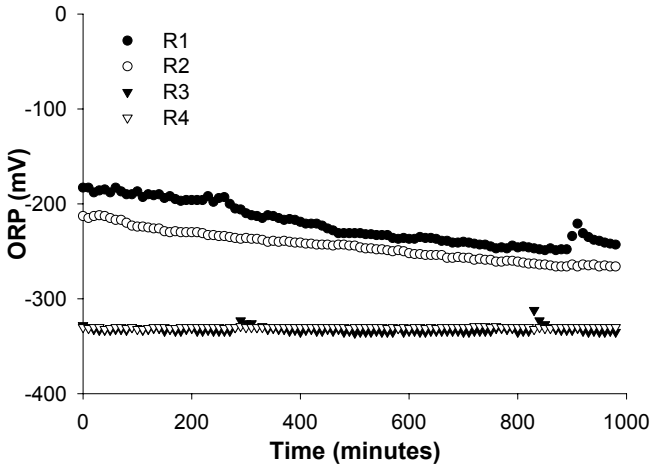

Figure 6.2. Different redox profiles (mV) of the EGSB reactors at 30ºC (R1 and R2) and 55ºC (R3 and R4) treating industrial wastewater. R1 and R3 are the reactors AQDS-free; R2 and R4 the reactors AQDS–supplemented. ORP values were corrected to the redox values of the reference electrode (+207 mV at 25ºC).

Table 6.2. Operational parameters and performance of the mesophilic (30ºC, R1 and R2) and thermophilic (55ºC, R3 and R4) EGSB reactors fed with both industrial textile wastewater and the model compound RR2.

Operational Parameters of the Reactors									
Period	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**
End of period (days)	**35**	**53**	**76**	**105**	**116**	**135**	**149**	**154**	**159**
Basal medium	**a**	**b**	**b**	**b**	**b**	**c**	**c**	**c**	**b**
HRT (h)	10	10	10	10	10	10	5	2.5	2.5
Co-substrate (gCOD/l)	2.5	2.5	2.5	2.5	2.5	2.5	1.25	0.625	0.625
$OLR_{sub.}$ (gCOD l^{-1} day^{-1})	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Dyebath : rinsing water (% v:v)	-	0: 100	0.1:99.9	0.5:99.5	2.0:98.0	-	-	-	0.1:99.9
RR2 (gRR2 l^{-1} day^{-1})	-	-	-	-	-	1.25	1.25	1.25	-
AQDS (μM) in R2 and R4	-	25	25	25	25	25	25	25	25
Performance of the Reactors									
Colour removal R1 (%)	-	85 (1.2)	85 (3.2)	85 (2.2)	86 (2.7)	56 (7.3)	37 (4.2)	13 (1.9)	65 (0.9)
Colour removal R2 (%)	-	87 (1.6)	87 (1.0)	85 (3.2)	88 (1.9)	88 (1.9)	79 (1.1)	47 (5.6)	78 (1.0)
Colour removal R3 (%)	-	84 (3.2)	87 (1.8)	89 (1.7)	91 (1.6)	95 (0.5)	85 (2.2)	69 (1.8)	81 (2.3)
Colour removal R4 (%)	-	84 (1.6)	88 (2.4)	89 (3.2)	92 (0.9)	95 (0.8)	93 (0.5)	79 (3.1)	81 (1.5)
COD removal R1 (%)	-	81 (2.1)	81 (1.7)	75 (4.0)	53 (15.3)	83 (4.6)	74 (1.9)	69 (0.8)	61 (1.0)
COD removal R2 (%)	-	78 (2.0)	70 (7.6)	74 (4.7)	72 (6.7)	84 (3.3)	76 (2.0)	70 (0.7)	65 (1.0)
COD removal R3 (%)	-	74 (2.8)	71 (3.6)	76 (3.7)	66 (11.5)	85 (3.1)	76 (2.6)	76 (0.1)	76 (1.0)
COD removal R4 (%)	-	60 (3.2)	64 (4.5)	65 (5.0)	59 (6.4)	70 (4.1)	76 (2.1)	74 (2.5)	74 (1.0)

[a] No dye nor AQDS was supplemented; [b] Industrial wastewater was fed to the reactors; [c] The dye model compound RR2 was fed to the reactors; Standard deviation is shown in parenthesis

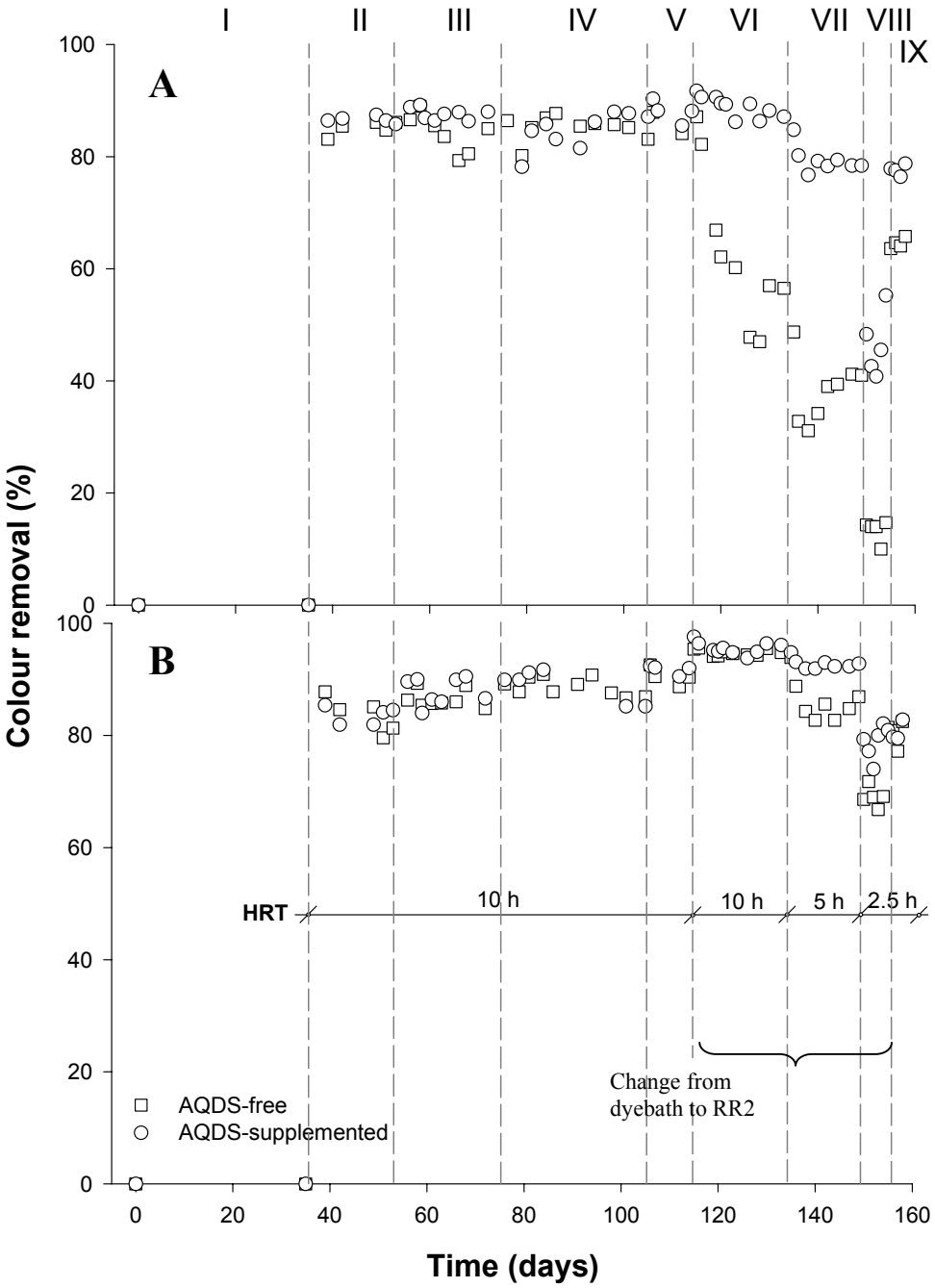

Figure 6.1. Performance of the continuous flow EGSB reactors in terms of colour removal at both 30ºC (A) and 55ºC (B), treating industrial wastewater and the model compound RR2 under different loading and hydraulic conditions. Reactors R1 and R3 were AQDS-free (controls) and the reactors R2 and R4 were supplemented with AQDS.

The apparent inefficiency of AQDS on colour removal could have been due to the presence of alternative electron acceptors such as nitrate or nitrite, which were not present in the wastewater (Table 6.1). In sludge-free bottles under anaerobic conditions, neither abiotic decolourisation due to the presence of reducing compounds like sulphide nor formation of precipitates was found in batch experiments. One important point to be observed in this

investigation is the high sulphate concentration in the influent, even for the rinsing water (Table 6.1). Therefore, in addition to the colour removal due to the biological activity during the primary electron donor oxidation, we should also consider the purely chemical decolourisation due to the sulphide produced via sulphate reduction, even while using a methanogenic sludge. The pure chemical decolourisation generally follows equations 1 and 2:

$$COD + SO_4^{-2} \rightarrow Sulphide + H_2O + 2HCO_3^- \quad (1)$$

$$Sulphide + 0.5\ R_1–N=N–R_2 \rightarrow Elemental\ Sulphur + 0.5\ R_1–NH_2 + 0.5\ R_2–NH_2 \quad (2)$$

Chemical decolourisation is significantly accelerated by redox mediators and temperature (Dos Santos et al., 2004a). Since colour removal efficiency values of the mesophilic and thermophilic bioreactors were similar, we postulated that the contribution of the chemical decolourisation was negligible in our experiments. On the other hand, when comparing Periods III (HRT of 10 h) and IX (HRT of 2.5 h) a clear impact of the applied HRT was demonstrated. The change in the dye-loading rate (Periods II-V) only slightly showed an effect of both temperature and mediator. In Period IX, there was a significantly (1.2-fold) higher colour removal in the mesophilic AQDS-supplemented reactor, R2, than in its AQDS-free counterpart, R1 (Table 6.2). Interestingly, there was negligible or no difference on colour removal observed at 55ºC between the reactors R3 and R4. Similar results were seen with COD removal and primary electron donor oxidation. Disregarding data obtained during operational difficulties, an average COD removal between 60 and 80% (Table 6.2) was measured. Trace amounts of VFA were found in the effluent, while the glucose fermentation products pyruvate, formate, lactate and alcohols were never detected. The COD removal with respect to the primary electron donors VFA and glucose was generally higher than 90%.

Investigation with the model compound RR2

Because of the results obtained with the wastewater at an HRT of 10 h (Period II-V), it was concluded that the wastewater was masking the effect of temperature and mediator on the decolourisation rates as explained before. In Periods VI-VIII, the textile wastewater was replaced by the model compound Reactive Red 2 (Table 6.2,), which is known to be rather recalcitrant to reductive transformations (Van der Zee et al., 2001b) . The difference on colour removal efficiencies between the reactors was immediate. Decolourisations at 30ºC (Fig. 6.1A) were considerably lower than those found at 55ºC (Fig. 6.1B, Periods VI-VIII). For instance, in Period VI, the decolourisation rates in the absence of AQDS were 1.7-fold higher

at 55ºC compared to 30ºC (Table 6.2). Furthermore in the same period, AQDS increased 1.6-fold the rates at 30ºC, but it had hardly or no impact on the rates at 55ºC (Table 6.2).

To assess the effect of the HRT on the performance of the reactors and to characterize the electron transfer limitation, the HRT was decreased from 10 h to 5 h (Period VII) and 2.5 h (VIII). As clearly evidenced in these periods, reactor R2 presented decolourisation rates 2.1-fold (Period VII) and 3.6-fold (Period VIII) higher in comparison with the mesophilic reactor, R1 (Table 6.2, Fig. 6.1A). The impact of HRT at 55ºC was hardly apparent (Fig. 6.1B), i.e. R4 presented decolourisation rates 1.09-fold (Period VII) and 1.14-fold (Period VIII) higher compared to the other thermophilic reactor, R3. The same degree of COD removal of around 75% indicates that the reducing equivalents were generated at a similar rate in all reactors. Apparently, the difference in the decolourisation rates was not related to the difference in the production rates of reducing equivalents. Consequently, the higher degree of colour removal was attributed to the impact of temperature and AQDS on electron shuttling.

Impact of pre-exposure to continuous AQDS dosage in bioreactors

The results shown in Figures 6.3A and 6.3B clearly evidences differences between the mesophilic and thermophilic sludges in the capacity of reducing AQDS. On one hand, the serum bottles containing mesophilic sludge coming from the AQDS-supplemented reactor (R2) reduced AQDS (1mM) much faster than those bottles containing sludge not exposed to AQDS (R1). On the other hand, there was virtually no difference in the capacity of reducing AQDS between sludges samples from the thermophilic reactors R3 and R4.

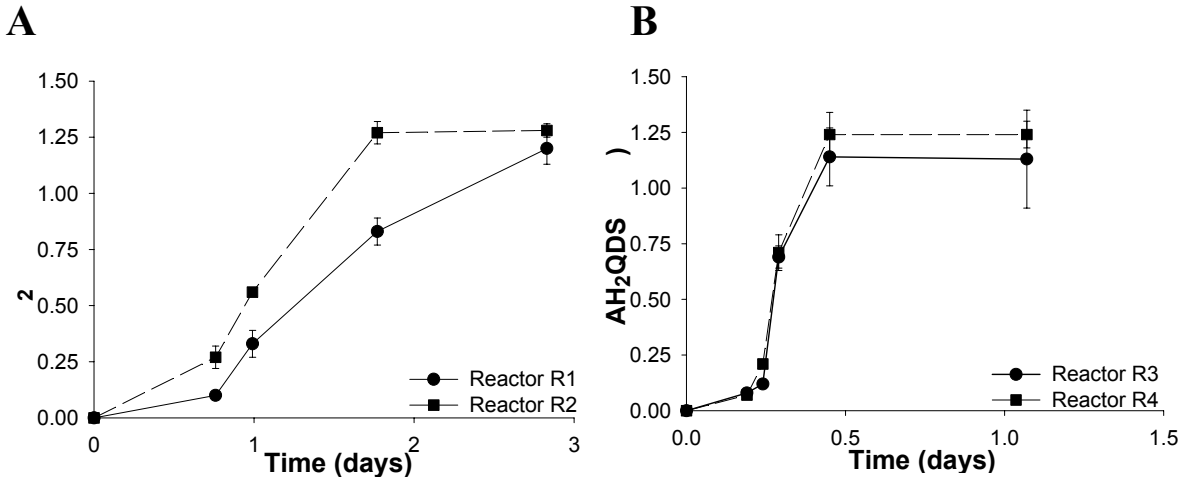

Figure 6.3. AH_2QDS formation (1 mM) at both 30ºC (A) and 55ºC (B), with sludge samples from the EGSB reactors. Reactors R1 and R2 are the mesophilic reactors AQDS-free and –supplemented, respectively (A). Reactors R3 and R4 are the thermophilic reactors AQDS-free and –supplemented, respectively (B). Glucose (1.5 gCOD/l) was used as co-substrate. The results are means of triplicate bottles.

This behaviour might be attributed to a different enrichment of AQDS-respiring microorganisms, which could couple glucose oxidation via AQDS reduction, at the two temperatures tested. However, the AQDS reducing capacity test solely does not provide compelling evidence of microbial AQDS-respiring enrichments in the anaerobic consortia. Furthermore, the rapid AQDS reduction of thermophilic "non-AQDS pre-exposed" sludge could also be attributed to a faster growth rate of thermophiles compared to mesophiles, which was sufficient to provide during the test a suitable population growth responsible for the AQDS reduction observed. Therefore, further investigations by molecular techniques are requested for a better evaluation of the different enrichments postulated.

Effect of different ratios of dyebath and rinsing water on colour removal and COD removal in the same bioreactor

Changes in decolourisation due to the increase in concentrated dyebath to rinsing water ratio were less pronounced than the effect of this gradient on methane formation (Table 6.3). For instance, comparing the ratios (% v:v) 0:100 and 2.0:98.0 in the absence of AQS, the first-order rate constants "k" for decolourisation were 0.32 day^{-1} and 0.19 day^{-1}, respectively, a 0.6-fold decrease (Table 6.3). This difference is not significant if we consider that the initial absorbance peak of the wastewater at 475 nm was 13-times higher at the ratio 2.0:98.0 compared to the ratio 0:100 (data not shown). Interestingly, the redox mediator AQS did not have any catalytic effect on colour removal for high concentrations of dyebath.

Table 6.3. Effect of different ratios of dyebath and rinsing water on both electron donor conversion and decolourisation rates by using standardized activity test at 30ºC.

Ratio Dyebath: Rinsing (% v:v)	Products (mgCOD/l)						k/VSS (day^{-1} g^{-1} l)
	Acetate	Propionate	Butyrate	Ethanol	Glucose	CH_4	
0:100 AQS-	-	15	22	-	-	1118	0.32
0:100 AQS+	-	190	15	-	-	990	0.37
0.1:99.9 AQS-	-	19	33	-	-	1103	0.30
0.1:99.9 AQS+	27	160	58	-	-	993	0.36
0.5:99.5 AQS-	595	22	0	-	-	485	0.28
0.5:99.5 AQS+	497	31	26	-	-	408	0.26
2.0:98.0 AQS-	831	14	26	-	-	232	0.19
2.0:98.0 AQS+	738	153	44	-	-	173	0.15
Control AQS-	-	29	26	-	-	924	-
Control AQS+	-	330	52	-	-	791	-

Glucose (1.5 gCOD/l) was used as electron donor. The results are means of duplicate bottles, in which the standard deviations are lower than 10% in all cases.

The effect of high concentrations of dyebath on methane formation is shown in Table 6.3, in which low concentrations of dyebath slightly stimulated methanogenesis. The specific methanogenic activity of the wastewater-free control was 4-times higher than that of the ratio 2.0:98.0 (Table 6.3). A 50% decrease in the CH_4 production compared to the control was found with 22 ml of dyebath for each 1000 ml of rinsing water (2.2:100). Acetate accumulation in the bottles occurred when the dyebath portion was increased in the influent. The CH_4 production in the AQS-free bottles was about 1.2-times higher than those supplemented with AQS. Furthermore, the oxidation of propionate was affected by the presence of AQS (Table 6.3).

Colour removal in the presence of a mix of mediators with different standard redox potentials (E_0')

Colour removal with RR2 at 30ºC in the presence of the redox mediator BQ (E_0' of +280 mV) was 1.1-fold higher compared to the rate of the mediator-free control (Fig. 6.4C). Contrary to 30ºC, the decolourisation rate at 55ºC was 0.1-fold lower than the mediator-free control rate (Fig. 6.4D). On the other hand AQS, a mediator with an E_0' of –225 mV, accelerated the decolourisation reactions at both 30ºC (Fig. 6.4A and 6.4C) and 55ºC (Fig. 6.4B and 6.4D). Colour removal with RR2 in the serum bottles supplemented with AQS was 3.8-fold and 2.3-fold higher at 30ºC and 55ºC, respectively, than those values in the absence of AQS. When the mediators BQ and AQS were simultaneously used no increase in the decolourisation rates was found, even though a broader range of the electron transfer mediating capacity could be covered in the solution (Fig. 6.4A-D). For instance, comparing the incubations supplemented with solely AQS (0.050 mM), and AQS (0.025 mM) together with BQ (0.025 mM), the decolourisation rates of RR2 with solely AQS are 1.1-fold higher at both 30ºC and 55ºC (Fig. 6.4C and 6.4D).

4. Discussion

Thermophilic anaerobic treatment as an effective approach for increasing the electron transfer capacity and subsequent colour removal in bioreactors

In the present investigation the effect of temperature, HRT and redox mediator on electron transfer and subsequent colour removal was assessed in mesophilic and thermophilic bioreactors. The results clearly show that compared to mesophilic anaerobic treatment,

thermophilic treatment at 55ºC is an effective approach for increasing the electron transfer capacity in bioreactors, and subsequent improvement of the colour removal efficiencies (Table 6.2). Furthermore, similar efficiency values on colour removal were found under thermophilic conditions between the AQDS-free and AQDS-supplemented reactors, whereas a significant difference on decolourisation rates occurred under mesophilic conditions. In fact, this comparative study between mesophilic and thermophilic bioreactors in the presence of redox mediators is reported for the first time in literature, hampering a sound comparison with the other previous investigations.

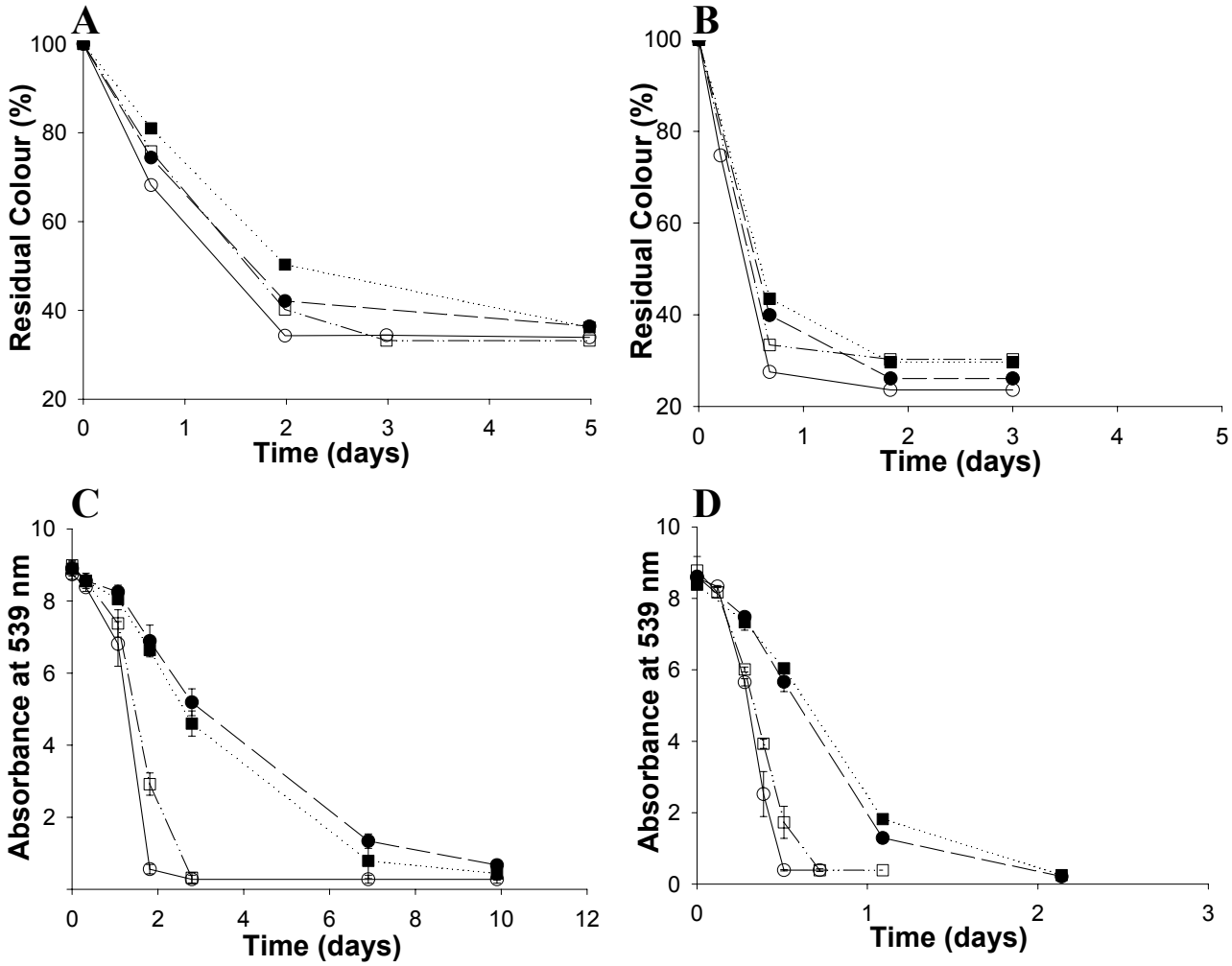

Figure 6.4. Colour removal of industrial wastewater at 30ºC (A) and 55ºC (B), and the azo dye RR2 at 30ºC (C) and 55ºC (D), in the presence of the redox mediators AQS (○), BQ (■) and BQ/AQS (□). Mediator-free bottles (●) controlled the impact of the redox mediators on colour removal. Negligible colour removal was verified in the sludge-free controls. Glucose (1.5 gCOD/l) was used as co-substrate. The mediators were tested both individually at concentrations of 0.050 mM or together at a ratio of 1:1, to give the same final concentration of 0.050 mM

Many different systems such as one or two-stage UASB reactors, EGSB reactors and rotating disk reactors have been tested at bench-scale for colour removal of textile wastewaters under mesophilic conditions (Tan et al., 2000; O'Neill et al., 2000b; Talarposhti et al., 2001; Libra and Sosath, 2003). In most studies however, long HRTs (up to 2 days) were required for colour removal efficiencies in the range of 60-80%. Recently, Van der Zee et al. (2001a) showed that 85% decolourisation of the azo dye RR2 was possible in a mesophilic UASB system operated at an HRT of 6 h and supplemented with 0.019 mM of AQDS. Furthermore, Cervantes et al. (2001) found about 88% colour removal while treating the azo dye Acid Orange 7 in a mesophilic UASB reactor supplemented with 0.003 mM of AQDS and operated at an HRT of only 2 h. Although addition of redox mediators distinctly accelerates decolourisation, for textile wastewaters with high dye concentrations the continuous redox mediator dosage will represent a high fixed cost to the wastewater treatment plants. Alternative approaches such as dialysis tubes/ion-exchanger (Laszlo, 2000), activated carbon (Van der Zee et al., 2003b) and henna leaves (Rau et al., 2002a) have been investigated to immobilize the redox mediators in reactors. In the current investigations we clearly demonstrate that the electron transfer capacity is significantly improved by increasing the operation temperature to the thermophilic range, thus decreasing the demand of a redox mediator. For instance at an HRT of 2.5 h and in the absence of AQDS, the colour removal was 5.3-fold higher at 55ºC. This phenomenon is of considerable interest if one realizes that textile wastewaters are generally discharged at high temperatures. Therefore, a very compact thermophilic reactor could be an option as a pre-treatment unit for textile wastewaters. Furthermore, the improvement on electron transfer capacity due to the temperature increase also brings good perspectives for the application of thermophilic anaerobic treatment to other reductive transformations such as nitroaromatics reduction and dehalogenations.

Feasibility of coupling colour removal and COD removal in bioreactors treating high concentration of dyes

In the current study at 30ºC granular sludge was used to assess colour removal at different ratios of dyebath and rinsing water. The objective of this experiment is to elucidate whether or not colour removal and COD removal can be coupled in anaerobic reactors treating high concentrations of dyes. Colour removal by using methanogenic anaerobic granular sludge is a result of complex biochemical interactions of microorganisms. The decolourisation can be the result of either a direct enzymatically catalysed reaction or an indirect mechanism via reduced

enzyme cofactors (Van der Zee et al., 2003a) of the different trophic groups present in the granular sludge, i.e. during the steps of hydrolysis, acidogenesis, acetogenesis and to some extent during methanogenesis. The full conversion of the primary electron donor into CH_4 is possible only if there is an equilibrium among those groups of microorganisms, in which the degree of dependence among them varies considerably (Schink, 2002).

Results clearly show that changes in decolourisation due to the increase in concentrated dyebath to rinsing water ratio were less pronounced than the effect of this increasing ratio on methane formation (Table 6.3). For low dyebath portions the CH_4 production was higher than in the dye-free control. However, the CH_4 production of the wastewater-free control was 4-times higher compared to the ratio 2.0:98.0. This stimulatory/inhibitory behaviour in terms of CH_4 production is in accordance with Beydilli et al. (1998) who found 20% increase in CH_4 production for 300 mg/l of RR2 followed by a sharp inhibition of the methanogens. The CH_4 production rate was about 59% of the dye-free control bottles after increasing the RR2 concentration to 500 mg/l of RR2. This sharp decrease was not surprising since methanogens are generally the most sensitive group to toxic compounds in the above-mentioned food chain (Van Lier et al., 1993b). Talarposhti et al. (2001) reported that the acetoclastic methanogens were inhibited during the treatment of a simulated textile wastewater in a two-phase anaerobic packed bed reactor with tapioca as co-substrate. The equivalent decolourisation rates in Table 6.3 suggest that the reducing equivalents required to cleave the azo bond were generated during glucose oxidation, as glucose oxidizers were not affect by the different ratios of wastewater tested. This is in agreement with Chinwetkitvanich et al. (2000) who mentioned that the acid forming bacteria could play an important role in the anaerobic decolourisation during a two-phase UASB system also using tapioca as co-substrate.

The CH_4 production in the AQS-free bottles was about 1.2-times higher than those supplemented with AQS (0.050 mM). We previously observed (Dos Santos et al., 2005a) that the anthraquinone dye Reactive Blue 19 (RB19) was not toxic to the acidogens, but extremely toxic to the acetate-utilizing methanogens in mesophilic granular sludge. As AQS is an anthraquinone-based molecule, its toxicity on methanogens is consistent with the observed toxicity of RB19, which is probably induced by the anthraquinone moiety. Interestingly, in the current continuous flow experiment at 30ºC, AQDS at a concentration of 0.025 mM did not have any toxic effect on methanogens, as the CH_4 productions between the reactors AQDS-free and AQDS-supplemented were similar. This is in agreement with Cervantes et al. (2003)

who observed a rapid methanogenic activity recovery even using an AQDS concentration that is about 500-times higher than the AQS concentration used in the present investigation. The question why AQS is more toxic to methanogens than AQDS remains unclear.

Colour removal in the presence of a mix of mediators with different standard redox potentials (E_0')

During indirect azo reduction, non-specific enzymes firstly reduce redox mediators, as the direct electron acceptor of the primary electron donor. Secondly, the electrons are chemically transferred to the azo dye, the terminal electron acceptor, with consequent mediator regeneration (Keck et al., 1997). A textile wastewater containing many dyes with different redox potentials was used in mesophilic and thermophilic incubations to assess the decolourisation rate-limiting step, i.e. the enzymatic mediator reduction versus the chemical electron transfer from the mediator to the dye. Compared to AQS (E_0' of -225 mV) supplemented assays, incubations supplemented with BQ (E_0' of 280 mV) did not accelerate colour removal with textile wastewater. This was probably due to the inability to transfer electrons from its reduced form (hydrobenzoquinone) to the dyes present in the wastewater, even though the first part of the reaction may have been accelerated by comparing the E_0' of AQS and BQ. This is an agreement with Rau et al. (2003) who concluded that mediators with E_0' higher than –50 mV will not efficiently reduce the azo bond at high rates.

When the mediators BQ and AQS were incubated together there was no improvement on the decolourisation rates in comparison with the bottles solely supplemented with AQS, even though a broader range of the electron transfer mediating capacity could be covered in the solution with dyes (Meckstroth et al., 1981). Therefore, either the concentration of mediator with negative E_0' (AQS) was driving the decolourisation rates, or the redox potential in the solution switched to a value that the reduced form of BQ could transfer the electrons more efficiently both directly to the dye and to AQS in a sequential reaction involving mediators and dye. The first hypothesis is in line with Laszlo (2000) who verified more negative redox potential values by increasing the AQS concentration in a reactor containing *Burkholderia cepacia*. Our present results showed that the use of mixed redox mediators with positive and negative E_0' under anaerobic conditions to cover the wide range of dyes-redox potential is not an efficient approach to improve colour removal in textile wastewaters.

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7

The transformation and toxicity of anthraquinone dyes during thermophilic (55ºC) and mesophilic (30ºC) anaerobic treatments

Abstract

We studied in batch assays the transformation and toxicity of anthraquinone dyes during incubations with anaerobic granular sludge under mesophilic (30ºC) and thermophilic (55ºC) conditions. Additionally, the electron shuttling capacity of the redox mediator anthraquinone-2-sulfonic acid (AQS) and subsequent increase on decolourisation rates was investigated on anthraquinone dyes. Compared to incubations at 30ºC, serum bottles at 55ºC presented distinctly higher decolourisation rates not only with the industrial textile wastewater containing anthraquinone dyes, but also with model compounds. Compared to batch assays at 30ºC, the first-order rate constant "k" of the Reactive Blue 5 (RB5) was enhanced 11-fold and 6-fold for bottles at 55ºC supplemented and free of AQS, respectively. However, the anthraquinone dye Reactive Blue 19 (RB19) demonstrated a very strong toxic effect on volatile fatty acids (VFA) degradation and methanogenesis at both 30ºC and 55ºC. The apparent inhibitory concentrations of RB19 exerting 50% reduction in methanogenic activity (IC_{50}-value) were 55 mg/l at 30ºC and 45 mg/l at 55ºC. Further experiments at both temperatures revealed that RB19 was mainly toxic to methanogens because the glucose oxidizers including acetogens, propionate-forming, butyrate-forming and ethanol-forming microorganisms were not affected by the dye toxicity.

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1. Introduction

Anthraquinone dyes constitute the second largest class of textile dyes, after azo dyes (Baughman and Weber, 1994). Normally to obtain a target colour, a mix of dyes, e.g. red, yellow and blue, is applied in dyebaths. These dyes might contain different chromophores, of which azo, anthraquinone and phtalocyanine dyes are the most important groups (Hao et al., 2000). Anthraquinone dyes give a wide range of colours in almost the whole visible spectrum, but they are most commonly used for violet, blue and green colours (Christie, 2001; Fontenot et al., 2002). Azo dyes, on the other hand, are mostly used for yellow, orange and red colours (Christie, 2001). Because anthraquinone dyes are widely applied, they might be present in considerably or relatively high concentrations in wastewaters originating from textile factories. Despite this, biological and physical-chemical investigations applied on decolourisation of anthraquinone dyes are not that frequent in comparison with those carried out with azo dyes. A large portion of textile wastewaters, particularly those coming from dyebaths, is discharged at high temperatures (40-70ºC). However, thermophilic anaerobic decolourisation of anthraquinone dyes by granular sludge has never been studied before.

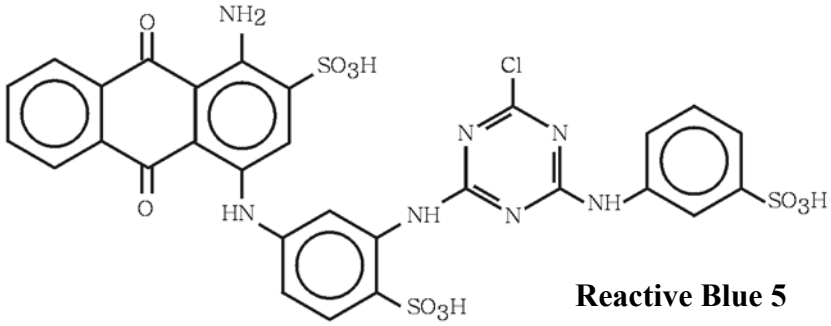
Quinone-based redox mediators have been shown to accelerate the transfer of reducing equivalents from a primary electron donor to an azo dye, and a distinct improvement on decolourisation rates has been observed in bioreactors (Cervantes et al., 2001; Dos Santos et al., 2003; Dos Santos et al., 2005b). These redox mediators are very effective for azo dye reduction, very likely due to the nature of the azo chromophore ─N═N─, which is electronically unstable and has the capacity to receive electrons from the reduced form of the mediator. However, anthraquinone dyes are electronically stable and as a result, the reduced form of the above-mentioned mediator will likely be less effective in transferring electrons to the dye. In the present investigation, the impact of a quinone-based redox mediator on colour removal of anthraquinone dyes is evaluated. Additionally, the transformation and toxicity of anthraquinone dye model compounds in incubations with anaerobic granular sludge under mesophilic (30ºC) and thermophilic (55ºC) conditions are investigated in batch assays. Finally, a comparative study between mesophilic and thermophilic decolourisation of anthraquinone dye-containing wastewater originating from a textile factory is performed.

2. Materials and Methods

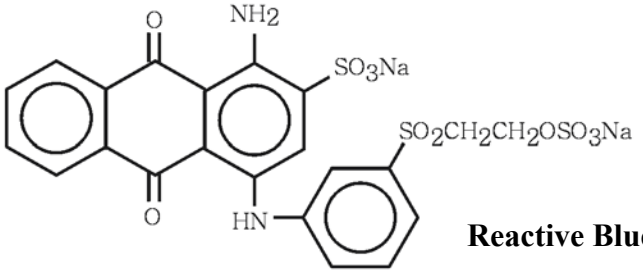
2.1. Chemicals

Reactive Blue 5 (RB5) and Reactive Blue 19 (RB19) were selected as anthraquinone dye model compounds (Figure 7.1). Anthraquinone-2-sulfonate (AQS) was selected as redox

mediator model compound (Chapter 5). Chemicals were purchased from Aldrich (Gillingham, UK), Sigma (Bornem, Belgium) or Acros (Geel, Belgium) and used without additional purification.



Reactive Blue 5



Reactive Blue 19

Figure 7.1. Molecular structure of the anthraquinone dye model compounds RB5 and RB19.

2.2. Seed inoculum and basal medium for decolourisation assays

Granular anaerobic sludge was collected from a full-scale mesophilic upflow anaerobic sludge blanket (UASB) reactor treating paper mill wastewater (Eerbeek, The Netherlands). The mesophilic sludge was acclimated to 55ºC as described in Chapter 3. For batch tests at 30ºC the same mesophilic granular sludge was first acclimated in an EGSB reactor (30ºC) as described in Chapter 3. Stable efficiencies in terms of COD removal for the mesophilic and thermophilic reactors during stable conditions were 95% and 85%, respectively. The influent stock solution was free of dye and AQS during the whole period. The basal medium for the tests with model compounds was described in Chapters 3,4,5. In decolourisation assays with the industrial wastewater, the wastewater was tested undiluted and without addition of nutrients or trace elements. The pH was adjusted to 7 with NaOH or HCl.

2.3. Activity Tests

In the activity tests 1.3 ± 0.1 gVSS/l of the previously described stabilized sludge were added to 117 ml serum bottles with 50 ml basal medium. The bottles were then sealed with butyl rubber stoppers and aluminium crimp caps. Anaerobic conditions were established by flushing

the headspace with N_2/CO_2 (70%:30%) and 2 gCOD/l co-substrate (VFA mixture or glucose), dyes (variable) and AQS (variable) were added. The neutralized VFA solution contained acetate, propionate and butyrate at a COD ratio of 1:1:1. Sterile controls were autoclaved once at 122ºC for 240 min and again following a 5 days incubation period, after which sterile co-substrate, mediator and dye stock solutions were added. The pH and the amount of VSS were determined after completion of the experiment.

Decolourisation assays with wastewater originating from a textile industry

The wastewater used for the decolourisation assays was a dark acid polyamide dye wastewater from a textile factory located near Gent, Belgium. It consisted of a mix of streams from the dyebath, fixation and rinsing steps. A co-substrate concentration of 2 gCOD/l (glucose:VFA mixture at a COD ratio of 1:3) ensured the supply of primary electron donors. To evaluate the electron shuttling effect of a redox mediator to anthraquinone dyes, AQS (0.5 mM) was added to some bottles. Both mesophilic and thermophilic acclimated sludges were incubated with the wastewater at a concentration of 1.3 ± 0.1 gVSS/l. Abiotic colour removal was assessed with both autoclaved sludge and sludge-free controls.

Decolourisation assays and standardized activity tests with model compounds

The anthraquinone dyes RB5 and RB19 were selected for decolourisation assays and added in varying concentrations in standardized activity tests under mesophilic and thermophilic conditions. A VFA mixture at a concentration of 2 gCOD/l was used as co-substrate. To evaluate the electron shuttling effect of a redox mediator to anthraquinone dyes, AQS (0.012 mM) was added to some bottles. Abiotic colour removal was assessed with both autoclaved sludge and sludge-free controls.

2.4. Analysis

For anthraquinone model compounds colour removal was determined photometrically (Spectronics 60, Milton-Roy Analytical Products Division, Belgium) as described in Chapter 3. The absorbance was read at the maximum absorbance wavelength, i.e. RB5 at 602 nm and RB19 at 593 nm. The extinction coefficients used (AU cm^{-1} per mM) were 8.2 and 9.5 for RB5 and RB19, respectively. Wastewater decolourisation was determined as described in Chapter 5.

Methane, VFA, methanol, ethanol, sucrose, fructose, glucose, lactate, formate and VSS determinations were described in Chapter 3.

3. Results

Decolourisation assays with anthraquinone dye-containing wastewater

The capacity of anaerobic granular sludge to decolourise an anthraquinone dye-containing wastewater was tested. The results demonstrate that the reductive decolourisations were enhanced at 55ºC compared to 30ºC (Fig. 7.2A and 7.2B). In the presence of the redox mediator, AQS, and assuming a first-order reaction, the first-order rate constant "k" was 0.19 day^{-1} at 55ºC and 0.08 day^{-1} at 30ºC, showing a 2.6-fold enhancement in decolourisation rates at 55ºC. In the absence of AQS the impact of temperature was even greater (6.7-fold increasing), as expressed in the k-value 0.20 day^{-1} at 55ºC and 0.03 day^{-1} at 30ºC. Metabolic activity of the inoculum was very likely the main mechanism of colour removal, as a negligible decolourisation was observed in the sludge-free controls, and higher rates of colour removal were observed in the bottles supplemented with co-substrate. As predicted, the redox mediator AQS at a concentration of 0.5 mM had no significant impact on colour removal rates under thermophilic conditions, although mesophilic decolourisation was increased 2.6-fold by AQS addition. Nevertheless, both rates at 30ºC were significantly lower than those obtained at 55ºC (Fig. 7.2A and 7.2B).

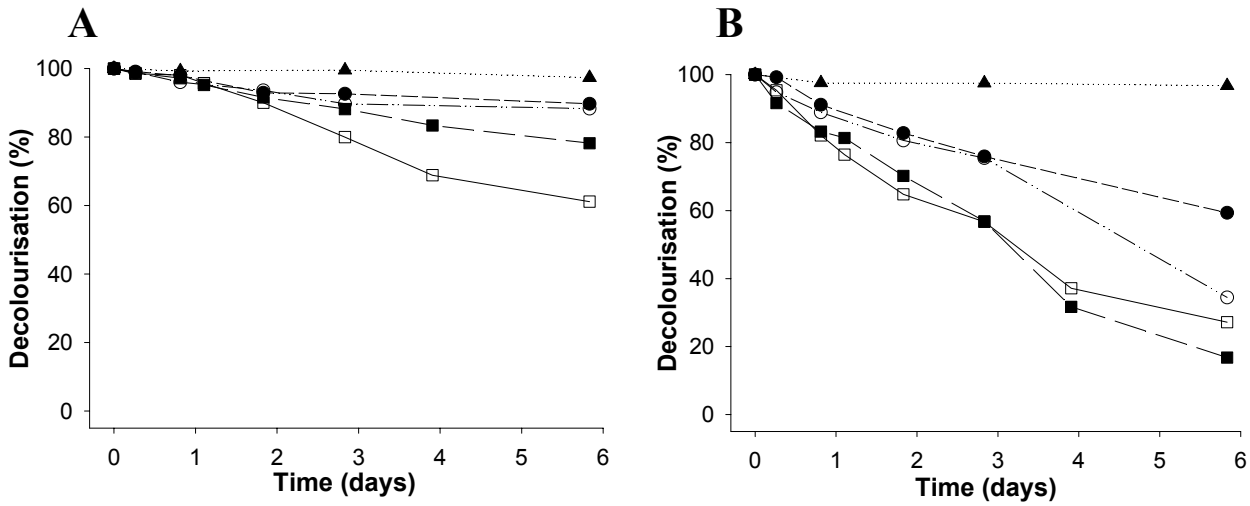


Figure 7.2. Decolourisation of the textile wastewater at 585 nm by mesophilic (A) and thermophilic (B) granular sludge added with wastewater (●), wastewater/AQS (○), wastewater/co-substrate (■), wastewater/co-substrate/AQS (□). Sludge-free bottles controlled the abiotic colour removal (▲). AQS (0.5 mM) was added to some bottles and 2 gCOD/l co-substrate. The results are means of duplicate incubations. The standard deviation was lower than 5% in all cases.

Decolourisation assays and standardized activity tests with model compounds

Decolourisation assays with Reactive Blue 5

Decolourisation assays with RB5 clearly confirm that a complete colour removal was only possible at 55ºC (Fig. 7.3). Assuming a first-order reaction in the presence of AQS, the k-values were 0.33 day^{-1} at 55ºC and 0.03 day^{-1} at 30ºC, a 11-fold enhancement due to the temperature increase. In the absence of AQS, the k-values were 0.18 day^{-1} at 55ºC and 0.03 day^{-1} at 30ºC, a 6-fold difference in decolourisation rates. The complete decolourisation at 55ºC was likely irreversible as no colour resurgence was seen after exposing the sample to oxygen (data not shown). Contrary to the experiments with textile wastewater, AQS at a concentration of 0.012 mM supplemented with 23.2 mg/l of RB5 enhanced 1.8-fold the decolourisation rate at 55ºC (Fig. 7.3). However, at high concentrations of RB5, e.g. 232 mg/l, the same concentration of AQS did not have any effect on colour removal (data not shown). In mesophilic bottles (30ºC) supplemented with 23.2 mg/l of RB5, the decolourisation rates were 0.035 day^{-1} and 0.044 day^{-1} for the AQS-free and AQS-supplemented incubations, respectively (Fig. 7.3). Therefore, AQS had a very slight impact on colour removal. Negligible (< 3%) colour removal occurred in sludge-free controls in the presence of AQS (results not shown) at 30ºC and 55ºC.

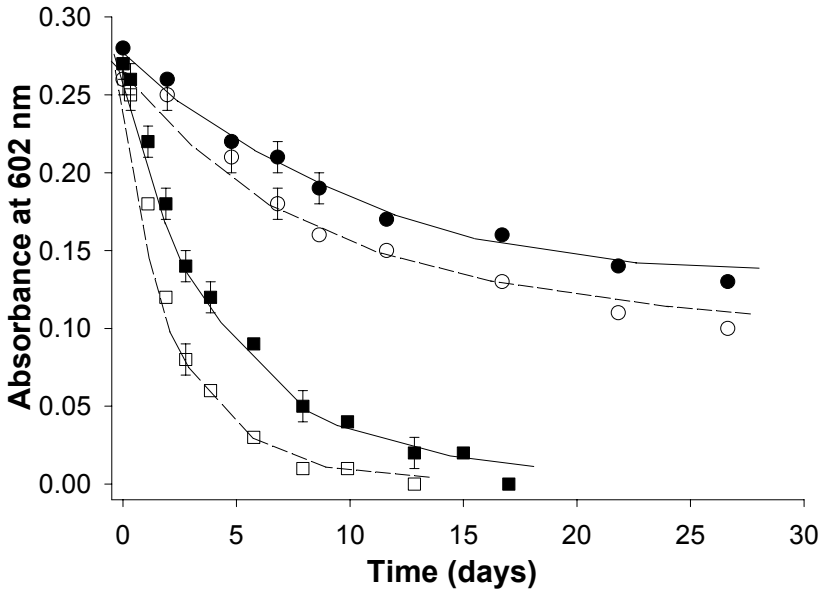


Figure 7.3. Colour removal of the anthraquinone dye Reactive Blue 5 (23.2 mg/l) under mesophilic and thermophilic conditions, either in the presence or absence of AQS (0.012 mM). Symbols for: AQS-free (●) and AQS-supplemented (○) at 30ºC; and AQS-free (■) and AQS-supplemented (□) at 55ºC. A VFA mixture (2 gCOD/l) was used as co-substrate. Sludge-free controls were used to assess the stability of the basal medium and contained RB5, AQS and co-substrate. The results are means of duplicate bottles and the bars indicate the standard deviation.

For the VFA mixture added as co-substrate, the conversion of acetate and butyrate to methane was almost complete at 30ºC and 55ºC (Table 7.1). Such a conversion corresponds to an excess of reducing equivalents required to completely reduce the dye. For instance, if just the conversion of acetate to methane is considered, consumption of less than 1% of the acetate present is required to reduce all dye molecules, if all reducing equivalents from acetate conversion are preferentially channelled to the anthraquinone group. Therefore, the difference between the potential for colour removal at 30ºC and 55ºC was not due to a lack of reducing equivalents, but it may be rather associated with differences in the rate of electrons transfer. Table 7.1 shows that the oxidation of propionate is affected by the presence of AQS (0.012 mM) at both temperatures. Moreover, propionate conversion at 55ºC ceased for concentrations of RB5 above 23.2 mg/l.

Table 7.1. Results of the standardized activity test for different concentrations of the anthraquinone dye Reactive Blue 5 during mesophilic (30º) and thermophilic (55º) incubations with anaerobic granular sludge.

Mesophilic

RB 5 (mg/l)	**COD Balance (mg/l)**				
	Acetate	**Propionate**	**Butyrate**	**Methane**	**Total**
Control	24	-	-	1900	1924
23.2	24	-	-	2280	2304
23.2+AQS	27	595	-	1568	2190
69.6	23	-	-	1840	1862
116.1	25	-	-	1857	1882

Thermophilic

RB 5 (mg/l)	**COD Balance (mg/l)**				
	Acetate	**Propionate**	**Butyrate**	**Methane**	**Total**
Control	45	46	-	1897	1987
23.2	24	115	-	2135	2273
23.2+AQS	76	682	-	1508	2265
69.6	75	668	-	1310	2052
116.1	59	696	-	1157	1911

A concentration of 2 gCOD/l of a VFA mixture (1:1:1 at a COD basis) was used as co-substrate. Anthraquinone-2-sulfonate (0.012 mM) was added to some of the bottles. Mean of duplicate bottles, with the standard deviation lower than 10% in all cases. The molecular weigh of RB5 is 774.2 g/mol.

In the absence of granular sludge, no colour removal was observed at 55ºC either in the presence or absence of AQS during a pure chemical reduction of RB5 (232 mg/l) with sulphide as potential reducing agent (4.5 mM, pH 7.2) (Figure 7.4). These results indicate that the biogenically produced sulphide does not significantly contribute to the decolourisation of this type of dye. Furthermore, this experiment suggests that although AQS may be reduced by sulphide, the expected reduced form of AQS (AH_2QS) does not have the capacity of transferring the electrons to the dye, making it the rate-limiting step in the process. On the other hand, a complete decolourisation of the azo dye Reactive Red 2 (RR2, 0.3 mM) was observed both in the presence and absence of AQS with the same sulphide concentration. The presence of AQS was found to increase the decolourisation rates 1.7-fold (Figure 7.4).

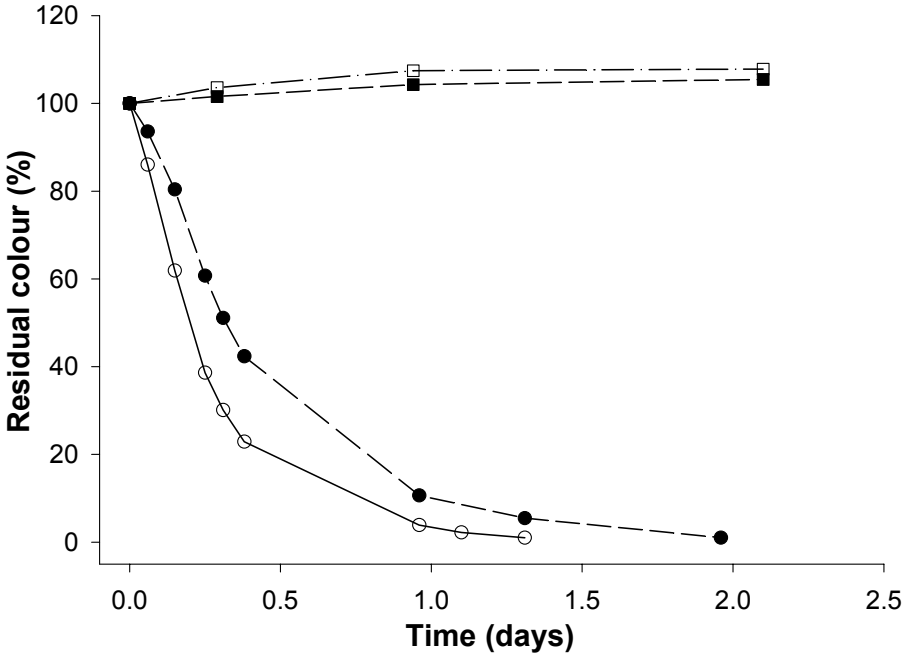


Figure 7.4. Chemical decolourisation at 55ºC in sludge-free bottles of the anthraquinone dye Reactive Blue 5 (0.3 mM) and the azo dye Reactive Red 2 (0.3 mM), by using sulphide (4.5 mM) as reducing agent. Symbols for: RR2 AQS-free (●) and AQS-supplemented (○); RB5 AQS-free (■) and AQS-supplemented (□). AQS (0.012 mM) was either present or absent in the bottles. The results are means of duplicate bottles. The standard deviation was lower than 5% in all cases.

Decolourisation assays with Reactive Blue 19

Although a complete colour removal of RB19 was achieved at both 30ºC and 55ºC (data not shown), the experiments revealed that decolourisation was mainly due to abiotic mechanisms.

Both a considerable adsorption on the granules and formation of precipitates in the medium were observed.

A very strong toxic effect on both the VFA degradation and methanogenesis was also observed for both inocula even when low concentrations of RB19 (37.5 mg/l) were supplied. Figure 7.5 demonstrates the methanogenic inhibition relative to the RB19-free control. The apparent inhibitory concentrations for RB19 exerting 50% reduction in methanogenic activity (IC_{50}-value) were about 55 mg/l at 30ºC and 45 mg/l at 55ºC. If the occurring precipitation of RB19 is taken into account, the actual IC_{50} is even lower than the mentioned apparent values. Despite the more complex molecular structure of RB5 compared to RB19 (Figure 7.1), the toxicity of RB19 in the inoculum was more pronounced than for RB5 (Figure 7.5 and Table 7.1).

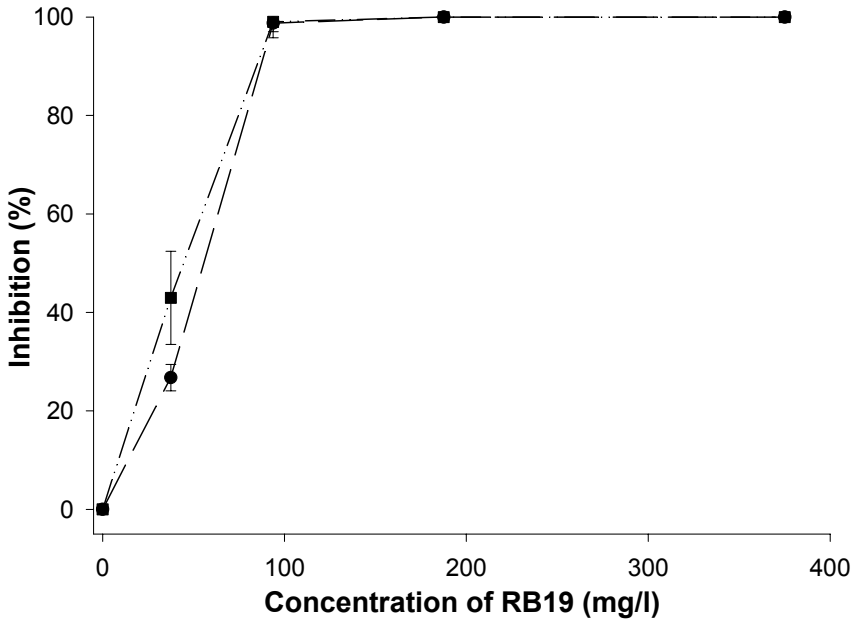


Figure 7.5. Methane production inhibition (%) relative to the control (dye-free) under mesophilic (●) and thermophilic (■) conditions after 11 days time elapsed, during experiments with RB19. A VFA mixture (2 gCOD/l) was used as co-substrate. The inhibitions are referred to the initial concentration of RB19. The results are means of duplicate bottles and the bars indicate the standard deviation.

Experiments were also conducted at 30ºC and 55ºC to verify the reversibility of the toxicity induced by RB19 (93.9 mg/l). The medium was replaced by a new medium free of RB19 and supplemented with VFA. The methane formation was compared to the control, i.e. free of RB19 for the whole period. As shown in Figure 7.6 for incubations at 30ºC, the

toxicity was indeed irreversible because no methane formation was detected after replacing the medium containing RB19. In experiments conducted at 55ºC the same toxic effect on methanogenesis was observed.

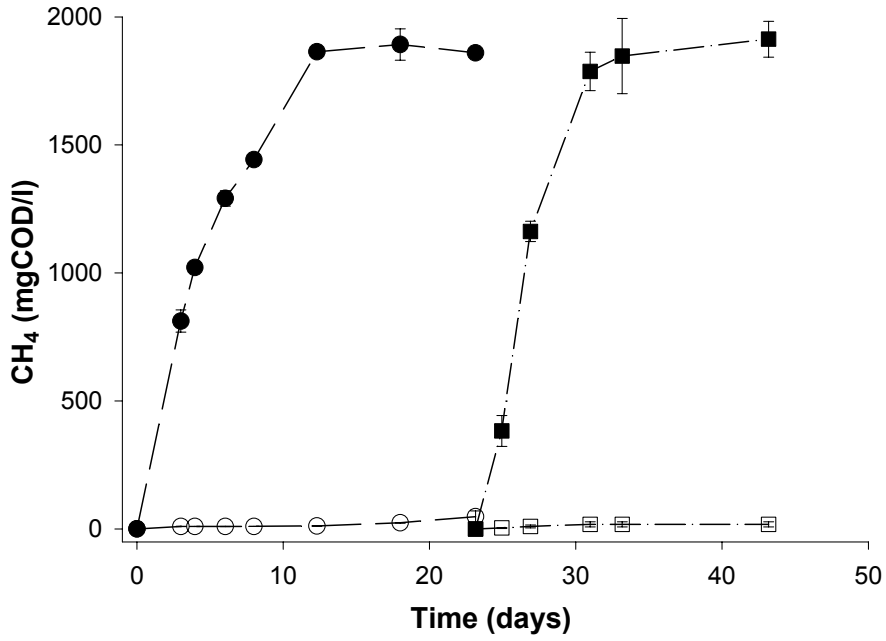


Figure 7.6. Evaluation of the permanent toxicity of Reactive Blue 19 under mesophilic conditions relative to the control (dye-free). Symbols for: dye-free control (●) and 93.9mg/l of RB19 (○); control after re-feeding (■) and RB19 after re-feeding (□). A VFA mixture (2 gCOD/l) was used as co-substrate. The results are means of duplicate bottles and the bars indicate the standard deviation.

Further experiments focused on the evaluation of which group of microorganisms was mainly affected by the toxicity of RB19. Glucose (1.5 gCOD/l) was selected as co-substrate and was tested at 30ºC either in the presence or absence of RB19 (93.9 mg/l) and AQS (0.012 mM) in standardized activity tests. Results revealed that the glucose oxidizers, including acetogens, propionate-forming, butyrate-forming and ethanol-forming microorganisms, were not affected by RB19, as all glucose was consumed in all bottles after 1 day of incubation. In the absence of RB19 the produced acetate (Figure 7.7A) was immediately converted into methane (Figure 7.7B). However, in the RB19-containing bottles acetate accumulated (Figure 7.7A), and methanogenesis ceased after 3 days of incubation (Figure 7.7B). This indicated that the acetate-utilizing methanogens was the most important group affected by RB19, which was also verified during experiments at 55ºC.

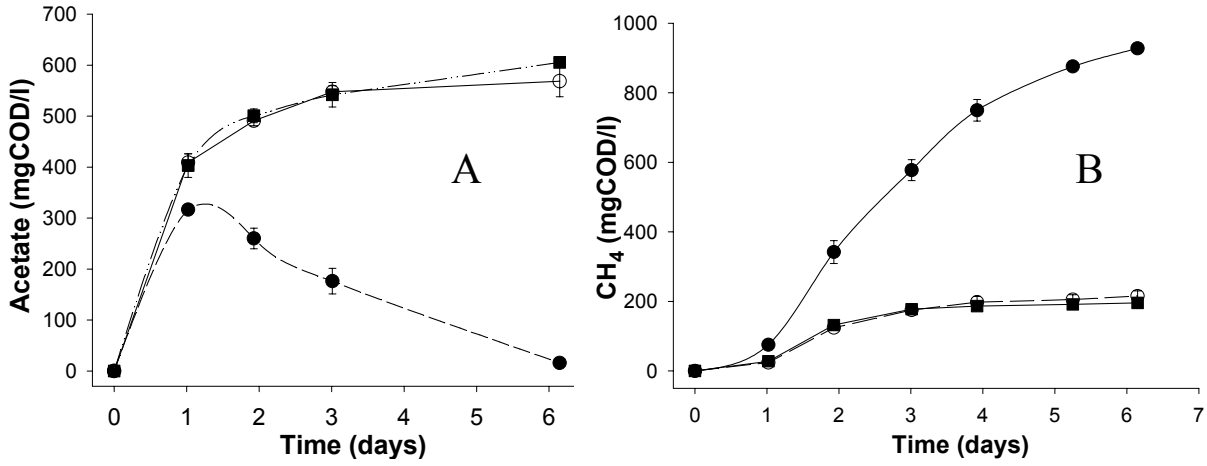


Figure 7.7. Acetate concentration (A) and CH_4 formation (B) during investigations with glucose (1.5 gCOD/l). RB19 (93.9 mg/l) and AQS (0.012 mM) were added to some bottles. Symbols for: dye-free control (●), RB19 AQS-free (○), RB19 AQS-supplemented (■). The results are means of triplicate bottles and the bars indicate the standard deviation.

4. Discussion

Results from batch experiments reveal that in comparison with mesophilic treatment at 30ºC, thermophilic treatment at 55ºC presents distinctly higher decolourisation rates with both textile wastewater and with the model compound RB5. A complete colour removal of the anthraquinone model compound RB5 was only possible at 55ºC. Additionally as hypothesized, the redox mediator AQS did not significantly increase the decolourisation rates of anthraquinone dyes. The few publications on anaerobic anthraquinone dye decolourisation under mesophilic conditions are not very consistent (Table 7.2). Our results under mesophilic conditions are in accordance with Carliell et al. (1994) who reported that compared to azo dyes, other dyes such as anthraquinone and phtalocyanine, are less susceptible to reduction and present very low colour removal capacity. Moreover, Fontenot et al. (2002) achieved an incomplete decolourisation, i.e. 78% colour removal of the anthraquinone dye Reactive Blue 4, in both amended and un-amended cultures at 35ºC. In our experiments with the industrial textile wastewater, the temperature increase from 30ºC to 55ºC distinctly enhanced the decolourisation rates. This was probably due to an improvement of the colour removal capacity not only for the anthraquinone dyes, but also for the azo dyes present in the wastewater. For instance, compared to anaerobic incubations at 30ºC, the decolourisation of the azo dye Reactive Red 2 at 55ºC was enhanced sixfold in the absence of the external mediator anthraquinone-2,6-disulfonate (AQDS) (Dos Santos et al., 2004c).

Table 7.2. Investigations on anaerobic colour removal and toxicity of anthraquinone dyes reported in the literature.

Dye	Decolour. (%)	Additional information	Reference
Reactive Blue 4	78	After 27.7 days. CH_4 production comparable to control	Fontenot et al., 2002
Reactive Blue 5	68	After 16 days. C = 20 mg/l	Luangdilok and Paswad, 2000
	37	After 16 days. C = 100 mg/l	
Reactive Blue 19	83	After 27.7 days. CH_4 production severely inhibited	Fontenot et al., 2002
	57, 32	After 16 days. C = 20 mg/l	Luangdilok and Paswad, 2000
	30-100	Average: 70%	Brown and Laboureur, 1983
Reactive Blue 49	7-10	After 2 h	Carliell et al., 1994
Acid Blue 80	4-16	Average: 7%	Brown and Laboureur, 1983
Acid Blue 25	36-100	Average: 67% Insoluble blue pigment formed	Brown and Laboureur, 1983
	100	After 21 days. 1:1 formation of 1-amino-4-phenyl-aminoanthraquinone (blue solid)	Brown and Hamburger, 1987
Basic Blue 22	46-86	Average: 62%	Brown and Laboureur, 1983
Disperse Red 159	0	After 3 days. C = range 300-2400 mg/l. Rate inhibited by 60% at 300 mg/l and by >90% at 2400 mg/l	Malpei et al., 1998
Disperse Blue 56	0	C = 30 mg/l dye made UASB reactor collapse	Delee et al., 1998

Our observation that the decolourisation of RB5 by a purely chemical reaction with sulphide was not possible confirms that the anthraquinone link at neutral pH is indeed electronically very stable, making the nucleophilic attack by sulphide inefficient. Particularly in the case of RB5, the hydrogen bond formed between the carbonyl group of the quinone substituent and the amino substituent likely precludes the RB5 molecule from receiving an additional electron. On the other hand, the complete decolourisation of the azo dye RR2 at neutral pH by sulphide suggests that the azo link is electronically less stable and therefore susceptible for reductive cleavage. It is reported that the biogenic sulphide may play an important role as a reductant and contribute to the decolourising processes (Yoo, 2002; Van der Zee et al., 2003a). However, for dyebaths composed of a mix of dyes, e.g. anthraquinone and azo dyes, the purely chemical decolourisation by the biogenic sulphide will occur mostly with azo dyes, as anthraquinone dyes are shown extremely stable. Generally, the low decolourisation rate of anthraquinone dyes is attributed to toxic effects, which display

different inhibition levels on anaerobic inocula (Table 7.2). It was verified that less than 100 mg/l of RB19 was enough to completely inhibit methanogenesis of both mesophilic and thermophilic inocula with a VFA co-substrate.

Contrarily, it was observed that glucose oxidizers were not affected by RB19 toxicity, as no trace of glucose (1.5 gCOD/l) was detected after one day of incubation when this sugar was supplemented as co-substrate. Anthraquinone dyes are also toxic to sulphate reducers (Lie et al., 1996), but the effects on methanogens seem to be less predictable (Cooling III et al., 1996). An explanation for the toxicity is that the active site of enzymes can be occupied by anthraquinone dye molecules, thus blocking the binding of substrate and cofactors (Prestera et al., 1992; Denizli and Piskin, 2001). Cooling III et al. (1996) proposed another inhibition mechanism, which was the uncoupling of electron transfer from ATP synthesis via an anthraquinone-mediated electron transfer reaction. Nevertheless, in the current experiment acetate was one of the most important glucose oxidation products that accumulated in the bottles when RB19 was present. This was an indication that the acetyl-CoA pathway used by almost all acetate forming microorganisms was not blocked by the dye (Diekert, 1991). On the other hand, methane production ceased in the RB19-supplemented bottles, suggesting the blockage of methanogenesis pathway by the dye. It was found that the toxicity was permanent in terms of methane production, and that just the removal of RB19 from the medium was not enough to re-establish the enzyme functionality. This is in accordance with the above-mentioned observation of Denizli and Pişkin (2001) that the dye-enzyme bond could not easily be broken. In the latter study the use of an elutant to break the enzyme bond did reverse the process. Delée et al. (1998) reported that the dye Disperse Blue 56 severely inhibited the inoculum of a lab scale UASB reactor, causing a process collapse, although it had been decolourising other dyes successfully. With RB19, a complete decolourisation at 30ºC and 55ºC was observed, which was mainly attributed to abiotic mechanisms. This conclusion is initially based on the observation that the reducing equivalents required to remove the colour were likely not formed, due to the inhibitory effect of RB19 on VFA conversion and methanogenesis. Therefore, the sole maintenance of reducing conditions was not enough to remove the colour. This contradicts the conclusions of Fontenot at al. (2002) who attributed the decolourisation of RB19 to the reduced conditions in the medium despite of a severe inoculum inhibition. The abiotic mechanism in the current experiment consisted of a considerable adsorption of RB19 on the granules and the formation of precipitates. This is in accordance with the results of Luangdilok and Paswad (2000) who reported that the decolourisation of RB 19 was only due to biomass adsorption, and that no biotransformation

of the dye could be observed. Brown and Hamburger (1987) also found a blue precipitate while investigating the reduction of Acid Blue 25 under anaerobic conditions. The formation of precipitates is a result of the pure chemical release of the reactive species of reactive anthraquinone dyes, e.g. the release of the free amine from the protonated group of anthraquinone dye incubated under neutral to alkaline solutions (Mccallum et al., 2000). As the pH of the current RB19 incubations was neutral, our results are in agreement with the latter.

In summary, anthraquinone dyes present in textile wastewaters represent a potential problem to the applicability of cost-effective anaerobic treatment technologies or even chemical treatment based on a nucleophilic attack. Therefore, a better insight in both the transformation and toxicity mechanisms of anthraquinone dyes is required for closing process water cycles of all dye-containing wastewaters to avoid their discharge in the environment.

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We would like to thank Teris van Beek for the valuable discussion. This work was supported by "Conselho Nacional de Desenvolvimento Científico e Technológico – CNPq" (Project number 200488/01-5), an organization of the Brazilian Government for the development of Science and Technology. A part of this work was done in cooperation with the Lettinga Associates Foundation, within the EU project TOWEF0 nº EVK1-CT-2000-00063.

8

The contribution of acidogenic bacteria and methanogenic archaea to azo dye reduction by a thermophilic anaerobic consortium

Abstract

We studied the contribution of acidogenic bacteria and methanogenic archaea to azo dye reduction by a thermophilic anaerobic consortium. Additionally, the competition for reducing equivalents between methanogenesis and azo dye reduction was assessed. Our results indicated that acidogenic bacteria and methanogenic archaea play important roles in this reductive process. Experiments with the thermophilic methanogens *Methanothermobacter thermoautotrophicus* ΔH and a *Methanothermobacter*-related strain NJ1 revealed that these strains were unable to reduce the dye in the absence of the redox mediator, riboflavin. This suggested that anaerobic dye reduction is not a universal property among methanogenic archaea and that redox mediators may play an important role for allowing some microbial groups, commonly found in wastewater treatment systems, to participate in reductive decolourisation.

🖫 Dos Santos AB, Madrid MP, de Bok, FAM, Stams AJM, Cervantes FJ and van Lier JB (2005). Enzyme and Microbial Technology. Submitted.

1. Introduction

Dyes make the world more beautiful through coloured substances, but on the other hand they represent a serious pollution problem worldwide. Particularly the release of coloured compounds into water bodies is undesirable not only because of their aesthetic appearance and the impact of dyes on photosynthesis of aquatic plants, but also because many of these dyes and their breakdown products are carcinogenic (Weisburger, 2002). Reductive decolourisation of azo dyes can be readily achieved under anaerobic conditions either chemically or biologically. Chemical decolourisation may involve biogenic reductants, such as sulfide, cysteine, ascorbate or Fe^{2+} (Stolz, 2001; Yoo, 2002). Microbial decolourisation, on the other hand, requires an unspecific enzymatic capacity ubiquitously found in a wide diversity of microorganisms (Chung and Stevens, 1993). This has been demonstrated with intestinal microorganisms such as *Clostridium, Salmonella, Bacillus, Eubacterium* and *Escherichia coli*, which are able to reduce dyes ingested through food, drugs and cosmetics (Brown and DeVito, 1993; Rau et al., 2002a; Chen et al., 2004). However, little is known about the microbiological aspects of anaerobic consortia from wastewater treatment plants in the reductive decolourisation of azo dyes, although the applicability of the cost-effective high-rate anaerobic reactors for azo dye reduction has been well demonstrated (Dos Santos et al., 2003; Dos Santos et al., 2004c). A large portion of textile wastewaters, mainly from the dyebath and rinsing steps, is discharged at high temperatures (40-70ºC). However, thermophilic anaerobic azo dye reduction by either pure cultures or mixed cultures has been only briefly examined (Willetts and Ashbolt, 2000).

It was found that redox mediators can enhance the shuttling of electrons between the primary electron donor and the azo dye, which is normally the rate-limiting step during anaerobic azo dye reduction (Rau and Stolz, 2003). Flavin-based compounds like FAD, FMN and riboflavin, as well as quinone-based compounds like anthraquinone-2-sulphonate (AQS), anthraquinone-2,6-disulphonate (AQDS) and lawsone are examples of compounds with catalytic properties (Semdé et al., 1998; Rau et al., 2002a; Dos Santos et al., 2004a). Therefore, the dosage of redox mediators may represent an interesting option to increase the rates of electron transfer and subsequent reductive decolourisation in bioreactors. Initially non-specific enzymes reduce the mediator, with its chemical reoxidation by the azo dyes (Keck et al., 1997). The reduction of the mediator in whole cells, which can occur either intracellularly (Rau and Stolz, 2003) or extracellularly by a membrane-bond enzyme (Kudlich

et al., 1997), will depend on many factors such as its size, polarity, standard redox potential (E_0') and other physical-chemical characteristics (Dos Santos et al., 2004a).

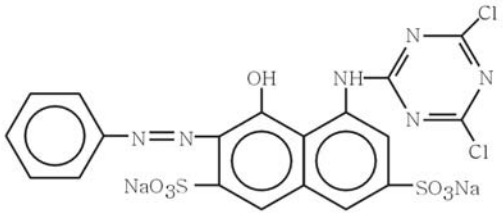
In the current investigation the contribution of acidogenic bacteria and methanogenic archaea to the reductive decolourisation of azo dyes by a thermophilic anaerobic consortium is studied. Additionally, the competition for reducing equivalents between methanogenesis and azo dye reduction is assessed in the presence of the methanogenic substrates acetate, H_2/CO_2, methanol and formate.

2. Materials and Methods

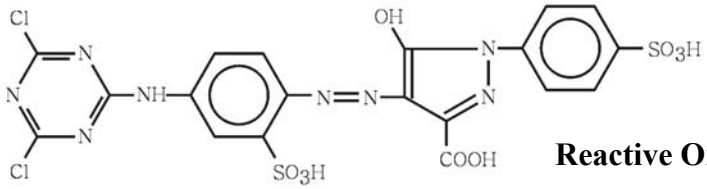
2.1. Chemicals

Reactive Red 2 (RR2), Reactive Red 4 (RR4) and Reactive Orange 14 (RO14) were selected as azo dye model compounds for the present study (Figure 8.1), because they presented the lowest rates of decolourisation among many azo dyes previously tested (Van der Zee et al., 2001b). Riboflavin (Vitamin B2) was selected as redox mediator model compound (Chapter 5). Vancomycin and 2-bromoethane sulphonic acid (BES) were used as inhibitors of acidogens and methanogens, respectively (Oremland and Capone, 1988; Paulo, 2002).

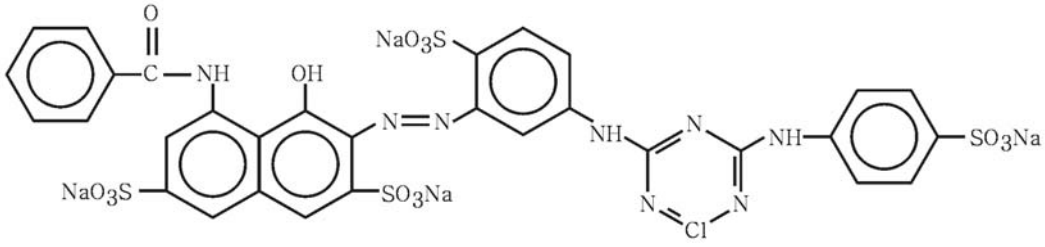
Chemicals were purchased from Aldrich (Gillingham, UK), Sigma (Bornem, Belgium) or Acros (Geel, Belgium) and used without additional purification.



Reactive Red 2 (RR2)



Reactive Orange 14 (RO14)



Reactive Red 4 (RR4)

Figure 8.1. Chemical structure of the azo dyes Reactive Red 2 (RR2), Reactive Red 4 (RR4) and Reactive Orange 14 (RO14), used as model compounds.

2.2. Seed inoculum and basal medium for decolourisation assays

Anaerobic granular sludge was collected from a full-scale mesophilic upflow anaerobic sludge blanket (UASB) reactor treating paper mill wastewater (Eerbeek, The Netherlands). The mesophilic sludge was acclimated at 55ºC until obtaining stable performance as described in Chapter 3.

2.3. Activity Test

In the activity tests 1.3 ± 0.1 g volatile suspended solids (VSS) l^{-1} of the thermophilic sludge was added to 57-ml serum bottles containing 25 ml basal medium, after which the bottles were sealed with butyl rubber stoppers. The composition of the medium is described in Chapter 4. Anaerobic conditions were established by flushing the headspace with N_2/CO_2 (70%:30%), after which the sludge was pre-incubated for 2-3 days with glucose (0.45 gCOD/l) as co-substrate. Then, the headspace was exchanged to N_2/CO_2 again (70%:30%), and the azo dyes (0.3 mM), riboflavin (0.012 mM) and different co-substrates (1.5 gCOD/l) were added. Sterile controls were autoclaved once at 122ºC for 240 min and again after 5 days incubation period, after which sterile electron donors, mediator and dye stock solutions were added. The pH and the amount of VSS were determined at the end of the experiment.

Contribution of acidogenic bacteria and methanogenic archaea to azo dye reduction

To evaluate the contribution of acidogens and methanogens to azo dye reduction, an experiment in the presence of vancomycin (1.0 g/l) and BES (10.5 g/l), was conducted at 55ºC. Three reactive azo dyes RR2, RR4 and RO14 at a concentration of 0.3 mM were tested either in the presence or the absence of specific inhibitors. Glucose (1.5 gCOD/l) was selected as primary electron donor and the redox mediator, riboflavin (0.012 mM), was added to some of the bottles to assess its effect on the rates of dye reduction. Abiotic decolourisation was evaluated in sludge-free and sterile controls, and endogenous (glucose-free) reduction activity was determined as well.

Effect of the electron donor concentration on azo dye reduction

To evaluate the effect of the primary electron donor concentration on azo dye reduction at 55ºC, different glucose concentrations were tested (mgCOD/l): 1500, 750, 250, 100, 50 and 0, either in the presence or the absence of riboflavin (0.012 mM). The azo dye RO14 (0.3 mM) was selected as model compound. Additionally, experiments in the presence of vancomycin

(1.0 g/l) and BES (10.5 g/l) were conducted at 55ºC with a low concentration of glucose (0.05 gCOD/l). Abiotic decolourisation was evaluated in sludge-free and sterile controls.

Competition for reducing equivalents between methanogenesis and azo dye reduction

To assess the competition for reducing equivalents between methanogenesis and azo dye reduction, the methanogenic substrates acetate, methanol, H_2/CO_2 and formate were tested at a concentration of 1.5 gCOD/l at 55ºC. Bottles were incubated in a rotary shaker at 50 rpm in the dark. Riboflavin (0.012 mM) was added to some of the bottles as well. BES (10.5 g/l) was also added to some of the bottles to assess the involvement of methanogens in dye reduction. When H_2/CO_2 (80%:20%) was tested as electron donor, a 117-ml bottle with 50-ml basal medium and a headspace of 1.8 bars was used. In that case, the rotation speed was increased from 50 to 200 rpm to improve the mass transfer of hydrogen to the liquid phase. The depletion of hydrogen and methane formation was followed in time. Abiotic decolourisation was evaluated in sludge-free and sterile controls, and endogenous (substrate-free) controls assessed the role of biological activity in dye reduction.

Growth and azo dye reduction by pure cultures of methanogens

Azo dye reduction by pure cultures of *Methanothermobacter thermoautotrophicus* ΔH (DSM 1053, T_{opt} 65ºC) and a *Methanothermobacter*-related strain NJ1 (T_{opt} 55ºC) was studied. These organisms were selected randomly, but they are commonly found in anaerobic granular sludge. They were inoculated to 117-ml serum bottles with 50 ml of a bicarbonate buffered mineral medium of which the composition is described elsewhere (De Bok et al., 2002). The Na_2S concentration was decreased from 1.0 mM to 0.25 mM to minimize the contribution of the chemical decolourisation. Resazurin was not included due to its mediating properties. H_2/CO_2 (80%:20%) applied at 1.8 bars was used as electron donor. For decolourisation assays, the azo dye RR2 (0.1 mM) was selected as model compound. Riboflavin (0.012 mM) and BES (5.3 g/l) were added to some of the bottles. Sterile controls with autoclaved-cells and bottles lacking cells were included to evaluate the abiotic decolourisation. Protein content and pH were measured at the end of the experiment.

2.4. Analysis

For azo dye model compounds, azo dye reduction was determined photometrically as described in Chapter 3. The absorbance was read at the maximum absorbance wavelength, i.e.

RR2 at 539 nm, RR4 at 521 nm and RO14 at 433 nm. The extinction coefficients used were 33.3, 24.0 and 11.6 AU cm^{-1} mM^{-1} for RR2, RR4 and RO14, respectively.

Methane, VFA, methanol, ethanol, sucrose, fructose, glucose, lactate, formate and VSS determinations were described in Chapter 3.

Protein content was determined by using the Bradford method (Bradford, 1976) with bovine serum albumin as standard. The whole cells were harvest by centrifugation at 15000 rpm for 10 min, and then the protein was extracted by boiling for 15 minutes in a 1M NaOH solution.

3. Results

Contribution of acidogenic bacteria and methanogenic archaea to azo dye reduction

A first-order kinetic with respect to the dye concentration was used, whereas the first-order rate constant "k" was determined as described in Chapter 3. In glucose-supplemented assays free of the redox mediator riboflavin, the reduction of azo dyes occurred at similar rates in BES-supplemented and BES-free bottles with the azo dyes RR4 and RO14 (Table 8.1). For the dye RR2, BES-supplemented cultures showed even a 1.4-fold higher decolourisation rate in comparison with the BES-free control. In contrast, the acidogenic inhibitor, vancomycin, showed a strong effect on azo dye reduction. In absence of riboflavin, the k-values in the inhibitor-free controls were 3.1, 3.3 and 6.5-fold higher compared to the vancomycin-supplemented bottles, for RR2, RR4 and RO14, respectively (Table 8.1). In the presence of riboflavin and absence of inhibitors, the reduction of RR2 and RO14 was enhanced 1.7 and 2.9-fold, respectively, compared to the mediator-free controls (Table 8.1). Interestingly, with RR4, there was no clear effect of riboflavin on azo dye reduction. For batch assays supplemented with riboflavin, the reductive decolourisation of all tested azo dyes was slightly lower in the BES-supplemented bottles (11-16%) than the values found in the absence of inhibitors.

Negligible decolourisation (< 5%) was found in the sludge-free and autoclaved controls (data not shown) during the same experimental period. For the endogenous controls (no addition of glucose), also a complete decolourisation was observed, but the rates were considerably (3.3 to 5.8-fold) lower than the values found in the presence of glucose. Acetate, propionate, butyrate and ethanol were the main fermentation products in the presence of BES, while negligible methane production occurred. In the vancomycin-supplemented bottles, there was a minor formation of glucose fermentation products such as VFA and alcohols (data not shown). Only traces of methane were found in the endogenous controls.

Table 8.1. Azo dye reduction at 55ºC by anaerobic granular sludge in the presence of the acidogens inhibitor vancomycin and methanogens inhibitor BES, with glucose as electron donor.

Azo dye	Riboflavin	Inhibitor		k / VSS
		BES	Vancomycin	(day^{-1} $gVSS^{-1}$ l)
RR2	-	-	-	0.84 (0.02)
RR2	-	+	-	1.18 (0.17)
RR2	-	-	+	0.27 (0.03)
RR2	+	-	-	1.43 (0.05)
RR2	+	+	-	1.27 (0.01)
RR2	+	-	+	0.56 (0.03)
RR4	-	-	-	1.74 (0.19)
RR4	-	+	-	1.61 (0.18)
RR4	-	-	+	0.52 (0.02)
RR4	+	-	-	1.82 (0.03)
RR4	+	+	-	1.53 (0.01)
RR4	+	-	+	0.69 (0.03)
RO14	-	-	-	0.84 (0.01)[b]/ 0.16 (0.01)[b]
RO14	-	+	-	0.85 (0.01)[b]/ 0.23 (0.04)[b]
RO14	-	-	+	0.13[b,c] / [b,d]
RO14	+	-	-	2.47 (0.03)[b]/ 0.61 (0.04)[b]
RO14	+	+	-	2.12 (0.01)[b]/ 0.90 (0.07)[b]
RO14	+	-	+	0.20[b,c]/ 0.11[b,c]

[a] The k-value was calculated assuming a first-order reaction; [b] Values (k' / k'') correspondent to glucose concentrations of 1.50 and 0.05 gCOD/l, respectively; [c] Calculated after a lag-phase of about 2 days; [d] Colour removal almost negligible during the time of the experiment; [e] SD is shown in parenthesis, which was calculated from triplicate assays.

Effect of primary electron donor concentration in azo dye reduction

The reduction rates of RO14 were increased by increasing the electron donor (glucose) concentration (Fig. 8.2). In the absence of riboflavin, the decolourisation rate was up to 4.5-fold higher in the glucose-supplemented bottles in comparison with the glucose-free controls (endogenous). In the presence of riboflavin (0.012 mM), the effect of glucose concentration

on RO14 reduction was more pronounced than in the absence of this mediator (Fig. 8.2). This effect was most apparent at the highest substrate concentrations. The decolourisation rates for the bottles containing glucose (1.5 gCOD/l) in the presence of riboflavin were 7.8-fold higher compared to the glucose-free controls (endogenous) supplemented with riboflavin. Negligible decolourisation was found in the sludge-free and sterile controls containing riboflavin.

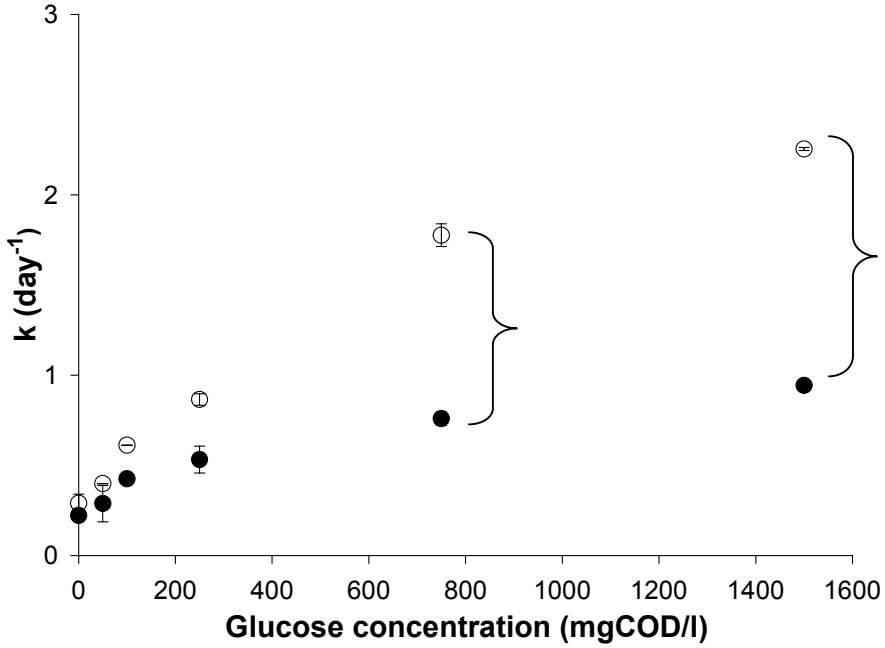


Figure 8.2. Azo dye reduction of RO14 at different concentrations of glucose, in both riboflavin-free (●) and riboflavin-supplemented (○) incubations. Sludge-free bottles controlled the stability of the dye at 55ºC. The brackets indicate that the effect of glucose concentration on RO14 reduction was more pronounced when riboflavin was supplemented as a redox mediator than in its absence. The results are means of triplicate incubations and the bars indicate the standard deviation.

The decolourisation rate of RO14 achieved at 0.05 gCOD/l glucose was only 1.1-fold higher in comparison with the glucose-free controls lacking both redox mediator and inhibitors (Table 8.1). Furthermore, at this concentration, the effect of BES on dye reduction was more pronounced. For instance, in assays lacking riboflavin, the rate in the BES-supplemented bottles was 1.4-fold higher in comparison with the BES-free controls (Table 8.1), which was in contrast to the similar rates found when glucose was provided in excess (at 1.5 gCOD/l). For the vancomycin-supplemented bottles lacking riboflavin, colour removal was almost negligible during the course of the experiment at 0.05 gCOD/l of glucose, but a complete decolourisation was found in the bottles containing this mediator (data not shown).

Competition for reducing equivalents between methanogenesis and azo dye reduction

Azo dye reduction rates at 55ºC in the presence of the methanogenic substrates acetate, H_2/CO_2, methanol and formate varied considerably (Table 8.2).

Table 8.2. Azo dye reduction at 55ºC by anaerobic granular sludge in the presence of the methanogenic substrates acetate, H_2/CO_2, methanol and formate.

Substrate	Azo dye	Riboflavin	Inhibitor		k / VSS
			BES	Vancomycin	(day^{-1} $gVSS^{-1}$ l)
Acetate	RR2	-	-	-	1.08 (0.08)
	RR2	-	+	-	1.04 (0.23)
	RR2	+	-	-	1.66 (0.09)
	RR2	+	+	-	1.68 (0.04)
Acetate	RO14	-	-	-	0.17 (0.01)
	RO14	-	+	-	0.16 (0.01)
	RO14	+	-	-	0.79 (0.04)
	RO14	+	+	-	0.56 (0.04)
H_2/CO_2	RR2	-	-	-	3.99 (0.05)
	RR2	-	+	-	5.29 (0.25)
	RR2	+	-	-	15.02 (1.08)
	RR2	+	+	-	14.74 (0.74)
H_2/CO_2	RO14	-	-	-	1.00 (0.01)
	RO14	-	+	-	1.72 (0.18)
	RO14	+	-	-	7.10 (0.39)
	RO14	+	+	-	6.47 (0.38)
Formate	RR2	-	-	-	1.59 (0.14)
	RR2	-	+	-	1.87 (0.09)
	RR2	+	-	-	3.11 (0.10)
	RR2	+	+	-	3.40 (0.35)
Methanol	RR2	-	-	-	0.69 (0.02)
	RR2	-	-	+	0.48 (0.02)[b]
	RR2	-	+	+	0.42 (0.11)[b]
	RR2	+	-	-	1.69 (0.01)
	RR2	+	-	+	0.76 (0.03)[b]
	RR2	+	+	+	0.72 (0.07)[b]

[a] The k-value was calculated assuming a first-order reaction; [b] To assess the contribution of methylotrophic methanogens; [c] The concentration of the electron donors tested was 1.5 gCOD/l; [d] SD is shown in parenthesis, which was calculated from triplicate assays.

Hydrogen and formate were good electron donors for the reductive decolourisation of RR2, whereas acetate and methanol were poor electron donors. The redox mediator riboflavin

accelerated RR2 reduction in the presence of the methanogenic substrates; the decolourisation rates were enhanced up to 7.1-fold compared to the riboflavin-free controls. Interestingly, in the presence of riboflavin, acetate and methanol became better electron donors, as indicated by the increase in the decolourisation rates (Table 8.2).

The effect of BES on azo dye reduction varied with the primary electron donor (Table 8.2). In the presence of acetate (Fig. 8.3) and methanol (data not shown) similar decolourisation rates of RR2 were found for BES-supplemented and BES-free bottles. However, there was a significant effect of BES on the reduction rates, i.e. BES addition stimulated dye reduction, when hydrogen (1.3-fold) and formate (1.2-fold) were used as electron donors (Fig. 8.4, Table 8.2). A delay in methanogenesis was usually found in the riboflavin-supplemented bottles with acetate (Fig. 8.3), hydrogen (Fig. 8.4), methanol (data not shown), and formate (data not shown) in comparison with controls lacking this compound. Furthermore, in the absence of riboflavin the conversion of the electron donors, which in terms of electron equivalents to reduce RR2 dyes were added in excess, was not directly coupled to the dye reduction (data not shown).

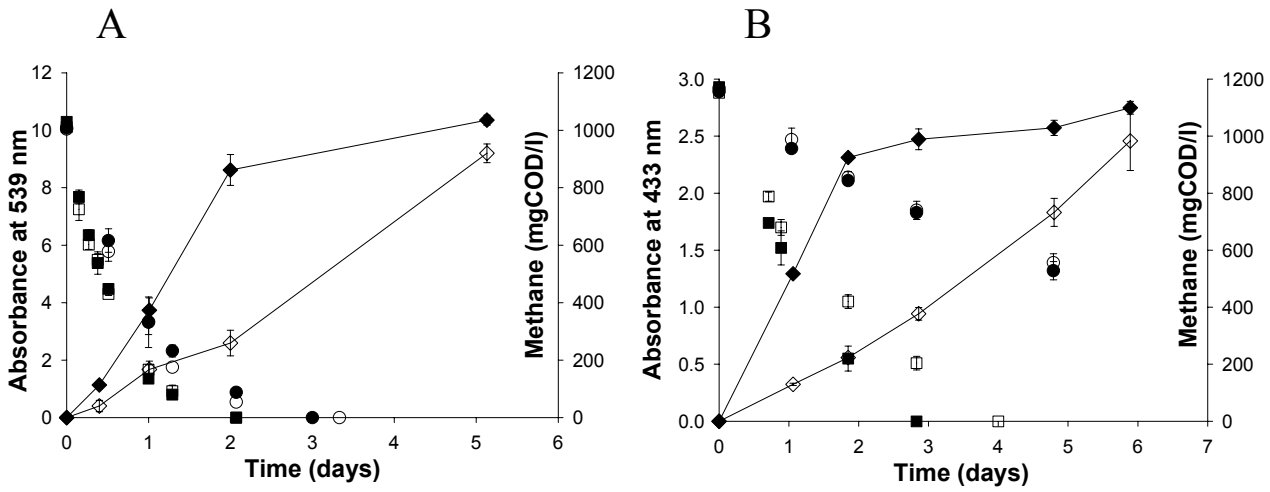


Figure 8.3. Azo dye reduction (left axis) of RR2 **(A)** and RO14 **(B)** in the presence of acetate (1.5 gCOD/l), with the CH_4 formation being shown on the right axis. Symbols for dye reduction in the bottles contained: acet. (●), acet.+BES (○), acet.+rib. (■), acet.+rib.+BES (□). Symbols for CH_4 formation in the bottles contained: acet. (◆), acet.+rib. (◇). BES and riboflavin were added to some bottles as methanogens inhibitor and redox mediator, respectively. Sludge-free bottles controlled the stability of the dye at 55ºC. The results are means of triplicate incubations and the bars indicate the standard deviation. Only traces of CH_4 were found in the BES-supplemented bottles and endogenous controls.

Results of the experiments conducted with the thermophilic organisms *M. thermoautotrophicus* ΔH and the strain NJ1 (Fig. 8.5A and 8.5B), and the mesophilic

organism *M. barkeri* (Fig. 8.5C) reveal that methanogens are able to reduce RR2. The rates of dye reduction considerably varied with the organism and the presence of riboflavin. In the absence of this redox mediator, no decolourisation was achieved with the thermophilic strains but it was possible with the mesophilic strain *M. barkeri* (Fig. 8.5C). In the presence of riboflavin, however, RR2 reduction was achieved with all strains.

The effect of BES on azo dye reduction varied considerably among the strains tested (Fig. 8.5A and 8.5B). With *M. thermoautotrophicus* ΔH, the decolourisation in the assays supplemented with both riboflavin and BES, was almost negligible (Fig. 8.5A). However, a complete decolourisation was found with strain NJ1 (Fig. 8.5B) and *M. barkeri* (Fig. 8.5C), in the presence of both mediator and inhibitor. Interestingly with *M. barkeri* in the presence of riboflavin, the rates of dye reduction between BES-free and BES-supplemented were comparable, while a considerable difference was found in the absence of riboflavin (Fig. 8.5C).

No methane formation was detected in the headspace in the BES-supplemented bottles during the course of the experiment (data not shown) neither with granular sludge nor with pure cultures. Negligible decolourisation was found in both autoclaved and cell-free controls (data not shown).

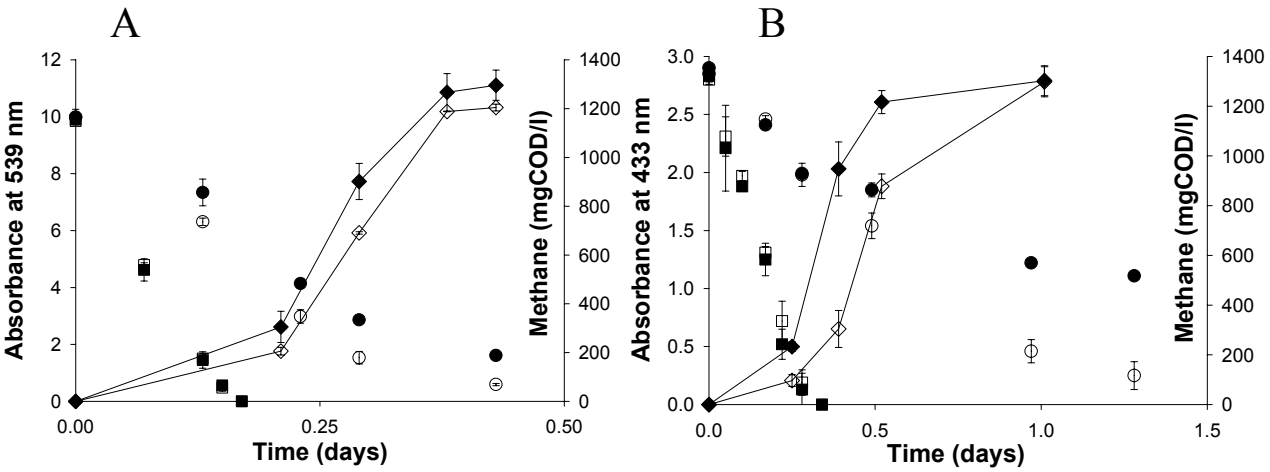


Figure 8.4. Azo dye reduction (left axis) of RR2 **(A)** and RO14 **(B)** in the presence of hydrogen (1.5 gCOD/l), with the CH_4 formation being shown on the right axis. Symbols for dye reduction in the bottles contained: H_2/CO_2 (●), H_2/CO_2+BES (○), H_2/CO_2+rib. (■), H_2/CO_2 +rib.+BES (□). Symbols for CH_4 formation in the bottles contained: H_2/CO_2 (◆), H_2/CO_2+rib. (◇). BES and riboflavin were added to some bottles as methanogens inhibitor and redox mediator, respectively. Sludge-free bottles controlled the stability of the dye at 55ºC. The results are means of triplicate incubations and the bars indicate the standard deviation. Only traces of CH_4 were found in the BES-supplemented bottles and endogenous controls.

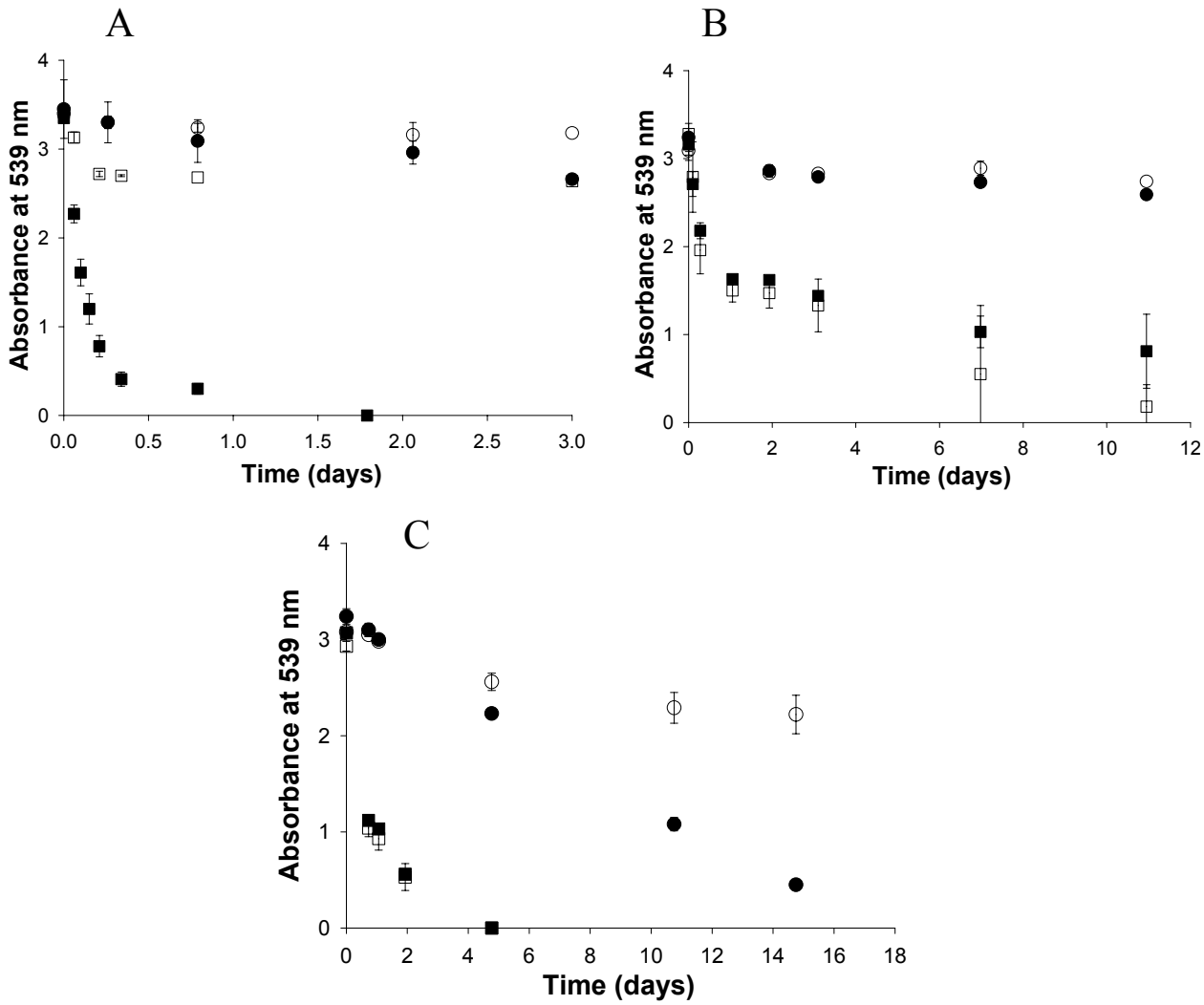


Figure 8.5. Azo dye reduction of RR2 by the thermophilic strains *M. thermoautotrophicus* ΔH **(A)** and NJ1 **(B)**, and in the mesophilic strain *M. barkeri* **(C)**. Symbols for dye reduction in the bottles contained: strain (●), strain+BES (○), strain+rib. (■), strain+rib.+BES (□). H_2/CO_2 (1.8 bars) was used as electron donor. BES and riboflavin were added to some bottles as methanogens inhibitor and redox mediator, respectively. Sludge-free bottles controlled the stability of the dye. The results are means of triplicate incubations and the bars indicate the standard deviation.

4. Discussion

In the current investigation the contribution of acidogenic bacteria and methanogenic archaea to the reductive decolourisation of azo dyes by a thermophilic anaerobic consortium was studied. The results indicate that both acidogenic bacteria and methanogenic archaea may play an important role in azo dye reduction. The presence of a redox mediator like riboflavin strongly enhanced the rates of decolourisation in channelling biogenic electrons to cleave the

azo bond. Moreover, experiments with thermophilic pure cultures of methanogens revealed that these strains were unable to reduce the dye in the absence of riboflavin.

With glucose as electron donor, the rates of dye reduction after inhibition of the methanogenic archaea by BES were comparable to the rates in incubations without this inhibitor. On the other hand, when the acidogenic bacteria were inhibited by vancomycin, a clear decrease in decolourisation rates was found, evidencing the important contribution of acidogens in the process. When riboflavin was supplemented as a redox mediator, the effect of glucose concentration on reductive decolourisation was more pronounced than in the absence of this mediator. Previous investigation with the VFAs, acetate, propionate, and butyrate, in the absence of redox mediators, showed that the rates of dye reduction were independent of the electron donors tested (Van der Zee et al., 2001a). This suggests that the low concentrations of hydrogen formed during propionate and butyrate oxidation could not sustain the dye reduction. Apparently in our research, riboflavin was mainly assisting the transfer of reducing equivalents from glucose fermentation to the dye, thus allowing the acidogens to participate more effectively in the reductive decolourisation. The results agree with the previously reported ability of fermentative bacteria to use humic acids as an electron acceptor instead of riboflavin, as well as to reduce azo dyes (Benz et al., 1998; Yoo et al., 2000; Dos Santos et al., 2003).

Riboflavin also considerably increased the rates of azo dye reduction when important intermediates of anaerobic bioconversion such as acetate, methanol, hydrogen and formate were tested, whereas the two latter were by far the best electron donors for sustaining reductive decolourisation. During the study of different electron donors in the carbon tetrachloride reduction, hydrogen was an effective electron donor, whereas methanol and acetate had a very poor electron donating capacity (Cervantes et al., 2004). In the same study, 20 μM of the redox mediator AQDS remarkably increased the reduction rates, making that even the poor electron donors became effective electron donors for dechlorination. A possible explanation for the low azo dye reduction rates of methanogenic archaea in the presence of acetate and methanol may be related to the enzymes involved in methanogenesis from these compounds, which do not utilize NAD^+ or $NADP^+$ as coenzymes. Instead some of the enzymes use a 5-deazaflavin F_{420} as coenzyme (Berk and Thauer, 1997). It is currently accepted that NADH or NAD(P)H are the primary electron donors for biological azo dye reduction (Zimmermann et al., 1982; Semdé et al., 1998; Stolz, 2001; Blumel and Stolz, 2003).

The effect of BES on azo dye reduction, in the presence of the methanogenic substrates acetate and methanol was negligible (Table 8.2). This is expected, since no electron transfer is involved after formation of methyl coenzyme M, which is blocked by BES (Thauer, 1998). Interestingly, riboflavin acting as a redox mediator, further enhanced azo dye reduction in BES-inhibited bioassays, so that the rates of decolourisation achieved were similar to non-inhibited controls including this redox mediator (up to 7-fold compared to controls lacking riboflavin). Remarkable results were obtained when hydrogen and formate were included as electron donors in the absence of riboflavin; the rates of decolourisation were considerably higher (up to 1.7-fold) in BES-supplemented bottles in comparison with non-inhibited controls. Apparently, the inclusion of BES supported azo dyes reduction to out-compete methanogenesis for the reducing equivalents available in hydrogen- and formate-supplemented cultures. Contrarily, no influence of BES in the rate of AQDS reduction was found when testing hydrogen and acetate as electron donors with different consortia, which suggested that methanogens were not involved in the quinone-respiration (Cervantes et al., 2000). However, many methanogenic archaea such as *M. barkeri*, *Methanococcus voltaei* and *Methanospirillum hungatei* JF1 can use AQDS as a terminal electron acceptor (Cervantes et al., 2002; Bond and Lovley, 2002). The role of riboflavin for stimulating reductive decolourisation is also reflected by a delay in methanogenesis from different electron donors (Fig. 8.3 and 8.4). Interestingly, only a very small amount of reducing equivalents was required to completely reduce RR2. Theoretically, 4 electrons are needed to reduce one molecule of RR2. Thus, to reduce 0.3 mmol of RR2 1.2 mmol electron equivalents (9.6 mgCOD) are needed, which represents 0.6% of the COD supplemented as electron donor (1500 mgCOD/l). Therefore, non-azo-dye-reducing methanogens and azo-dye-reducing methanogens present in the consortium were competing for the same reducing equivalents, in which riboflavin helped the transport of electrons to the azo bond cleavage. Recently it was reported that some methanogens preferentially oxidize some electron donors linked to the reduction of Fe(III) instead of methanogenesis, also indicating the competition for electrons (Van Bodegom et al., 2004).

Experiments with pure cultures of the thermophilic methanogens *M. thermoautotrophicus* ΔH and the strain NJ1 reveal that these strains were unable to reduce the azo dye RR2 in the absence of the redox mediator riboflavin. In contrast, Stolz (2001) suggested that almost all azo dyes tested so far are biologically reduced under anaerobic conditions. For instance, a complete reduction of the sulfonated azo dye Amaranth by α,β and

γ-proteobacteria (Gram negative bacteria)*, low and high GC* (Gram positive bacteria), and the archaeon *Halobacterium salinarum*, was recently reported (Rau et al., 2002a). Based on our results with methanogenic archaea, anaerobic azo dye reduction is not a universal characteristic as it has been repetitively reported. Interestingly, in the presence of riboflavin, we found that both thermophilic strains were able to reduce RR2. Because hydrogen was the best electron donor for reductive decolourisations in the bioassays with the thermophilic granular sludge studied, very likely other hydrogenotrophic methanogens rather than the strains tested could have been directly involved in the azo cleavage. Another possible explanation for the fast reductive decolourisation of the riboflavin-free bottles with hydrogen as an electron donor could be due to redox mediators naturally produced in the sludge, which allows methanogens to participate in this reductive process. Quinone-based compounds have not been found in methanogens, but phenazine derivatives have been shown to fulfil a quinone-like role in the electron transfer of *Methanosarcina mazei* Gö1 (Abken et al., 1998). It might well be that riboflavin or related compounds are formed and excreted by anaerobic bacteria in the sludge. The effect of riboflavin in enabling methanogens to participate in azo dye reduction suggests transfer of intracellular reduction equivalents to the outside of the cell and subsequent dye reduction. Reductive decolourisation of the sulfonated dye model compound RR2 is unlikely to occur intracellularly, because no relation between reduction rate and molecular weight was found (Table 8.1, Fig. 8.1) and because of the limited membrane permeability, which is affected by the dye polarity (Kudlich et al., 1997; Russ et al., 2000; Van der Zee et al., 2001b). The redox mediator lawsone, i.e. a quinone-based compound, was reduced intracellularly by *Escherichia coli*, after which reducing equivalents were transported to the outside of the cell and subsequently reduced the azo dye Amaranth, probably involving a similar reduction mechanism as riboflavin (Rau and Stolz, 2003). An enzyme of *Paracoccus denitrificans*, called FerA, which catalysed the reduction of a number of Fe(III) complexes by NADH, required the addition of FMN or riboflavin for activity on Fe(III) substrates (Mazoch et al., 2004). Many similarities between the mechanisms of iron reduction and azo dye reduction can be found.

The results of this investigation have ecological implications because in many countries where discharge regulations of coloured wastewaters is not so strict, azo dyes can reach freshwater and marine sediments, and are reduced by microorganisms present there. Methanogenic archaea are known to be extremely important for the degradation of organic matter in sediments, and acetate and hydrogen are important intermediates in anaerobic

digestion (Scholten et al., 2000). However, most acetate-utilizing methanogens seem do not couple the conversion of acetate to the reduction of azo dye, whereas hydrogen-utilizing methanogens are very likely involved in this reductive process. The capacity of humus as an alternative terminal electron acceptor for the above-mentioned intermediates of the carbon flow (Cervantes et al., 2000), with the subsequent chemical transfer of the reducing equivalents to the dye molecule (Field et al., 2000; Stolz, 2001), suggests the indirect contribution of quinone-respiring microorganisms to the reduction of azo dyes in the above-mentioned environments.

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9

General Discussion and Conclusions

1. Ecological and public health concerns of textile wastewaters

A huge number of chemicals from textile industries, which have a negative effect on the environment and public health, are released through the wastewaters, representing one of the main contributors to the severe pollution problems worldwide (Hao, 2000). Particularly the release of coloured compounds into water bodies is undesirable not only because of their aesthetic appearance and the impact of dyes on photosynthesis of aquatic plants, but also because many of these dyes and their breakdown products are potentially toxic and/or mutagenic to life (Weisburger, 2002).

2. Technological challenge for decolourisation of textile wastewaters

Colour removal, especially from textile wastewaters, has been a big challenge over the last decades, and currently there is no single and economically attractive treatment that can decolourise dyes. The problems of textile wastewater decolourisation are mainly related to the high variety of dyes used in the dyeing process and because of the recalcitrant nature of some dyes and/or their reduced products. Moreover, the composition of the wastewater is continuously changing not only as a result of dye switching, but also due to the presence of other compounds like sodium hydroxide, surfactants and formaldehyde. Therefore, the wastewater treatment systems have to be highly adaptable to these changes. **Chapter 2** explains the advantages and disadvantages of the different treatment processes for colour removal.

The use of biotechnology applied to textile wastewater treatment, either oxidative or reductive processes, has progressed greatly in the last years, not only for colour removal but also for the complete biodegradation of dyes. Different microbes such as aerobic and

anaerobic bacteria, fungi, and actinomycetes have shown their ability to decolourise both azo and anthraquinone dyes (**Chapter 2**). In the following discussion **we will mostly focus on colour removal of azo dyes** because they are the most important class of dyes used in textile industry, representing about 70% by weight. Under *aerobic conditions* with bacteria, usually low colour removal efficiencies are achieved (10-30%) because oxygen is a more effective electron acceptor, therefore having a greater preference for reducing equivalents than azo dyes (Stolz, 2001). On the other hand, under *anaerobic conditions*, the reducing equivalents are more efficiently channelled to the azo dyes, which are generally the main terminal electron acceptors. **The colour removal achieved under anaerobic conditions is also called azo dye reduction**. The cleavage involves a transfer of four-electrons, which proceeds through two stages at the azo linkage, i.e. hydrazo intermediate and aromatic amines formation. Figure 9.1 shows the steps involved in the fate of azo dyes. *Step 1* is the anaerobic reductive cleavage of the azo bond and subsequent production of the aromatic amines, which are anaerobically recalcitrant. Thus, azo dyes cannot be completely biodegraded but only reduced. *Step 2* is the required post-treatment in which the reduced products are ultimately degraded.

$$(R_1{-}N{=}N{-}R_2) + 4e^- + 4H^+ \rightarrow R_1{-}NH_2 + R_2{-}NH_2 \quad \text{(Step 1)}$$

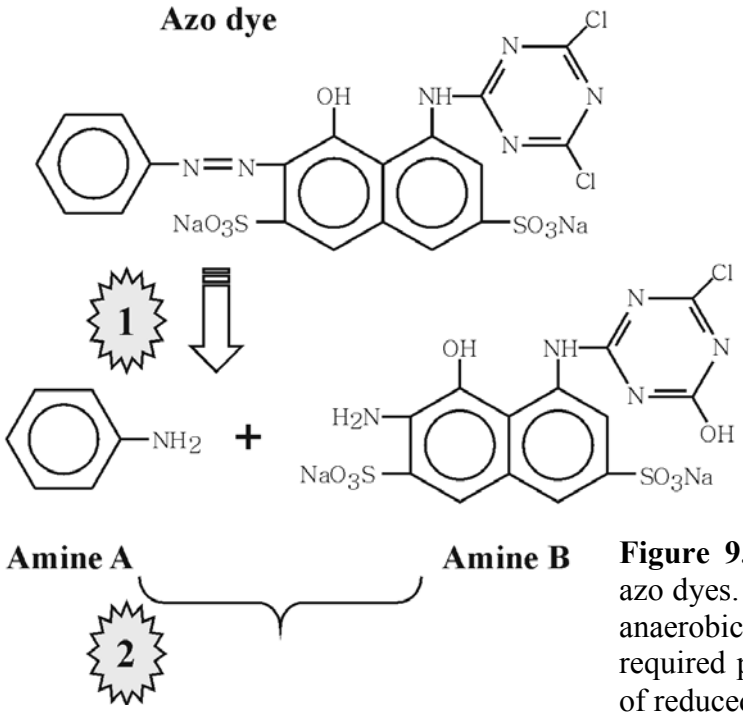


Figure 9.1. Steps involved in the fate of azo dyes. Step 1 is the reduction step under anaerobic conditions, and Step 2 is the required post-treatment for the degradation of reduced products.

The conversion of aromatic amines proceeds faster compared to the **reductive cleavage of the azo dyes**, which generally is **the rate-limiting step** in the overall reaction.

Many post-treatment methods, following the cost-effective 'high-rate' anaerobic bioreactors have been investigated. For instance, a sequential anaerobic-aerobic treatment strategy has been repeatedly proposed in which the aerobic microorganisms could use the aromatic amines as nitrogen and carbon source (Field et al., 1995; O'Neill et al., 2000a; Tan, 2001). Although in theory this set-up is feasible, many other investigations show contradictory findings and the full-scale potential of this integrated set-up remains unclear for industrial textile wastewaters, which are composed of many different dyes and consequently many different reduction products (Tan et al., 2000; Stolz, 2001; Libra and Sosath, 2003). Alternative physical-chemical post-treatments such as ozone, photocatalytic oxidation, Fenton reagents, etc. have shown good prospects, but treatment costs are still considerably high (Vandevivere et al., 1998; Libra and Sosath, 2003). This **Thesis** focuses on the rate-limiting decolourisation step and discusses strategies to increase the rates of dye reduction.

3. The state of the art on colour removal of azo dyes in bioreactors by anaerobic granular sludge

Even though anaerobic azo dye reduction could be readily achieved with different microorganisms, there is no strain reported so far that is able to decolourise a broad range of azo dyes. Therefore, the use of a specific strain or enzymes on azo dye reduction does not make much sense in treating textile wastewater, which is composed of many kinds of dyes (Laszlo, 2000). The use of mixed cultures, such as anaerobic granular sludge, which is composed of stable microbial pellets with a high activity, is probably a more logic alternative. Different reactor configurations, such as the widely used upflow anaerobic sludge bed (UASB) system and expanded granular sludge bed (EGSB) system, are used to immobilize high concentrations of biomass (Lettinga et al., 1980; Lettinga, 1995; Van Lier et al., 2001). Indeed, the different microbial consortia present in anaerobic granular sludge can carry out tasks that no individual pure culture can undertake successfully (Nigam et al., 1996; Pearce et al., 2003).

Many researchers have been investigating azo dye reduction, as well as the fate of aromatic compounds in both anaerobic and aerobic bioreactors. Initially Razo-Flores (1997) found that the reduction of the azo dye Mordant Orange 1 (MO1) could be achieved in a UASB reactor fed with glucose or VFA, with efficiencies reaching values up to 99%. Interestingly, batch experiments showed that the azo dye MO1 was 200-fold and 2140-fold more toxic to the anaerobic microorganisms than its expected reduction products 5-

aminosalicylic acid and 1,4-phenyledediamine, respectively. Tan (2001) investigated the use of an integrated and sequential anaerobic/aerobic systems for the biodegradation of azo dyes. In the integrated anaerobic/aerobic system with ethanol as co-substrate, the concentration of oxygen was shown to play an important role in the reduction of the azo dye MO1. Moreover, the fate of MO1 products under anaerobic conditions was not achieved, contrarily to the findings of Razo-Flores (1997). With a sequential azo dye reduction, i.e. anaerobic and aerobic steps taking place in two different reactors, higher rates of dye reduction were achieved in the anaerobic phase, as well as the mineralization of the aromatic amines in the aerobic phase. Van der Zee (2002) researched the understanding of the azo dye reduction mechanisms in batch and continuous flow systems. No correlation between molecular size and rates of decolourisation was found, which indicates that dye penetration into the cell wall is not playing a major role in the reductive decolourisation, and therefore the latter is likely to occur outside of the cell or is membrane-bond. Additionally, low rates of reduction and severe toxicity of the azo dye Reactive Red 2 were found in a UASB reactor operated at an HRT of 6 hours and fed with a VFA mixture as electron donor. The addition of catalytic concentrations of the redox mediator AQDS was an efficient strategy to recover the reactor in terms of both colour removal and COD removal. Cervantes et al. (2001) showed that decolourisation efficiencies around 90% were achieved by testing the azo dye Acid Orange 7 in a UASB reactor operated at an HRT of 2 h, fed with VFA and 3μM of AQDS. It was observed that the effect of the HRT decrease was more apparent in the absence of AQDS, in which a sharp decrease in the efficiencies was found. This indicates that AQDS is assisting the transfer of reducing equivalents to the azo dye, which apparently is the rate-limiting factor. All the investigations previously reported were conducted under mesophilic conditions and used single azo dye model compounds.

Chapter 2 explains that azo dye reduction is composed of a biological part, i.e. the reducing equivalents are biologically generated, and a chemical part, i.e. the formed electrons reduce chemically the azo dye. In general, the chemical reaction follows the Arrhenius equation, indicating that an increase in temperature will proportionally increase the collision frequency of the reactants, thus improving the kinetics of the reaction. Therefore, the use of thermophilic treatment could be advantageous for the improvement of this chemical part of the overall reaction. Furthermore, the use of redox mediators as an electron shuttle to accelerate azo dye reduction under thermophilic conditions could be advantageous, not only for the expected faster enzymatic reduction of the mediator compared to mesophilic

conditions, but also for the faster regeneration of reduced form of the mediator by the chemical reaction with azo dyes. In this respect it is important to note that a large portion of textile wastewaters, particularly those coming from dyebath, is discharged at high temperatures. Thus far, redox mediators in conjunction with thermophilic conditions have not been investigated yet.

3.1. Thermophilic colour removal of azo dyes by anaerobic granular sludge

Our results clearly show that thermophilic treatment with anaerobic granular sludge accelerates the reduction of azo dyes. **Chapter 3** describes that the metabolic activities of the inoculum induced a fast reduction of RR2, indicating a biotic predominance in the process. The supplemented co-substrate, acting as a primary electron donor, is important not only for improving the colour removal rates but also for supplying the high maintenance requirements of the thermophilic microorganisms. Biological treatment at 55ºC shows a fast initial generation of reducing compounds via co-substrate conversion, which we verified by comparing the k-values of batch assays free of sulphide with incubations containing 2 mM of sulphide, in the presence of granular sludge. The pseudo-exponential increase at 55ºC on the decolourisation rates by increasing VSS concentration is another example of the fast initial generation and/or transfer of reducing compounds via co-substrate conversion at 55ºC. In addition, the more negative redox potential values obtained at 55ºC compared to those at 30ºC confirm a better reduction capacity at 55ºC. As a result of the afore-mentioned factors, sixfold and twofold increases on the decolourisation rates were verified in the absence and presence of AQDS, respectively, between thermophilic (55ºC) and mesophilic (30ºC) conditions by using the same sludge source. Therefore, most likely the transfer of reducing equivalents at 30ºC was the rate-limiting step.

The results of **Chapter 4** show that thermophilic EGSB bioreactors are extremely efficient in treating the recalcitrant azo dye RR2, as well as having a high stability when high loading rates of RR2 are applied (up to 2.7 gRR2 l^{-1} day^{-1}). Furthermore, the impact of the redox mediator AQDS on colour removal is less apparent than in the previous investigations conducted under mesophilic conditions (Van der Zee et al., 2001a; Cervantes et al., 2001). Very likely this difference is also related to the higher HRT of 10 h used in the current experiment at 55ºC compared to the HRT of 6 h previously used at 30ºC. Long-term experiments revealed that the AQDS-free reactor achieved efficiencies of around 91% in

comparison with the efficiencies around 95% for the AQDS-supplemented reactor. The higher decolourisation rates achieved at 55ºC, compared to those obtained at 30ºC, are important for the conversion process not only directly for the colour removal, but also indirectly because a much lower fraction of the sludge, i.e. in and around the biofilm, is exposed to possible toxic concentrations of RR2. Azo dyes are known to be more toxic to methanogens than their cleavage products, i.e. aromatic amines (Donlon et al., 1997). The thermophilic EGSB bioreactors were also extremely stable with regard to the full co-substrate conversion into methane, which was in contrast to the results of Van der Zee et al. (2001a). They reported a decrease in the colour removal efficiencies to 25% after 40 days of feeding with non-hydrolysed RR2 in the absence of AQDS during anaerobic treatment at 30ºC. Furthermore, the co-substrate (VFA) removal efficiencies dropped to around 10%. Nevertheless, the system capacity for colour removal and co-substrate conversion was recovered after the reactor was supplied with 19μM of AQDS. In our experiments no toxicity effects were observed, which might be attributed to the use of a hydrolysed RR2 opposed to the non-hydrolysed RR2 of the previous work. The toxicity effect of the former RR2 could be more pronounced as indicated by the results of Chung and Stevens (1993) who reported microbial respiration inhibition due to the presence of chloride or bromide as functional groups. The differences in reactor type, for instance comparing UASB and EGSB for mass transfer limitations, is likely to be of minor importance for the toxicity of azo dyes, particularly if appropriate primary electron donors and HRT are used. Our results show that applying thermophilic treatment and redox mediators to overcome toxicity problems in azo dye reduction is an effective strategy than big increases in HRT.

The significant enhancement of the electron transfer capacity and subsequent increase on the reductive decolourisation of azo dyes, simply by applying high temperature, is demonstrated in **Chapter 6**. For instance, at an HRT of 2.5 h and in the absence of AQDS, the colour removal is 5.3-fold higher at 55ºC in comparison with the efficiency achieved at 30°C. Furthermore, the catalytic effect of the redox mediator AQDS on the reductive decolourisation is even decreased 3.2-fold compared to experiments carried out at 30ºC. Very likely, this indifference to the presence of AQDS is the consequence of the high efficiency reached at 55°C, which masks the impact of AQDS on colour removal. The same degree of COD removal of about 75% indicates that the reducing equivalents are generated at a similar rate in the mesophilic and thermophilic bioreactors. Apparently, the difference in the decolourisation

rates is not related to the difference in the production rates of reducing equivalents. Consequently, we conclude that the higher degree of colour removal is attributed to the impact of temperature and AQDS on electron shuttling. In **Chapter 6** we researched at 30ºC whether or not colour removal and COD removal can be coupled in anaerobic reactors treating high concentration of dyes. Results show that changes in decolourisation rates due to the increase in the ratio of concentrated dyebath to rinsing water are less pronounced than the effect of this gradient on methane formation. For instance, comparing the ratios (% v:v) 0:100 and 2.0:98.0 in the absence of AQS, the k-values for decolourisation were 0.32 day^{-1} and 0.19 day^{-1}, respectively, a 0.6-fold decrease. The difference in the decolourisation rates is not significant if we consider that the initial absorbance peak of the wastewater at 475 nm was 13-times higher at the ratio 2.0:98.0 compared to the ratio 0:100. High concentrations of dyebath result in an accumulation of acetate and a severe inhibition of methanogenesis. The specific methanogenic activity of the wastewater-free control is 4-times higher than that of the ratio 2.0:98.0. A 50% decrease in the CH_4 production compared to the control is found with 22 ml of dyebath for each 1000 ml of rinsing water (2.2:100).

3.2. Redox mediators

Redox mediators and colour removal

Flavin-based compounds as well as quinone-based compounds have been extensively reported as redox mediators. In these cases, non-specific enzymes biologically reduce the mediator, as the initial electron acceptor for the primary electron donor conversion. Secondly, the electrons are chemically transferred to the azo dye, the terminal electron acceptor, with consequent mediator regeneration (**Chapter 2**).

In **Chapter 5** we demonstrate that the normal rate limiting step, the transfer of reducing equivalents to the azo dye, is accelerated under thermophilic conditions. Both biotic and abiotic mechanisms involved in the biochemistry of colour removal are enhanced at 55ºC. The faster biological reduction of the redox mediator, AQDS, achieved by sludge incubations at 55°C in comparison with 30°C evidences the biological contribution in enhancing the rate of electron transfer. For instance, about 1 mM of AQDS is completely reduced at 55ºC after 0.7 days of incubation, whereas mesophilic reduction after 0.7 days is just 12.9% of this value. Furthermore, no lag-phase is found at 55ºC. The abiotic chemical reduction of RR2 by sulphide, as expected, follows the Arrhenius equation, and the decolourisation rates are

accordingly accelerated by the temperature increase. Furthermore, the AQS-supplemented incubations present a lower Ea requirement. The calculated Ea values are 27.9 kJ/mol and 22.9 kJ/mol for the AQS-free and AQS-supplemented incubations, respectively. Therefore, the activation energy is decreased 1.2-fold due to the addition of 0.012mM of AQS.

Theoretically, useful redox mediators for biological azo dye reduction must have redox potentials between the half reactions of the azo dye and the primary electron donor (Van der Zee et al., 2003b). In a screening of redox potential values for different azo dyes, it was found that standard redox potential (E_0') values are generally between –0.430 and –0.180 V (Dubin and Wright, 1975). Rau et al. (2002) cites that the NAD(P)H cofactor, which has the lowest E_0' value of –0.320 V, seems to set the limits of redox mediators application. The reason for this is that mediators with a more negative E_0' value will not be reduced by the cells and mediators with E_0' greater than –0.05 V will not efficiently reduce the azo bond at high rates. Although this observation was confirmed with a model compound (Rau et al., 2002a), it was never verified using a complex textile wastewater, which is composed of many dyes with different E_0' values.

In **Chapter 6** a textile wastewater containing many dyes with different redox potentials is used under mesophilic and thermophilic conditions to assess the rate-limiting step of the decolourisation, i.e. the enzymatic mediator reduction versus the chemical electron transfer from the mediator to the dye. Compared to AQS (E_0' of -225 mV) supplemented assays, bottles supplemented with BQ (E_0' of +280 mV) do not accelerate colour removal from textile wastewater. Probably, this is related to the inability to transfer electrons from its reduced form (hydrobenzoquinone) to the dyes present in the wastewater, even though the first part of the reaction may have been accelerated by comparing the E_0' of AQS and BQ. When the mediators BQ and AQS are incubated together there is no improvement on the decolourisation rates in comparison with the bottles solely supplemented with AQS, even though a broader range of the electron transfer mediating capacity could be covered in the solution with dyes (Meckstroth et al., 1981). Therefore, either the concentration of mediator with negative E_0' (AQS) is driving the decolourisation rates, or the redox potential in the solution switches to a value that the reduced form of BQ could transfer the electrons more efficiently both directly to the dye and to AQS in a sequential reaction involving mediators and dye. Our present results show that the use of mixed redox mediators with positive and negative E_0' under anaerobic conditions to cover the wide range of dyes-redox potential, is not an efficient approach to improve colour removal in textile wastewaters.

Redox mediators and the influence of steric and electro-chemical factors on colour removal by granular sludge

The E_0' is a good indicator of a compound's capacity to function as redox mediator. However, apparently in addition to E_0', other factors are of importance as well. Different decolourisation rates in the presence of mediators with similar E_0' values have been reported, as well as similar decolourisation rates with mediators with different E_0' values. For instance, Brown (1981) tested the polymeric nitro dye Poly Y-607 and found that both methyl viologen and benzyl viologen increased the decolourisation rates 4.5-fold, even though the E_0' of methyl viologen is much lower than that of benzyl viologen, i.e. –0.440 V and –0.360 V, respectively (Figure 9.2).

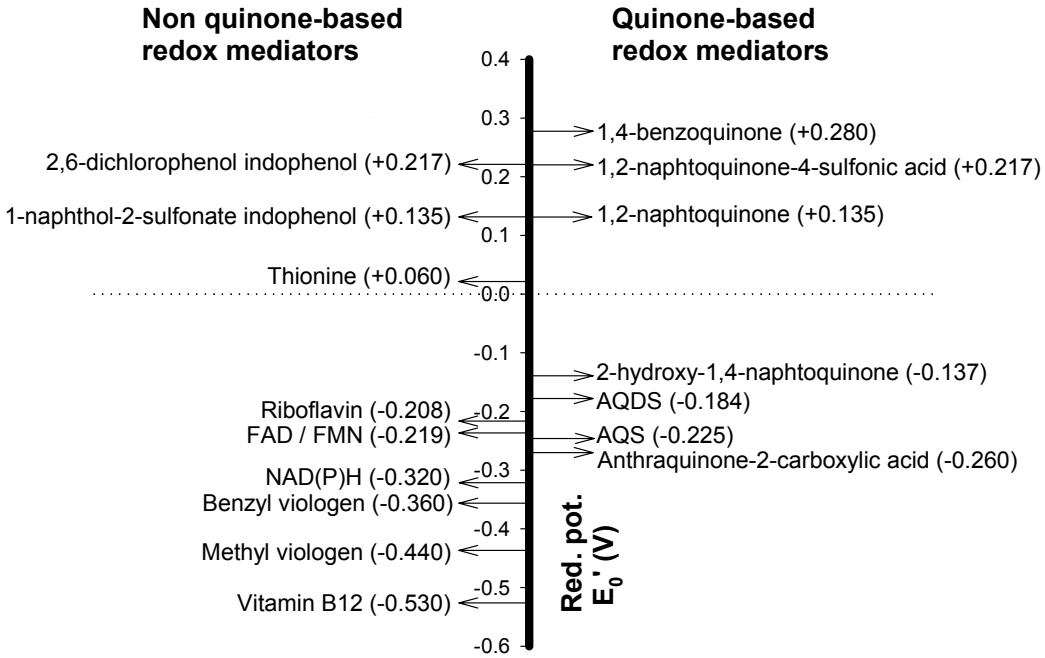


Figure 9.2. E_0' values both for quinone-based and non quinone-based redox mediators (Adapted from Rau et al., 2002a; Fultz and Durst, 1982).

In **Chapter 4** we show that catalytic concentrations of the low molecular weight redox mediators AQS, AQDS and riboflavin incubated with granular sludge at 55ºC improve up to 8-fold the decolourisation rates of the azo dye model compounds compared to the mediator-free bottles. Mediator addition does not affect the reaction-order. Riboflavin is by far the best mediator with the reactive azo dye RR2, increasing the k_1-value 3-fold compared to the AQS-supplemented incubation. However, the same tendency is not observed with AO7 and MY10, for which AQS has a higher catalytic capacity than riboflavin. Comparing the decolourisation

rates between AQS- and AQDS-supplemented bottles, it is observed that the mediating capacity of AQS is much higher than AQDS, i.e. with RR2 1.8-fold and AO7 1.4-fold. Surprisingly, with MY10 all mediators catalyse the colour removal at similar rates. However, as expected vitamin B12 is a very poor redox mediator with the azo dye model compounds, which is probably due to its highly negative redox potential E_0' of –0.530 V.

Riboflavin and AQS have similar mediating properties, which is most likely related to the more or less equal redox potentials (E_0') of riboflavin and AQS (–0.208 V and –0.225 V, respectively, Figure 9.2). However, riboflavin is a far better mediator than AQS with regard to the azo dye RR2 (Figure 9.3).

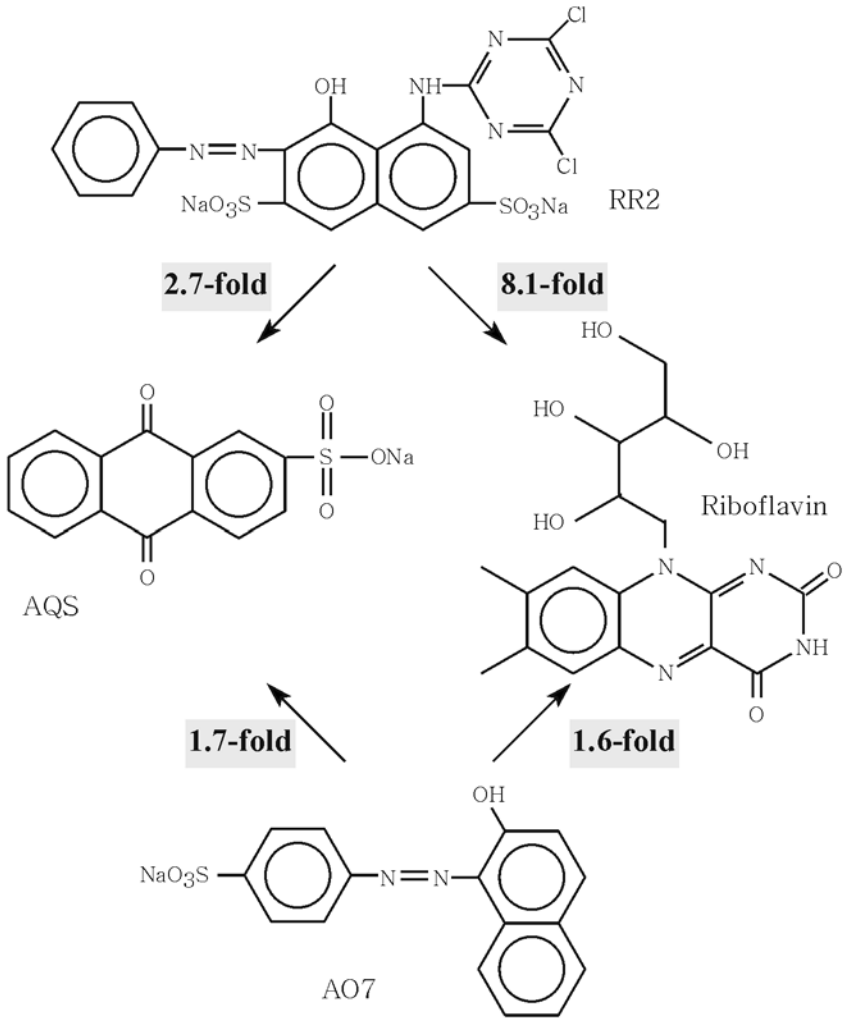


Figure 9.3. Apparent differences in the reduction rates of the azo dyes RR2 and AO7, in the presence of the redox mediators riboflavin and AQS, showing that other factors like steric and electro-chemical factors between mediator and azo dyes, rather than E_0' of the mediators, play an important role in the reaction.

This result suggests that other factors like steric hindrance between azo dye and redox mediator, also determine the decolourisation rates in the system. Walker and Ryan (1971)

postulated that decolourisation rates are related to the electron density in the azo bond region. They suggested a colour removal increase by lowering the electron density in the azo link. Therefore, the use of redox mediators would not only tend to accelerate the transfer of reducing equivalents to the terminal electron acceptor (azo dye), but also to minimize the steric hindrance of the dye molecule (Moir et al., 2001). Apparently, differences in steric and electro-chemical factors between mediator and azo dye need also to be considered when the suitability of a specific mediator is evaluated. Furthermore, the penetration of redox mediators through the cytoplasm and subsequent transference of reducing equivalents from the inside to the outside of the cell also plays an important role in accelerating azo dye reduction (**Chapter 8**).

Natural redox mediators and indirect contribution to azo dye reduction

Chapter 3 shows that AQDS (0.012 mM) increases the colour removal rates (k-value) when co-substrate is added. However, there is no effect on the observed k-values of the endogenous controls, suggesting that co-substrate conversion coupled to electron transfer through AQDS is required for the enhanced decolourisation. Moreover, the impact of AQDS as redox mediator at high VSS concentrations is less evident than at low VSS concentrations. Based on these results, we suggest that intracellular components with mediating properties are released to the medium by the addition of biomass. In general, compared to mesophilic microorganisms, thermophilic microorganisms present higher metabolic rates (2-3 times), maintenance energy requirements, and sludge turnover rates (Van Lier et al., 1993b).

Keck et al. (2002) reported that during aerobic degradation of naphthalene-2-sulphonate (2NS) at 30ºC by *Sphingomonas xenophaga* strain BN6, redox mediators were produced, which increased the efficiency of the strain to reduce azo dyes anaerobically. Furthermore, quinone-based compounds have not been found in methanogens, but phenazine derivatives have shown to fulfil a quinone-like role in the electron transfer of *Methanosarcina mazey* Gö1 (Abken et al., 1998). It is very likely that this indirect contribution of naturally formed redox mediators to azo dye reduction is higher at 55ºC than at 30ºC due to the higher sludge turnover rate and cell lysis at 55ºC than at 30ºC. Thus, intracellular mediating compounds, previously inactive due to inability to cross the cell membrane, can actively participate in the reductive decolourisation. In addition, cell lysis and sludge disruption can provide the medium with reducing agents, e.g. sulfide, iron and other reduced cofactors, which may reduce azo dyes chemically.

Redox mediators and toxicity towards the trophic groups present in granular sludge

In **Chapter 3** a negligible increase in colour removal capacity of thermophilic sludge at high AQDS concentrations is found. Furthermore, high AQDS concentrations inhibit the thermophilic acetate conversion, as acetate accumulates and methanogenesis concomitantly decreases. Propionate oxidation is also affected by an increase in the AQDS concentration, in which the propionate conversion almost ceases at AQDS concentrations exceeding 1.2 mM. On the other hand, glucose fermentation is not affected by high AQDS concentrations as evidenced by the negligible detection of this substrate at the end of the incubation period.

In **Chapter 6** the CH_4 production in the AQS-free bottles is about 1.2-times higher than those supplemented with AQS (0.050 mM). In **Chapter 7** the anthraquinone dye Reactive Blue 19 (RB19) is found to be non-toxic to acidogens, but it is extremely toxic to the acetate-utilizing methanogens in mesophilic granular sludge. Likely because AQS is an anthraquinone-based molecule, its toxicity on methanogens corroborates with that observed with RB19 and the toxic effect is probably induced by the anthraquinone moiety. Interestingly, in **Chapter 6**, AQDS at a concentration of 0.025 mM does not have any toxic effect towards methanogens, as the CH_4 productions between the reactors AQDS-free and AQDS-supplemented are similar. This is in agreement with Cervantes et al. (2003) who observed a rapid methanogenic activity recovery even using an AQDS concentration of about 500-times higher than the AQS concentration used in the present investigation. The question why AQS is more toxic to methanogens than AQDS remains unclear.

4. Toxicity and transformation of anthraquinone dyes by mesophilic and thermophilic consortia

In **Chapter 7** we demonstrate that in comparison with incubations at 30ºC, incubations at 55ºC present distinctly higher decolourisation rates not only with real wastewater, but also with the model compound Reactive Blue 5 (RB5). The k-value of RB5 at 55ºC is enhanced 11.1-fold in the presence of AQS, and sixfold in the absence of AQS, upon comparison with mesophilic incubations at 30ºC. However, the anthraquinone dye RB19 exhibits a very strong toxic effect on VFA degradation and methanogenesis at both 30ºC and 55ºC. The apparent inhibitory concentrations of RB19 exerting 50% reduction in methanogenic activity (IC_{50}-value) are 55 mg/l at 30ºC and 45 mg/l at 55ºC. Further experiments at both temperatures

revealed that RB19 is mainly toxic to methanogens, because the glucose oxidizers including acetogens, propionate-forming, butyrate-forming and ethanol-forming microorganisms are not affected by the dye toxicity.

As hypothesized, the redox mediator AQS does not significantly increase the decolourisation rates of anthraquinone dyes with granular sludge. Moreover, a pure chemical decolourisation of RB5 even with high concentrations of sulphide as reducing agent is not achieved either in the presence or absence of AQS (Figure 9.4).

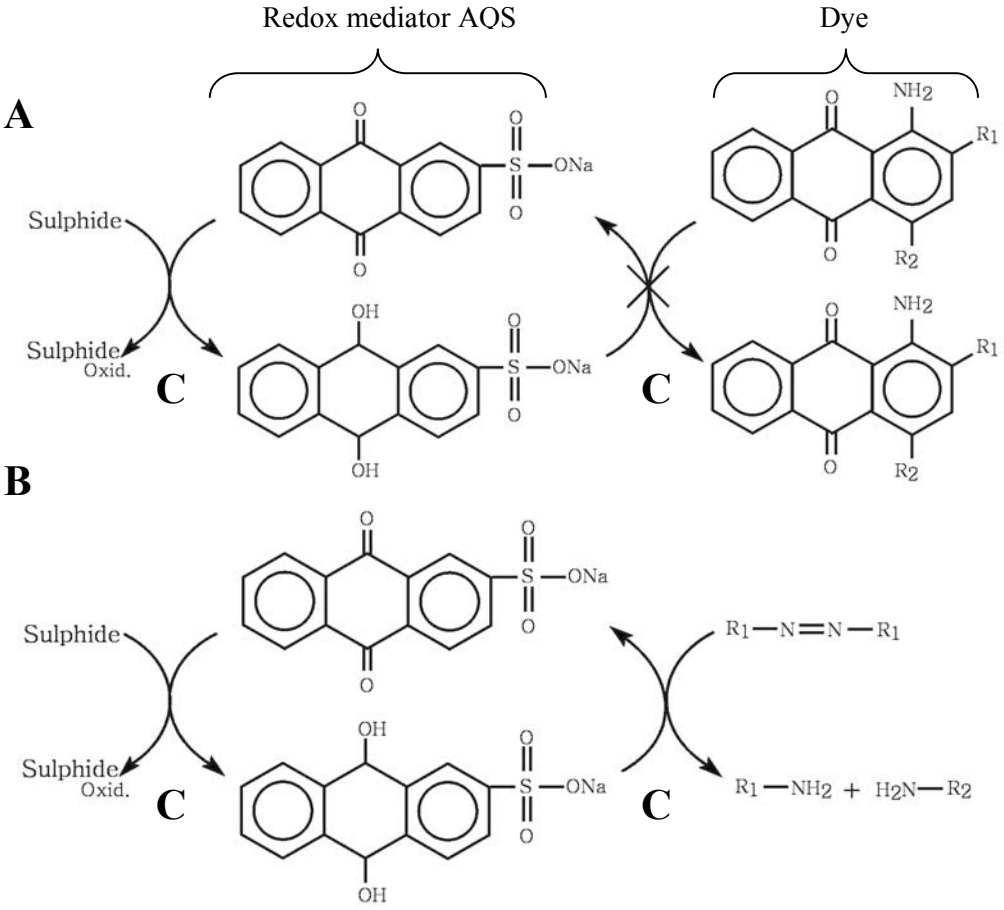


Figure 9.4. Chemical decolourisation (letter C) of anthraquinone (A) and azo (B) dyes by sulphide mediated by AQS.

The latter confirms that the carbonyl moiety of the anthraquinone link at neutral pH is indeed electronically very stable, making the nucleophilic attack by sulphide inefficient. Particularly in the case of RB5, the hydrogen bond formed between the carbonyl group of the quinone substituent and the amino substituent likely precludes the RB5 molecule from receiving an additional electron. In contrast, the complete decolourisation of the azo dye RR2

at neutral pH by sulphide demonstrates that the azo link is electronically less stable and therefore susceptible for reductive cleavage (Figure 9.4). Thus, based on the results presented for dyebaths containing both anthraquinone and azo dyes, the purely chemical decolourisation by the biogenic sulphide will occur mostly with azo dyes, as anthraquinone dyes are shown extremely stable under these conditions.

5. Microbiological aspects of azo dye reduction in methanogenic anaerobic granular sludge

Microbial decolourisation requires an unspecific enzymatic capacity ubiquitously found in a wide diversity of microorganisms (Chung and Stevens, 1993). This has been demonstrated with intestinal microorganisms such as *Clostridium, Salmonella, Bacillus, Eubacterium* and *Escherichia coli*, which are able to reduce dyes ingested through food, drugs and cosmetics. However, little is known about the exact role (contribution) of anaerobic consortia, from wastewater treatment plants in azo dye reduction, when a redox mediator is either present or absent.

In **Chapter 4** the decolourisation rates at 55ºC are found to be co-substrate dependent, in which a VFA mixture is the least efficient electron donor. The highest decolourisation rate is achieved in the presence of hydrogen. AQDS has a considerable impact on the decolourisation rate either when a glucose–VFA mixture or glucose is solely used as a co-substrate, whereas the rates for both electron donors are the same in the absence of AQDS. This indicates that AQDS is facilitating the electron transfer to the reductive decolourisation, most probably hydrogen- and formate-oxidizing species, since both intermediates are generated via glucose fermentation. The redox couple AQDS/AH_2QDS (reduction potential E_0' equal to -0.18 V) is thermodynamically more favourable than the redox couple CO_2/CH_4 (E_0' equal to -0.24 V) indicating the preference of AQDS as an electron acceptor (Cervantes, 2002). Hydrogen-oxidising rather than acetate-utilising microorganisms seem to be more actively involved in quinone-respiration under thermophilic and hyperthermophilic conditions (Field et al., 2000). Considering the increasing role of H_2 (or formate) at high temperatures (Van Lier et al., 1993a; Stams, 1994) and their effectiveness as electron donor for dye reduction, thermophilic anaerobic treatment seems to be advantageous either on the direct reductive decolourisation or on the hydroquinone generation, which is sometimes the rate-limiting step, when quinone-based redox mediators are used to accelerate azo dye reduction (Rau et al., 2002a).

In **Chapter 8** the contribution of acidogenic bacteria and methanogenic archaea to the reductive decolourisation of azo dyes by a thermophilic anaerobic consortium is studied. The results indicate that both acidogenic bacteria and methanogenic archaea may play an important role in azo dye reduction. The presence of a redox mediator like riboflavin strongly enhances the rates of decolourisation in channelling biogenic electrons to cleave the azo bond. Moreover, experiments with pure cultures of thermophilic methanogens reveal that these strains are unable to reduce the dye in the absence of riboflavin.

With glucose as an electron donor, the rates of dye reduction after inhibition of the methanogenic archaea by BES are comparable to the rates in incubations without this inhibitor. On the other hand, when the acidogenic bacteria are inhibited by vancomycin, a clear decrease in decolourisation rates is found, evidencing the important contribution of acidogens in the process. When riboflavin is supplemented as a redox mediator, the effect of glucose concentration on reductive decolourisation is more pronounced than in the absence of this mediator. Previous investigation with the VFAs, acetate, propionate, and butyrate in the absence of redox mediators showed that the rates of dye reduction were independent of the electron donors tested (Van der Zee et al., 2001a). This suggests that the low concentrations of hydrogen formed during propionate and butyrate oxidation cannot sustain the dye reduction. Apparently in our research, riboflavin is mainly assisting the transfer of reducing equivalents from glucose fermentation to the dye, thus allowing the acidogens to participate more effectively in the reductive decolourisation. These results agree with the previously reported ability of fermentative bacteria to use humic acids as an electron acceptor instead of riboflavin, as well as to reduce azo dyes (Benz et al., 1998; Yoo et al., 2000; Dos Santos et al., 2003).

Riboflavin also considerably increases the rates of azo dye reduction when important intermediates of anaerobic bioconversion such as acetate, methanol, hydrogen and formate are tested, whereas the latter two are by far the best electron donors for achieving reductive decolourisation. During the study of different electron donors in the carbon tetrachloride reduction, hydrogen was an effective electron donor, whereas methanol and acetate had a very poor electron donating capacity (Cervantes et al., 2004). In the same study, 20 μM of the redox mediator AQDS remarkably increased the reduction rates, making even the poor electron donors become effective electron donors for dechlorination. A possible explanation for the low azo dye reduction rates of methanogenic archaea in the presence of acetate and methanol may be related to the enzymes involved in methanogenesis from these compounds,

which do not utilize NAD^+ or $NADP^+$ as coenzymes. Instead some of the enzymes use a 5-deazaflavin F_{420} as coenzyme (Berk and Thauer, 1997). It is currently accepted that NADH or NAD(P)H are the primary electron donors for biological azo dye reduction (Semdé et al., 1998; Stolz, 2001; Blumel and Stolz, 2003).

In **Chapter 8** experiments with pure cultures of the thermophilic methanogens *M. thermoautotrophicus* ΔH and the strain NJ1 reveal that these strains are unable to reduce the azo dye RR2 in the absence of the redox mediator riboflavin. In contrast, Stolz (2001) suggested that almost all azo dyes tested so far are biologically reduced under anaerobic conditions. For instance, a complete reduction of the sulfonated azo dye Amaranth by α,β and γ-proteobacteria (Gram negative bacteria)*, low and high GC* (Gram positive bacteria), and the archaeon *Halobacterium salinarum*, was recently reported (Rau et al., 2002a). Based on our results with methanogenic archaea, anaerobic azo dye reduction is not a universal characteristic as it has been repetitively reported. Interestingly, in the presence of riboflavin, we found that both thermophilic strains are able to reduce RR2, suggesting the transfer of intracellular reduction equivalents to the outside of the cell and subsequent dye reduction.

Because hydrogen is the best electron donor for reductive decolourisations in the bioassays with thermophilic granular sludge, very likely other hydrogenotrophic methanogens rather than the strains tested could have been directly involved in the azo cleavage. Another possible explanation for the fast dye decolourisation of the riboflavin-free bottles with hydrogen as an electron donor could be due to redox mediators naturally produced in the sludge, which allow methanogens to participate in this reductive process.

6. Concluding remarks

Compared to the application of specific bacterial strains or specific enzymes, the anaerobic colour removal by granular sludge as a pre-treatment for coloured wastewaters from dyebath and rinsing steps seems to be a very attractive technology. In comparison with mesophilic anaerobic treatment, decolourisation rates of both azo and anthraquinone dyes are distinctly accelerated under thermophilic conditions. The significant enhancement of the electron transfer capacity and subsequent increase in colour removal of azo dyes simply by applying high temperature (55ºC) is demonstrated in continuous flow experiments at different HRT. The use of redox mediators as an electron shuttle is shown to be extremely effective in

enhancing azo dye reduction at 30ºC, but their catalytic effect on decolourisation rates is distinctly decreased at 55ºC. Such an effect is likely a combination of both biotic, i.e. the increased role of H_2 and higher sludge turnover rates (and subsequent increase of mediating and/or reducing compounds) at 55ºC compared to 30ºC, and abiotic mechanisms, i.e. increasing the collision frequency of the reactants dyes by the temperature increase, thus improving the kinetics of the reduction. The clear picture for the effectiveness of thermophilic treatment by anaerobic granular sludge on the complex biochemistry of dye reduction is not possible yet, and therefore further research is necessary.

7. New challenges, perspectives and recommendations to improve colour removal from textile wastewaters

The choice of the best redox mediator will depend on the dyes present in the wastewater, and ways to either immobilize redox mediators in bioreactors or to achieve their recovery when continuously dosed, still represent a challenge. Research to a further understanding of the toxicity mechanisms of anthraquinone dyes in anaerobic microorganisms, mainly with acetoclastic methanogens, is also suggested. The study of azo dye reduction in pure cultures of methanogens must be performed with other redox mediators to assess the effects of these compounds in the transport of reducing equivalents from inside to the outside of the cell and subsequent dye reduction. Furthermore, the identification of the natural redox mediators formed due to sludge turnover rates and cell lysis, as well as ways to increase the production of these compounds by molecular techniques, also represent a challenge.

The investigation of colour removal by thermophilic anaerobic granular sludge under extreme conditions of pH and salt concentration are suggested because such conditions are commonly found in textile wastewaters. To overcome the problems of biofilm/granule instability under the above-mentioned conditions, the application of membrane bioreactors could be considered. Because hydrogen was the best electron donor for colour removal, a gas-lift anaerobic bioreactor with hydrogen as a gas carrier may represent an alternative for future investigations. Furthermore, a comparative study between UASB and EGSB bioreactors operated under similar conditions, except for the up-flow velocity, is recommended to evaluate if mass transfer plays a distinct role in the decolourisation process. Different post-treatment methods for anaerobic effluents, e.g. aerobic biological post-treatment, ozone, and fenton reagents are suggested to be investigated under mesophilic and thermophilic conditions

to evaluate the temperature impact on the mineralization of aromatic amines. Because of the improvement on electron transfer capacity due to the temperature increase, thermophilic anaerobic treatment is suggested to other reductive transformations like nitroaromatics reduction and dehalogenations.

The most important recommendation is the development and standardization of a simple and efficient method for measuring colour removal of textile wastewaters, which is currently a real drawback for emissions control.

9'

Algemene Discussie en Conclusies

1. Bezwaren van textielafvalwater lozingen voor ecologie en volksgezondheid

Een groot aantal in de textielindustrie gebruikte chemicaliën, die een negatief effect hebben op het milieu en de volksgezondheid, komen vrij via het afvalwater en vertegenwoordigen gezamenlijk één van de belangrijkste bijdrages aan de ernstige milieuvervuiling wereldwijd (Hao, 2000). Het in het milieu terechtkomen van kleurstoffen is niet alleen ongewenst vanwege de verkleuring van ontvangende oppervlaktewateren, die de fotosynthese van aquatische planten kan beïnvloeden, maar ook omdat veel kleurstoffen en hun afbraak producten potentieel toxisch en/of mutageen zijn (Weisburger, 2002).

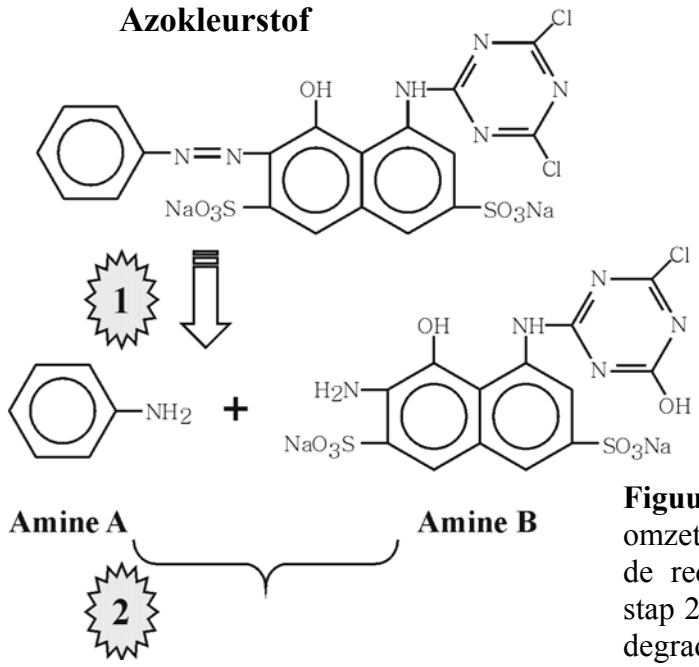
2. Technologische uitdaging voor de ontkleuring van textielafvalwater

Ontkleuring, vooral van textielafvalwater, is een grote uitdaging geweest gedurende de laatste decennia, desondanks is er momenteel geen enkele economisch attractieve behandeling voor de ontkleuring van kleurstoffen. De problemen van textielafvalwater ontkleuring zijn vooral gerelateerd aan de grote verscheidenheid in gebruikte kleurstoffen en aan de recalcitrante aard van een aantal kleurstoffen en/of hun gereduceerde producten. Bovendien verandert de samenstelling van het afvalwater continu, niet alleen als gevolg van het gebruik van verschillende kleurstoffen, maar ook vanwege de aanwezigheid van andere stoffen zoals natrium hydroxide, oppervlakte actieve stoffen en formaldehyde. Daarom moeten deze afvalwaterbehandelingsystemen flexibel zijn. **Hoofdstuk 2** beschrijft de voordelen en nadelen van de verschillende ontkleuringprocessen.

Het gebruik van biotechnologie voor de behandeling van textielafvalwater, zowel van oxidatieve als van reductieve processen, is de laatste jaren sterk toegenomen, en niet uitsluitend voor de ontkleuring, maar ook voor de complete biologische afbraak van kleurstoffen. Van verschillende micro-organismen zoals aërobe en anaërobe bacteriën, schimmels en actinomyceten is aangetoond dat ze zowel azo- als anthrachinon-kleurstoffen kunnen ontkleuren (**Hoofdstuk 2**). In de volgende discussie **zullen we ons voornamelijk richten op ontkleuring van azokleurstoffen** omdat ze de belangrijkste klasse van kleurstoffen vormen die gebruikt worden in

de textielindustrie, namelijk ongeveer 70% van het totale gewicht. Onder aërobe condities worden met bacteriën meestal lage ontkleuringefficiënties bereikt (10-30%), omdat de aanwezige zuurstof een betere elektronen acceptor is en daardoor een grotere voorkeur geniet voor de reductie-equivalenten dan de azokleurstoffen (Stolz, 2001). In tegenstelling, onder anaërobe condities worden de reductie-equivalenten efficiënter naar de azokleurstoffen, in dit geval de belangrijkste terminale elektronen acceptoren, geleid. **De ontkleuring onder anaërobe condities wordt ook wel azokleurstofreductie genoemd.** Azokleurstofsplitsing behelst een overdracht van vier elektronen, die via twee stadia op de azoverbinding verloopt, te weten vorming van een hydrazo-intermediair en uiteindelijk aromatische aminen. Figuur 9.1 laat de stappen zien die betrokken zijn bij de omzetting van azokleurstoffen. *Stap 1* geeft de anaërobe reductieve splitsing van de azoverbinding en daaropvolgende productie van de aromatische aminen, die anaëroob recalcitrant zijn. Biologisch kunnen azokleurstoffen dus niet volledig afgebroken worden, maar slechts gereduceerd. *Stap 2* geeft de vereiste nabehandeling, waarin de gereduceerde producten uiteindelijk worden afgebroken.

$$(R_1{-}N{=}N{-}R_2) + 4e^- + 4H^+ \rightarrow R_1{-}NH_2 + R_2{-}NH_2 \quad \text{(Step 1)}$$



Figuur 9.1. Stappen betrokken bij de omzetting van azokleurstoffen. Stap 1 geeft de reductie onder anaërobe condities, en stap 2 is de vereiste nabehandeling voor de degradatie van gereduceerde produkten.

De omzetting van aromatische aminen verloopt sneller dan de **reductieve splitsing van de azokleurstoffen**, hetgeen meestal **de snelheidsbepalende stap** van de totale reactie is. Veel nabehandeling methoden, volgend op de kosteneffectieve 'high-rate' anaërobe bioreactoren zijn onderzocht. Bijvoorbeeld, een sequentiële anaërobe-aërobe behandelingsstrategie is meerdere malen voorgesteld waarin de aërobe micro-organismen de aromatische aminen kunnen gebruiken

als stikstof en koolstof bron (Field et al., 1995; O'Neill et al., 2000a; Tan, 2001). Hoewel deze opzet in theorie mogelijk is hebben veel andere onderzoekers tegenstrijdige resultaten gevonden en momenteel is de toepasbaarheid van zo'n geïntegreerde opzet op grote schaal in de textielindustrie onduidelijk, mede vanwege de grote verscheidenheid aan gebruikte kleurstoffen, hetgeen tevens resulteert in een grote verscheidenheid aan gereduceerde producten (Tan et al., 2000; Stolz, 2001; Libra and Sosath, 2003). Alternatieve fysisch-chemische nabehandelingmethoden waarbij gebruik gemaakt wordt van ozon, fotokatalytische oxidatie, Fenton reagens, etc., laten goede vooruitzichten zien al zijn de behandelingskosten nu nog tamelijk hoog (Vandevivere et al., 1998; Libra and Sosath, 2003). Dit **Proefschrift** richt zich op de snelheidsbepalende ontkleuringstap en bediscussieerd strategieën om de snelheid van kleurstofreductie te verhogen.

3. Huidige stand van kennis over kleurverwijdering van azokleurstoffen in bioreactoren door anaëroob korrelslib

Hoewel anaërobe azokleurstofreductie met verschillende micro-organismen vlot behaald kan worden, is er tot dusver geen stam beschreven die een breed scala aan azokleurstoffen kan ontkleuren. Daarom is het gebruik van een specifieke stam of enzymen voor azokleurstofreductie bij de behandeling van textielafvalwater, met daarin vele soorten kleurstoffen, weinig zinvol (Laszlo, 2000). Het gebruik van gemengde cultures, zoals in het geval van anaëroob korrelslib, bestaande uit stabiele microbiële korrels met een hoge activiteit, is waarschijnlijk een logischer alternatief. Verschillende reactor configuraties, zoals de veelvuldig toegepaste 'upflow anaerobic sludge bed' (UASB) en 'expanded granular sludge bed' (EGSB) systemen, worden gebruikt om hoge concentraties biomassa te immobiliseren (Lettinga et al., 1980; Lettinga, 1995; Van Lier et al., 2001). Bovendien kunnen de verschillende microbiële consortia aanwezig in anaëroob korrelslib taken uitvoeren die geen enkele individuele reincultuur succesvol kan uitvoeren (Nigam et al., 1996; Pearce et al., 2003).

Veel onderzoekers hebben azokleurstofreductie onderzocht, alsmede het lot van aromatische stoffen in zowel anaërobe als aërobe bioreactoren. Als eerste ontdekte Razo-Flores (1997) dat de reductie van de azokleurstof Mordant Orange 1 (MO1), met efficiënties tot 99%, bereikt kan worden in een UASB reactor gevoed met glucose of VFA. Interessant is dat batch experimenten aangetoond hebben dat de azokleurstof MO1 200-maal en 2140-maal toxischer voor de anaërobe micro-organismen was dan de verwachte reductieproducten 5-aminosalicyl zuur en 1,4-phenylelediamine, respectievelijk. Tan (2001) onderzocht het gebruik van een geïntegreerd en opeenvolgend anaëroob/aëroob systeem voor de biologische afbraak van azokleurstoffen. In het

geïntegreerde anaërobe/aërobe systeem, met ethanol als co-substraat, speelde de zuurstofconcentratie een belangrijke rol in de reductie van de azokleurstof MO1. Met een opeenvolgend azokleurstof afbraak systeem, waarbij de anaërobe en aërobe stappen plaatsvinden in twee verschillende reactoren, werden zowel hogere kleurstofreductiesnelheden in de anaërobe fase alsmede hogere aromatische-amine mineralisatiesnelheden behaald. Van der Zee (2002) onderzocht de reductiemechanismen van azokleurstoffen in batch en continu-doorstroomde systemen. Er werd geen correlatie gevonden tussen de moleculaire grootte en ontkleuringsnelheid, hetgeen aangeeft dat kleurstof penetratie door de celwand geen belangrijke rol speelt in reductieve ontkleuring, en dat deze waarschijnlijk extracellulair of membraan gebonden optreedt. Bovendien werden lage reductiesnelheden en ernstige toxische effecten gevonden voor de azokleurstof Reactive Red 2 gevonden in a UASB reactor gevoed met een VFA mengsel als elektronen donor en bedreven bij een HRT van 6 uur. De toevoeging van katalytische concentraties van de redox mediator AQDS is een efficiënte strategie gebleken om de reactor prestaties te herstellen in termen van zowel ontkleuring als COD verwijdering. Cervantes et al. (2001) liet zien dat ontkleuringefficiënties van ongeveer 90% bereikt kunnen worden met de azokleurstof Acid Orange 7 in een UASB reactor bedreven bij een HRT van 2 uur en gevoed met VFA en 3μM AQDS. Het effect van de HRT verlaging was duidelijker in de afwezigheid van AQDS, resulterend in een sterke daling van de efficiënties. Dit wijst erop dat AQDS helpt in de overdracht van reductie-equivalenten naar de azokleurstof, welke klaarblijkelijk de snelheidsbepalende factor is. Alle hiervoor genoemde onderzoeken werden uitgevoerd onder mesofiele condities en gebruikten één enkele azokleurstof als model.

Hoofdstuk 2 verklaart dat azokleurstofreductie bestaat uit een biologisch deel, de biologische generatie van reductie-equivalenten, en een chemisch deel, waarin de gevormde elektronen chemisch de azokleurstof reduceren. In het algemeen volgt de chemische reactie de Arrhenius vergelijking, hetgeen aanduidt dat een temperatuursverhoging de botsfrequentie van de reactanten proportioneel verhoogd en daarmee de kinetiek van de reactie. Daarom zou het gebruik van thermofiele behandeling voordelig kunnen zijn voor de verbetering van het chemisch deel van de totale reactie. Bovendien zou het gebruik van redox mediatoren als elektronen shuttle voor de versnelling van azokleurstofreductie onder thermofiele omstandigheden voordelig kunnen zijn, niet alleen voor de verwachte snellere enzymatische reductie van de mediator ten opzichte van mesofiele omstandigheden, maar ook voor de snellere regeneratie van de gereduceerde vorm van de mediator door de chemische reactie met azokleurstoffen. In dit zicht is het belangrijk te weten dat een groot deel van textielafvalwater, vooral komend van het verfbad, vrijkomt bij hoge temperaturen. Tot dusver zijn redox mediatoren in combinatie met thermofiele omstandigheden nog niet onderzocht.

3.1. Thermofiele ontkleuring van azokleurstoffen door anaëroob korrelslib

Onze resultaten laten duidelijk zien dat door thermofiele behandeling met anaëroob korrelslib de azokleurstofreductie versneld wordt. **Hoofdstuk 3** beschrijft dat de metabole activiteit van het inoculum een snelle reductie van RR2 initieert, hetgeen duidt op een overwegend biotische reactie in het proces. Het toegevoegde co-substraat, optredend als een primaire elektronen donor, is niet alleen belangrijk voor de verbetering van de ontkleuringsnelheden, maar tevens voor verstrekking van de hoge onderhoudsvereisten van de thermofiele micro-organismen. Biologische behandeling op 55ºC vertoont een snelle initiële productie van reducerende stoffen door co-substraat omzetting, hetgeen we geverifieerd hebben in batch experimenten met korrelslib door de k-waarden in incubaties vrij van sulfide te vergelijken met incubaties in de aanwezigheid van 2 mM sulfide. De pseudo-exponentiële verhoging van de ontkleuringsnelheden op 55ºC door de VSS concentratie te verhogen geeft een ander voorbeeld van de snelle initiële productie en/of overdracht van reducerende stoffen door co-substraat omzetting. Daarnaast bevestigen de negatievere redox potentialen verkregen op 55ºC vergeleken met die op 30ºC een betere reductie capaciteit op 55ºC. Als gevolg van de hiervoor genoemde factoren werden zesvoudige en tweevoudige verhogingen van de ontkleuringsnelheden aangetoond in de afwezigheid en aanwezigheid van AQDS, respectievelijk, tussen thermofiele (55ºC) en mesofiele (30ºC) omstandigheden gebruikmakend van dezelfde slib bron. Daardoor is hoogstwaarschijnlijk de overdracht van reductie-equivalenten op 30ºC de snelheidsbepalende stap.

De resultaten van **Hoofdstuk 4** laten zien dat thermofiele EGSB bioreactoren zeer efficiënt zijn voor de behandeling van de recalcitrante azokleurstof RR2, alsmede zeer stabiel presteren bij hoge belasting met RR2 (tot 2,7 gRR2 l^{-1} dag^{-1}). Bovendien is de invloed van de redox mediator AQDS op de ontkleuring minder duidelijk dan in eerdere onderzoeken, uitgevoerd onder mesofiele condities, is aangetoond (Van der Zee et al., 2001a; Cervantes et al., 2001). Zeer waarschijnlijk is dit verschil ook gerelateerd aan de toegepaste hogere HRT van 10 uur in dit experiment op 55ºC dan de HRT van 6 uur eerder toegepast op 30ºC. Langdurige experimenten hebben laten zien dat de AQDS-vrije reactor efficiënties kon bereiken van zo'n 91%, terwijl de reactor met AQDS efficiënties behaalde van zo'n 95%. De hogere ontkleuringsnelheden op 55ºC, vergeleken met die behaald op 30ºC, zijn belangrijk voor het omzettingsproces, niet alleen voor de directe ontkleuring, maar ook indirect omdat een veel lagere slibfractie, in en rondom de biofilm, blootgesteld is aan mogelijk toxische concentraties RR2. Van azokleurstoffen is bekend dat ze toxischer zijn voor methanogenen dan hun splitsingproducten, de aromatische aminen (Donlon et al., 1997). De thermofiele EGSB bioreactoren waren bovendien zeer stabiel met betrekking tot de volledige omzetting van het co-substraat in methaan, hetgeen in contrast staat tot de resultaten van

Van der Zee et al. (2001a). Zij rapporteerden een afname van de ontkleuringefficiënties tot 25% na 40 dagen voeden met niet-gehydrolyseerd RR2 in de afwezigheid van AQDS bij anaërobe behandeling op 30ºC. Bovendien zakte de co-substraat (VFA) verwijderingefficiëntie naar zo'n 10%. Niettemin konden de ontkleuringcapaciteit en co-substraat omzetting terug verkregen worden nadat 19μM AQDS aan de reactor was toegevoegd. In onze experimenten zijn geen toxische effecten gesignaleerd, hetgeen mogelijk toegeschreven kan worden aan het gebruik van gehydrolyseerd RR2 in tegenstelling tot gebruik van niet-gehydrolyseerd RR2 in eerdere studies. Het toxische effect van de eerder gebruikte RR2 kan meer uitgesproken zijn, zoals aangegeven in de resultaten van Chung en Stevens (1993), die remming van microbiële respiratie beschreven als gevolg van de aanwezigheid van chloride of bromide als functionele groepen. De verschillen in reactor type, bijvoorbeeld wanneer UASB en EGSB reactoren worden vergeleken voor massa overdrachtsbeperkingen, is waarschijnlijk van ondergeschikt belang voor de toxiciteit van azokleurstoffen, vooral als geschikte primaire elektronen donoren en HRT worden toegepast. Onze resultaten tonen dat de toepassing van thermofiele behandeling en redox mediatoren om toxiciteit problemen te overwinnen een effectievere strategie is dan grote verhogingen van de HRT.

De significante verbetering van de elektronen overdrachtscapaciteit en daaropvolgende verhoging van de reductieve ontkleuring van azokleurstoffen, eenvoudigweg door het toepassen van een hoge temperatuur, is gedemonstreerd in **Hoofdstuk 6**. Zo is bijvoorbeeld, bij een HRT van 2,5 uur in de afwezigheid van AQDS, de ontkleuring 5,3-maal hoger op 55ºC in vergelijking met de efficiëntie behaald op 30°C. Bovendien is het katalytisch effect van de redox mediator AQDS op de reductieve ontkleuring zelfs afgenomen met een factor 3,2 in vergelijking met experimenten uitgevoerd bij 30ºC. Meest waarschijnlijk is het verminderde effect van AQDS het gevolg van de hoge efficiëntie die behaald wordt op 55°C en maskeert dit de invloed van AQDS op de ontkleuring. Dezelfde mate van COD verwijdering van zo'n 75% geeft aan dat de productie van reductie-equivalenten op soortgelijke snelheden plaatsvindt in mesofiele en thermofiele bioreactoren. Klaarblijkelijk heeft het verschil in ontkleuringsnelheden niet gerelateerd aan het verschil in productie van reductie equivalenten. Daarom concluderen we dat de hogere mate van ontkleuring het gevolg is van de invloed van temperatuur en AQDS op de overdracht van elektronen. In **Hoofdstuk 6** hebben we onderzocht of op 30ºC ontkleuring en COD verwijdering gekoppeld kan worden in de behandeling van hoge concentraties kleurstoffen in anaërobe reactoren. De resultaten laten zien dat variaties in ontkleuringsnelheden als gevolg van een verhoging van de verhouding tussen geconcentreerd verfbadafvalwater en spoelwater minder duidelijk zijn dan het effect van deze verhouding op de methaanvorming. Bijvoorbeeld bij de

vergelijking van de verhoudingen (% v:v) 0:100 en 2:98 in de afwezigheid van AQS, lieten de k-waarden voor ontkleuring, 0,32 dag^{-1} en 0,19 dag^{-1}, respectievelijk, een 0,6-voudige afname zien. Het verschil in ontkleuringsnelheid is niet significant wanneer we in beschouwing nemen dat de initiële absorptie piek op 475 nm 13-maal hoger was bij de verhouding 2:98 in vergelijking met de verhouding 0:100. Hoge concentraties van verfbadafvalwater resulteren in een ophoping van acetaat en een ernstige remming van de methanogenese. De specifieke methanogene activiteit van de afvalwatervrije controles is 4-maal hoger dan die bij een verhouding van 2:98. Een afname van 50% in CH_4 productie ten opzichte van de controle is gevonden met 22 ml van het verfbadafvalwater in iedere 1000 ml spoelwater (2.2:100).

3.2. Redox mediatoren

Redox mediatoren en ontkleuring

Flavine-bevattende stoffen alsmede chinon-verbinding bevattende stoffen zijn uitvoerig beschreven als redox mediatoren. In deze gevallen reduceren aspecifieke enzymen de mediator biologisch, die als de initiële elektronen acceptor fungeert voor de omzetting van de primaire elektronen donor. Vervolgens worden de elektronen chemisch overgebracht op de azokleurstof, de uiteindelijke elektronen acceptor, waarbij de mediator geregenereerd wordt (**Hoofdstuk 2**).

In **Hoofdstuk 5** tonen we aan dat de snelheidsbepalende stap, de overdracht van reductie-equivalenten naar de azokleurstof, versneld wordt onder thermofiele condities. Zowel de biotische als de abiotische mechanismen betrokken in de biochemie van ontkleuring versnellen op 55ºC. De snellere biologische reductie van de redox mediator, AQDS, in incubaties met slib op 55°C in vergelijking met 30°C geeft bewijs voor de biologische bijdrage in de verhoging van de elektronen overdrachtssnelheid. Een voorbeeld hiervan is dat 1 mM AQDS volledig gereduceerd was op 55ºC na 0,7 dag, terwijl mesofiel de reductie na 0,7 dag slechts 12,9% bedroeg. Bovendien werd op 55ºC geen lag-fase gevonden. De abiotische chemische reductie van RR2 door sulfide volgt, als verwacht, de Arrhenius vergelijking, en de ontkleuringsnelheden worden overeenstemmend hiermee verhoogd door de temperatuursverhoging. Bovendien vertonen de incubaties met AQS een lagere Ea behoefte. De berekende Ea waarden zijn 27,9 kJ/mol en 22,9 kJ/mol in de AQS-vrije en AQS verstrekte incubaties, respectievelijk. Daarmee wordt de activering energie 1,2-voudig verlaagd door de toevoeging van 0,012 mM AQS.

Theoretisch moeten bruikbare redox mediatoren voor biologische azokleurstofreductie redox potentialen hebben tussen de half reacties van de azokleurstof en de primaire elektronen donor (Van der Zee et al., 2003b). In een vergelijkend onderzoek naar redox potentiaal waarden van verschillende azokleurstoffen is gevonden dat de waarden voor de standaard redox potentialen

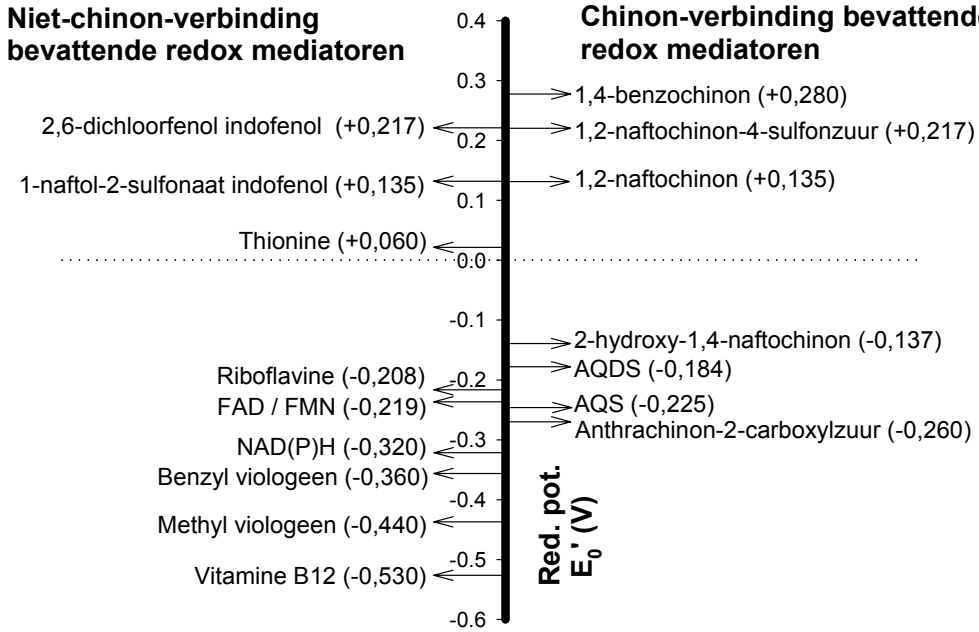
(E_0') in het algemeen tussen –0,430 en –0,180 V liggen (Dubin and Wright, 1975). Rau et al. (2002) voert aan dat de NAD(P)H cofactor, die de laagste E_0' waarde van –0.320 V bezit, de grens aangeeft voor de toepassing van redox mediatoren. De reden hiervoor is dat mediatoren met een meer negatieve E_0' waarde niet door de cellen gereduceerd kunnen worden, terwijl mediatoren met een E_0' groter dan –0,05 V de azoverbinding niet efficiënt kunnen reduceren. Hoewel deze observatie bevestigd is met een model stof (Rau et al., 2002a), is het nooit geverifieerd voor een complex textielafvalwater bestaande uit een mengsel van veel kleurstoffen met verschillende E_0' waarden.

In **Hoofdstuk 6** is een textielafvalwater met veel kleurstoffen met verschillende redox potentialen getest onder mesofiele en thermofiele condities om de snelheidsbepalende stap van de ontkleuring te beoordelen, d.w.z. de enzymatische mediator reductie of de chemische elektronen overdracht van de mediator naar de kleurstof. Vergeleken met AQS (E_0' van -225 mV) bevattende tests, laten flessen met BQ toegevoegd (E_0' van +280 mV) geen versnelde ontkleuring van textielafvalwater zien. Waarschijnlijk is dit gerelateerd aan het onvermogen om elektronen van zijn gereduceerde vorm (hydrobenzochinon) naar de kleurstoffen aanwezig in het afvalwater over te dragen, ondanks dat het eerste deel van de reactie mogelijk wel versneld is als gevolg van het verschil in E_0' van AQS en BQ. Wanneer beide mediatoren, BQ en AQS, gezamenlijk geïncubeerd worden is geen verbetering van de ontkleuringsnelheden gevonden in vergelijking met de flessen met uitsluitend AQS, ondanks dat een bredere spreiding van de elektronen overdrachtcapaciteit in de oplossing met kleurstoffen bestreken zou kunnen worden (Meckstroth et al., 1981). Daarom is ofwel de concentratie van de mediator met negatieve E_0' (AQS) bepalend voor de ontkleuringsnelheden, ofwel de redox potentiaal in de oplossing gaat naar een waarde zodat de gereduceerde vorm van BQ de elektronen efficiënter kan overbrengen naar zowel direct de kleurstof danwel naar AQS in een sequentiële reactie waarbij mediatoren en kleurstof betrokken zijn. Onze huidige resultaten laten zien dat het gebruik van mengsels van redox mediatoren met positieve en negatieve E_0' onder anaërobe condities om een grote spreiding van kleurstof redox potentialen te omvatten, geen efficiënte benadering is om ontkleuring van textielafvalwater te verbeteren.

Redox mediatoren en de invloed van sterische en elektrochemische factoren op ontkleuring door korrelslib

De E_0' is een goede indicator voor de capaciteit van een stof om te functioneren als redox mediator. Echter zijn, schijnbaar in aanvulling op E_0', andere factoren ook van belang. Verschillende ontkleuringsnelheden in de aanwezigheid van mediatoren met gelijkaardige E_0' waarden zijn gerapporteerd, alsmede gelijkaardige ontkleuringsnelheden met mediatoren met

verschillende E_0' waarden. Brown (1981) heeft bijvoorbeeld de polymerische nitrokleurstof Poly Y-607 getest en vond dat zowel methyl viologeen als benzyl viologeen de ontkleuringsnelheid met een factor 4,5 verhoogde, ondanks dat de E_0' van methyl viologeen veel lager is dan die van benzyl viologeen, namelijk –0,440 V and –0,360 V, respectievelijk (Figuur 9.2).

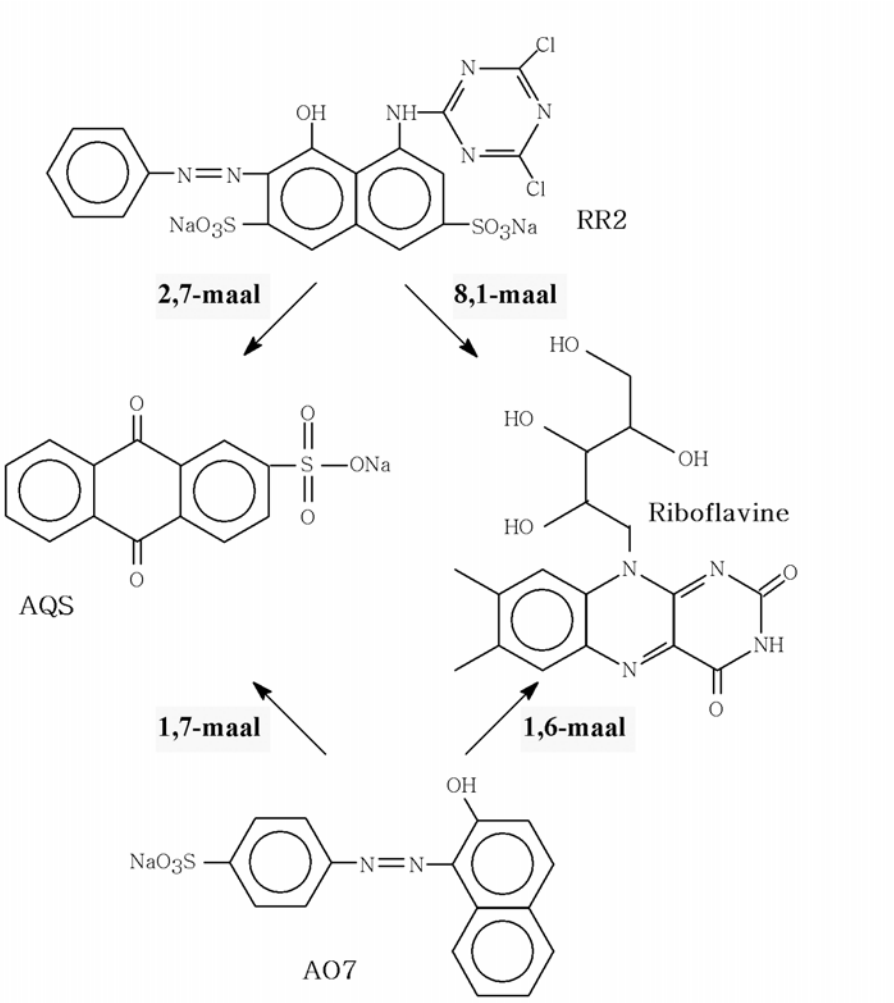


Figuur 9.2. E_0' waarden voor zowel chinon als niet-chinon-verbinding bevattende redox mediatoren (Aangepast van Rau et al., 2002a; Fultz and Durst, 1982).

In **Hoofdstuk 4** laten we zien dat katalytische concentraties van de redox mediatoren AQS, AQDS en riboflavine geïncubeerd met korrelslib op 55ºC de ontkleuringsnelheden van model azokleurstoffen tot 8-maal verbeteren in vergelijking met mediator-vrije flessen. Toevoeging van een mediator heeft geen invloed op de orde van de reactie. Riboflavine is verreweg de beste mediator voor de reactieve azokleurstof RR2 en verhoogd de k_1-waarde 3-maal in vergelijking met de incubatie met AQS. Dezelfde trend is echter niet gevonden met AO7 en MY10, waarvoor AQS een hogere katalytische capaciteit laat zien dan riboflavine. Wanneer we de ontkleuringsnelheden tussen flessen waaraan AQS- en AQDS toegevoegd zijn vergelijken, zien we dat de capaciteit van AQS veel hoger is dan van AQDS, namelijk voor RR2 1,8-maal en voor AO7 1,4-maal. Verrassend genoeg laten alle mediatoren bij de ontkleuring van MY10 gelijkaardige snelheden zien. Echter, zoals verwacht is vitamine B12 een erg slechte redox mediator voor deze azokleurstoffen, hetgeen waarschijnlijk veroorzaakt wordt door zijn sterke negatieve redox potentiaal E_0' van –0,530 V.

Riboflavine en AQS vertonen gelijkaardige mediator eigenschappen, waarschijnlijk gerelateerd aan de min of meer gelijke redox potentialen (E_0') van riboflavine en AQS (–0,208 V

and –0,225 V, respectievelijk, Figuur 9.2). Riboflavine is echter een veel betere mediator voor ontkleuring van de azokleurstof RR2 dan AQS (Figuur 9.3).



Figuur 9.3. Klaarblijkelijke verschillen in reductiesnelheden van de azokleurstoffen RR2 and AO7, in de aanwezigheid van de redox mediatoren riboflavine en AQS, hetgeen aantoont dat andere factoren, zoals sterische en elektrochemische factoren, tussen mediator en azokleurstof, waarschijnlijk een grotere rol spelen dan de E_0' van de mediatoren.

Dit resultaat suggereert dat andere factoren zoals sterische hinder tussen de azokleurstof en redox mediator de ontkleuringsnelheden in het systeem mee bepalen. Walker en Ryan (1971) stelden dat ontkleuringsnelheden gerelateerd hebben aan de elektronen dichtheid in de azoverbindingregio. Zij suggereerden een verbetering van de ontkleuring door de elektronendichtheid bij de azoverbinding te verlagen. Daardoor zou het gebruik van redox mediatoren niet alleen de overdracht van reductie-equivalenten naar de terminale elektronen acceptor (azokleurstof) versnellen, maar tevens de sterische hinder van de kleurstof molecuul minimaliseren (Moir et al., 2001). Schijnbaar is het nodig om verschillen in sterische en elektrochemische factoren tussen mediator en azokleurstof eveneens in acht te nemen wanneer

men kijkt naar de geschiktheid van een specifieke mediator. Bovendien speelt de penetratie van redox mediatoren door het cytoplasma en de daaropvolgende overdracht van reductie equivalenten van binnen de cel naar buiten een belangrijke rol in het versnellen van azokleurstofreductie (**Hoofdstuk 8**).

Natuurlijke redox mediatoren en indirecte bijdrages aan azokleurstofreductie

Hoofdstuk 3 toont dat AQDS (0,012 mM) de ontkleuringsnelheden verhoogd (k-waarde) wanneer co-substraat wordt toegevoegd. Er is echter geen effect op de k-waarden van endogene controles geconstateerd, wat suggereert dat co-substraat omzetting gekoppeld aan elektronen overdracht door AQDS nodig is voor verbetering van de ontkleuring. Bovendien is de invloed van AQDS als redox mediator bij hoge VSS concentraties minder duidelijk dan bij lage VSS concentraties. Gebaseerd op deze resultaten stellen we dat intracellulaire componenten met mediator eigenschappen vrijkomen in het medium door de toevoeging van biomassa. In het algemeen vertonen thermofiele micro-organismen, in vergelijking met mesofiele micro-organismen, hogere metabolische snelheden (2-3 maal), onderhoudsenergie vraag, en slib vernieuwingsnelheden (Van Lier et al., 1993b).

Keck et al. (2002) rapporteerde dat tijdens aërobe afbraak van naftaleen-2-sulfonaat (2NS) bij 30ºC door *Sphingomonas xenophaga* stam BN6, redox mediatoren geproduceerd werden, die de efficiëntie van de stam om azokleurstoffen anaëroob te reduceren verhoogde. Chinon-verbinding bevattende stoffen zijn niet aangetroffen in methanogenen, maar van fenazine-derivaten is aangetoond dat ze een chinon-achtige rol in de elektronen overdracht van *Methanosarcina mazey* Gö1 kunnen uitvoeren (Abken et al., 1998). Het is erg waarschijnlijk dat deze indirecte bijdrage van natuurlijke vorming van redox mediatoren aan azokleurstofreductie hoger is op 55ºC dan op 30ºC vanwege een hogere slib vernieuwingsnelheid en cel lysis op 55ºC dan op 30ºC. Dus intracellulaire mediator stoffen, eerder inactief vanwege het onvermogen om de celmembraan te passeren, kunnen actief bijdragen aan de reductieve ontkleuring. In toevoeging daarop kunnen cel lysis en slib afbraak het medium voorzien met reducerende stoffen, bijv. sulfide, ijzer en andere gereduceerde co-factoren, die de azokleurstoffen chemisch kunnen reduceren.

Redox mediatoren en toxiciteit naar de trofische groepen aanwezig in korrelslib

In **Hoofdstuk 3** werd een verwaarloosbare toename in de ontkleuringcapaciteit van thermofiel slib gevonden bij hoge AQDS concentraties. Daarnaast remmen hoge AQDS concentraties thermofiele acetaat omzetting, omdat acetaat accumuleerde en methanogenese overeenstemmend afnam.

Propionaat oxidatie wordt ook beïnvloed door een toename van de AQDS concentratie, waarbij de propionaat omzetting vrijwel stopt bij AQDS concentraties hoger dan 1,2 mM. Aan de andere kant wordt glucose fermentatie niet beïnvloed door hoge AQDS concentraties, hetgeen aangetoond is door de niet noemenswaardige detectie van dit substraat aan het einde van de incubatie periode.

In **Hoofdstuk 6** is de CH_4 productie in de AQS-vrije flessen ongeveer 1,2-maal hoger dan in die met AQS (0,050 mM). In **Hoofdstuk 7** is gevonden dat de anthrachinon kleurstof Reactive Blue 19 (RB19) niet toxisch is voor acidogenen, maar extreem toxisch voor acetaatgebruikende methanogenen in mesofiel korrelslib. Waarschijnlijk, omdat AQS een anthrachinon-soort molecuul is, bevestigd dat deze toxiciteit voor methanogenen, net als bij RB19, waarschijnlijk veroorzaakt wordt door de anthrachinon structuur. Interessant is dat in **Hoofdstuk 6**, een AQDS concentratie van 0,025 mM geen enkel toxisch effect heeft op methanogenen, omdat de CH_4 productie van de AQDS-vrije en AQDS-toegevoegde reactor gelijkaardig zijn. Dit is in overeenstemming met de resultaten van Cervantes et al. (2003), waarin een snel herstel van de methanogene activiteit gevonden werd bij het gebruik een AQDS concentratie die zelfs ongeveer 500-maal hoger is dan de AQS concentratie gebruikt in het onderhavige onderzoek. De vraag waarom AQS toxischer is dan AQDS voor methanogenen blijft onbeantwoord.

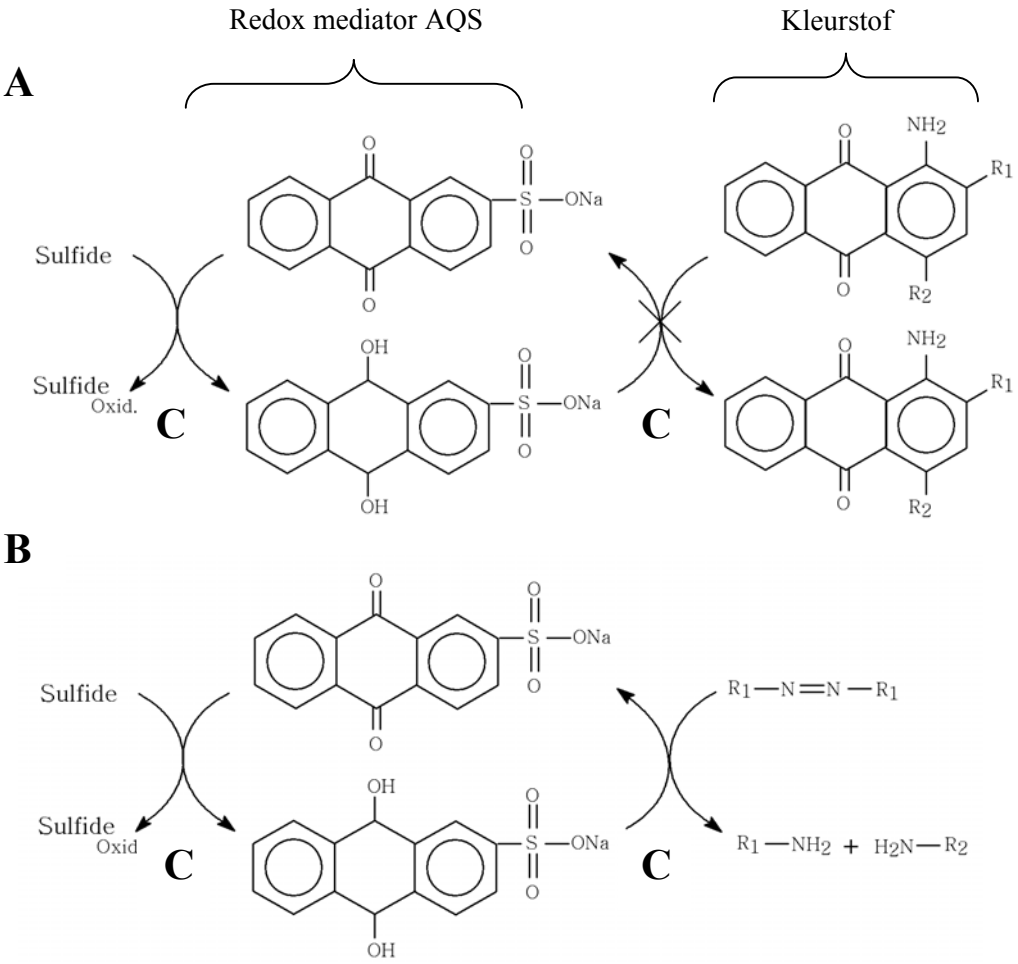
4. Toxiciteit en transformatie van anthrachinon kleurstoffen door mesofiele en thermofiele consortia

In **Hoofdstuk 7** demonstreren we dat in vergelijking met incubaties op 30ºC, incubaties op 55ºC aanzienlijk hogere ontkleuringsnelheden laten zien en niet uitsluitend met echt afvalwater, maar tevens met model kleurstof Reactive Blue 5 (RB5). De k-waarde van RB5 op 55ºC is 11,1-maal toegenomen in de aanwezigheid van AQS, en zesvoudig in de afwezigheid van AQS, ter vergelijking met mesofiele incubaties op 30ºC. De anthrachinon kleurstof RB19 geeft echter een sterk toxisch effect op VFA afbraak en methanogenese bij zowel 30ºC als 55ºC. De klaarblijkelijke remmende concentraties van RB19 waarbij 50% reductie in methanogene activiteit optreedt (IC_{50}-waarde) zijn 55 mg/l bij 30ºC en 45 mg/l bij 55ºC. Verdere experimenten op beide temperaturen lieten zien dat RB19 vooral toxisch is voor methanogenen, omdat de glucose oxideerders waaronder acetogenen, propionaat-vormende, butyraat-vormende en ethanol-vormende micro-organismen niet getroffen worden door de toxiciteit van de kleurstof.

Zoals voorgesteld neemt de ontkleuringsnelheid van anthrachinon kleurstoffen in korrelslib niet significant toe door de redox mediator AQS. Bovendien kan een puur chemische

ontkleuring van RB5 zelfs in de aanwezigheid van hoge concentraties sulfide als reducerende stof niet bereikt worden in de aan- of afwezigheid van AQS (Figuur 9.4).

Het laatste bevestigt dat de carbonyl structuur van de anthrachinon verbinding bij neutrale pH inderdaad elektronisch erg stabiel is en waardoor de nucleofiele aanval van sulfide inefficiënt is. Dit is in het bijzonder het geval voor RB5, waar de waterstofbinding gevormd tussen de carbonyl-groep van de chinon-substituent en de amino-substituent waarschijnlijk het RB5 molecuul belet om een extra elektron te ontvangen. In tegenstelling hierop toont de complete ontkleuring van de azokleurstof RR2 bij neutrale pH door sulfide dat de azoverbinding elektronisch minder stabiel is en daardoor gevoelig voor reductieve splitsing (Figuur 9.4). Dus, gebaseerd op de resultaten gepresenteerd voor verfbaden die zowel anthrachinon en azokleurstoffen bevatten, verloopt de pure chemische ontkleuring door het biologisch gevormde sulfide voornamelijk met azokleurstoffen, omdat anthrachinon kleurstoffen zeer stabiel gebleken zijn onder deze condities.



Figuur 9.4. Chemische ontkleuring (letter C) van anthrachinon (A) en azo- (B) kleurstoffen door sulfide met AQS als mediator.

5. Microbiologische aspecten van azokleurstofreductie in methanogene anaëroob korrelslib

Microbiële ontkleuring vereist een aspecifieke enzymatische capaciteit die veelvuldig in een grote diversiteit aan micro-organismen wordt aangetroffen (Chung and Stevens, 1993). Dit heeft men aangetoond met inwendige micro-organismen, zoals *Clostridium, Salmonella, Bacillus, Eubacterium* en *Escherichia coli*, welke kleurstoffen kunnen reduceren die ingenomen zijn door voedsel, medicijnen en schoonheidsmiddelen. Er is echter maar beperkte kennis van de exacte rol (bijdrage) van anaërobe consortia in afvalwaterbehandelinginstallaties in azokleurstofreductie, wanneer een redox mediator aanwezig danwel afwezig is.

In **Hoofdstuk 4** is gevonden dat de ontkleuringsnelheden op 55ºC co-substraat afhankelijk zijn, en waarbij een VFA mengsel de minst efficiënte elektronen donor is gebleken. De hoogste ontkleuringsnelheid is behaald in de aanwezigheid van waterstof. AQDS heeft een aanzienlijke invloed op de ontkleuringsnelheid wanneer ofwel een glucose–VFA mengsel of glucose als enig co-substraat gebruikt wordt, terwijl de snelheden voor beide elektronen donoren gelijk zijn in de afwezigheid van AQDS. Dit geeft aan dat AQDS de elektronenoverdracht naar de reductieve ontkleuring vereenvoudigd, meest waarschijnlijk waterstof- en formaat-oxiderende soorten, omdat beide intermediairen gevormd worden via fermentatie van glucose. Het redox koppel AQDS/AH_2QDS (reductie potentiaal E_0' gelijk aan -0.18 V) is thermodynamisch gunstiger dan het redox koppel CO_2/CH_4 (E_0' gelijk aan -0.24 V) hetgeen de voorkeur voor AQDS als elektronen acceptor duidelijk maakt (Cervantes, 2002). Waterstof-oxiderende micro-organismen lijken meer actief betrokken te zijn in chinon respiratie onder thermofiele en hyperthermofiele condities dan acetaatgebruikende micro-organismen (Field et al., 2000). In beschouwing nemend dat H_2 (of formaat) een toenemende rol speelt op hoge temperaturen (Van Lier et al., 1993a; Stams, 1994) en hun effectiviteit als elektronen donor voor kleurstofreductie, lijkt thermofiele anaërobe behandeling voordelig te zijn zowel voor de directe reductieve ontkleuring als voor de hydrochinon productie, hetgeen soms de snelheidsbepalende stap is wanneer chinon redox mediatoren gebruikt worden om de azokleurstofreductie te versnellen (Rau et al., 2002a).

In **Hoofdstuk 8** is de bijdrage van acidogene bacteriën en methanogene archaea aan de reductieve ontkleuring van azokleurstoffen door een thermofiel anaëroob consortium bestudeerd. De resultaten geven aan dat zowel acidogene bacteriën en methanogene archaea een belangrijke rol kunnen vervullen bij de azokleurstofreductie. De aanwezigheid van een redox mediator zoals riboflavine verbetert de ontkleuringsnelheden sterk door het kanaliseren van de biologisch gevormde elektronen voor de splitsing van de azoverbinding. Bovendien hebben experimenten met reinculturen van thermofiele methanogenen laten zien dat deze stammen de kleurstof niet in de afwezigheid van riboflavine kunnen reduceren.

Met glucose als elektronen donor zijn de snelheden van de kleurstofreductie na remming van de methanogene archaea met BES vergelijkbaar met de snelheden in incubaties zonder deze remmer. Aan de andere kant, wanneer de acidogene bacteriën geremd worden met vancomycine wordt een duidelijke afname in ontkleuringsnelheid gevonden, hetgeen een belangrijk bewijs vormt voor de bijdrage van acidogenen in het proces. Wanneer riboflavine toegevoegd wordt als redox mediator, is het effect van de glucose concentratie op de reductieve ontkleuring meer uitgesproken dan in de afwezigheid van deze mediator. Eerder onderzoek met de VFAs, acetaat, propionaat, en butyraat in de afwezigheid van redox mediatoren gaf aan dat de snelheid van kleurstofreductie onafhankelijk was van de geteste elektronen donor (Van der Zee et al., 2001a). Dit suggereert dat de kleine hoeveelheden waterstof gevormd tijdens propionaat en butyraat oxidatie de kleurstofreductie niet kan ondersteunen. Kennelijk helpt, in ons onderzoek, riboflavine voornamelijk de overdracht van reductie-equivalenten van de glucose fermentatie naar de kleurstof, en daarmee de acidogenen toestaan om effectiever deel te nemen aan de reductieve ontkleuring. Deze resultaten zijn in overeenstemming met het eerder beschreven vermogen van fermentatieve bacteriën om humuszuren als elektronen acceptor te gebruiken in plaats van riboflavine, alsmede om azokleurstoffen te reduceren (Benz et al., 1998; Yoo et al., 2000; Dos Santos et al., 2003).

Riboflavine verhoogt tevens de snelheid van azokleurstofreductie aanzienlijk wanneer belangrijke intermediairen van anaërobe bioconversies, zoals acetaat, methanol, waterstof en formaat worden getest, waarbij de laatste twee verreweg de beste elektronen donoren zijn voor reductieve ontkleuring. In de studie naar verschillende elektronen donoren voor de reductie van koolstof tetrachloride is gevonden dat waterstof een effectieve elektronen donor is, terwijl methanol en acetaat een erg matige elektronen donerende capaciteit hebben (Cervantes et al., 2004). In hetzelfde onderzoek met 20 μM AQDS lieten de reductiesnelheden een opmerkelijke toename zien, hetgeen erop wijst dat in de aanwezigheid van AQDS zelfs matige elektronen donoren, effectieve elektronen donoren voor dechlorinatie kunnen worden. Een mogelijke verklaring voor de lage azokleurstofreductiesnelheden van methanogene archaea in de aanwezigheid van acetaat en methanol kan gerelateerd zijn aan de enzymen die betrokken zijn in methanogenese van deze stoffen, aangezien deze geen NAD^+ of $NADP^+$ als co-enzymen gebruiken. In plaats daarvan gebruiken sommige van de enzymen een 5-deazaflavine F_{420} als co-enzym (Berk and Thauer, 1997). Het wordt momenteel algemeen aangenomen dat NADH of NAD(P)H de primaire elektronen donoren zijn voor biologische azokleurstofreductie (Semdé et al., 1998; Stolz, 2001; Blumel and Stolz, 2003).

In **Hoofdstuk 8** tonen experimenten met reinculturen van de thermofiele methanogenen *M. thermoautotrophicus* ΔH en stam NJ1 dat deze stammen de azokleurstof RR2 in de afwezigheid van riboflavine niet kunnen reduceren. In tegenstelling hiermee stelt Stolz (2001) dat bijna alle azokleurstoffen tot dusver getest biologisch gereduceerd kunnen worden onder anaërobe condities. Zo is bijvoorbeeld een complete reductie van de gesulfoneerde azokleurstof Amarant door α,β en γ-*Proteobacteriën* (Gram negatieve bacteriën)*, lage en hoge GC* (Gram positieve bacteriën), en door de archaeon *Halobacterium salinarum*, recentelijk beschreven (Rau et al., 2002a). Gebaseerd op onze resultaten met methanogene archaea is anaërobe azokleurstofreductie niet zo'n universele karakteristiek als herhaaldelijk gerapporteerd. Interessant is dat in de aanwezigheid van riboflavine we gevonden hebben dat beide thermofiele stammen RR2 kunnen reduceren, hetgeen duidt op de overdracht van intracellulaire reductie equivalenten naar buiten de cel en de daaropvolgende kleurstofreductie.

Omdat waterstof de beste elektronen donor voor de reductieve ontkleuring is gebleken in de biologische testen met thermofiel korrelslib, is het zeer waarschijnlijk dat andere hydrogenotrofe methanogenen direct betrokken kunnen zijn in de azosplitsing in plaats van de geteste reinculturen. Een andere mogelijke verklaring voor de snelle ontkleuring in de riboflavinevrije flessen met waterstof als elektronen donor zou het gevolg kunnen zijn van natuurlijke, in het slib, geproduceerde redox mediatoren, welke de methanogenen in staat stellen deel te nemen aan dit reductieve proces.

6. Afsluitende opmerkingen

Vergeleken met de toepassing van specifieke bacterie stammen of specifieke enzymen lijkt de anaërobe ontkleuring met korrelslib als voorbehandeling van gekleurd afvalwater van verfbaden en spoelstappen een erg aantrekkelijke technologie. In vergelijking met mesofiele anaërobe behandeling verloopt de ontkleuring van zowel azo- als anthrachinon kleurstoffen aanzienlijk sneller onder thermofiele condities. De significante verbetering van de elektronenoverdrachtcapaciteit en de resulterende verbetering van de ontkleuring van azokleurstoffen door eenvoudigweg het toepassen van een hoge temperatuur (55ºC) is gedemonstreerd in continue-doorstroom-experimenten bij verschillende hydraulische verblijftijden (HRT). Het gebruik van redox mediatoren als elektronen shuttle is extreem effectief gebleken bij verbetering van azokleurstofreductie bij 30ºC, maar hun katalytisch effect op de ontkleuringsnelheden is aanzienlijk minder bij 55ºC. Zo'n effect is waarschijnlijk het gevolg van een combinatie van zowel biotische, d.w.z. de toegenomen rol van H_2 en hogere slib vernieuwingsnelheden (en daaropvolgende toenamen van mediator en/of reduceer stoffen) op

55ºC vergeleken met 30ºC, alsmede abiotische mechanismen, d.w.z. toename van de botsfrequentie van de reactanten door de temperatuursverhoging, en daarmee de kinetiek van de reductie verbeterd. Een compleet beeld van de effectiviteit van thermofiele behandeling door anaëroob korrelslib op de complexe biochemie van kleurstofreductie is nog niet mogelijk en daarom is verder onderzoek noodzakelijk.

7. Nieuwe uitdagingen, perspectieven en aanbevelingen om ontkleuring van textiel afvalwater te verbeteren

De keuze voor de beste redox mediator zal afhangen van de kleurstoffen aanwezig in het afvalwater en de mogelijkheden om redox mediatoren te immobiliseren in bioreactoren danwel hun terugwinning te bewerkstelligen bij continue dosering. Onderzoek naar een beter begrip van de toxische mechanismen van anthrachinon kleurstoffen voor anaërobe micro-organismen, voornamelijk voor acetoclastische methanogenen, verdient ook aanbeveling. Het onderzoek naar azokleurstofreductie in methanogene reinculturen zou uitgevoerd moeten worden met andere redox mediatoren om het effect van deze stoffen op het transport van reductie equivalenten van binnenin naar buiten de cel and daaropvolgende kleurstofreductie te kunnen beoordelen. Bovendien vormt de identificatie van natuurlijke redox mediatoren, gevormd door slib vernieuwing en cel lysis, alsmede manieren om de productie van deze stoffen te verhogen een uitdaging.

Onderzoek naar ontkleuring met thermofiel anaëroob korrelslib bij extreme pH en zoutconcentraties wordt aanbevolen, omdat dergelijke condities vaak aangetroffen worden in textiel afvalwater. Om de problemen gerelateerd aan biofilm/korrel instabiliteit te overkomen onder bovengenoemde condities kan het gebruik van membraan bioreactoren overwogen worden. Omdat waterstof een van de beste elektronen donoren voor de ontkleuring is gebleken, kan het gebruik van een anaërobe gas-lift bioreactor gevoed met waterstof een alternatief zijn voor toekomstig onderzoek. Daarnaast wordt aanbevolen om een vergelijkende studie uit te voeren tussen een UASB en EGSB reactor, die onder vergelijkbare condities bedreven worden afgezien van de opstroomsnelheid, om te evalueren of massa overdracht mogelijk een rol speelt in het ontkleuringproces. Verschillende nabehandeling methoden voor anaërobe effluenten, bijv. aërobe biologische nabehandeling of behandeling met ozon en Fenton reagens, zouden nader onderzocht moeten worden onder zowel mesofiele als thermofiele omstandigheden om de temperatuursinvloed op de mineralisatie van aromatische aminen te beoordelen. Vanwege de verbeterde elektronen overdrachtscapaciteit als gevolg van de temperatuursverhoging, wordt

thermofiele anaërobe behandeling ook voorgesteld voor andere reductieve transformaties zoals reductie van nitroaromaten en dehalogenering.

De belangrijkste aanbeveling is de ontwikkeling en standaardisatie van een eenvoudige en efficiënte methode voor het bepalen van de mate van ontkleuring van textiel afvalwater, hetgeen momenteel een groot nadeel vormt voor emissiebeheersing.

References

Abadulla E, Tzanov T, Costa S, Robra KH, Cavaco PA and Guebitz GM, 2000. Decolorization and detoxification of textile dyes with a laccase from *Trametes hirsuta*. *Applied and Environmental Microbiology* 66: 3357-3362.

ABIT. 2003. Exportações de Produtos Têxteis, São Paulo, Brasil. Associação Brasileira de Indústria Têxtil e de Confecções.

Abken HJ, Tietze M, Brodersen J, Bauemer S, Beifuss U and Deppenmeier U, 1998. Isolation and characterization of methanophenazine and function of phenazines in membrane-bound electron transport of *Methanosarcina mazei* Go1. *Journal of Bacteriology* 180: 2027-2032.

Ahring BK, 1995. Methanogenesis in thermophilic biogas reactors. *Antonie van Leeuwenhoek* 67: 91-102.

Alaton IA, Balcioglu IA and Bahnemann DW, 2002. Advanced oxidation of a reactive dyebath effluent: Comparison of O3, H2O2/UV-C and TiO2/UV-A processes. *Water Research* 36: 1143-1154.

APHA. 1998. Standard methods for the examination of water and wastewater, 20 ed. American Public Health Association, Washington D.C.

Baughman GL and Weber EJ, 1994. Transformation of dyes and related compounds in anoxic sediment: Kinetics and products. *Environmental Science and Technology* 28: 267-276.

Benz M, Schink B and A. B, 1998. Humic acid reduction by *Propionibacterium freudenreichii* and other fermenting bacteria. *Applied and Environmental Microbiology* 64: 4507-4512.

Berk H and Thauer RK, 1997. Function of coenzyme F420-dependent NADP reductase in methanogenic archaea containing and NADP-dependent alcohol dehydrogenase. *Archives of Microbiology* 168: 396-402.

Beydilli MI, Pavlostathis SG and Tincher WC, 1998. Decolorization and toxicity screening of selected reactive azo dyes under methanogenic conditions. *Water Science and Technology* 38: 225-232.

Bisschops IAE and Spanjers H, 2003. Literature review on textile wastewater characterisation. *Environmental Technology* 24: 1399-1411.

Blumel S and Stolz A, 2003. Cloning and characterization of the gene coding for the aerobic azoreductase from *Pigmentiphaga kullae* k24. *Applied Microbiology and Biotechnology* 62: 186-190.

Boncz M. 2002. Selective oxidation of organic compounds in wastewater by ozone-based oxidation processes. Ph.D. Wageningen University, Wageningen, The Netherlands.

Bond DR and Lovley DR, 2002. Reduction of Fe(III) oxide by methanogens in the presence and absence of extracellular quinones. *Environmental Microbiology* 4: 115-124.

Bradford MM, 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248-254.

Bragger JL, Lloyd AW, Soozandehfar SH, Bloomfield SF, Marriott C and Martin GP, 1997. Investigations into the azo reducing activity of a common colonic microorganism. *International Journal of Pharmaceutics* 157: 61-71.

Bromley-Challenor KCA, Knapp JS, Zhang Z, Gray NCC, Hetheridge MJ and Evans MR, 2000. Decolorization of an azo dye by unacclimated activated sludge under anaerobic conditions. *Water Research* 34: 4410-4418.

Brown D and Hamburger B, 1987. The degradation of dyestuffs: Part III - Investigations of their ultimate degradability. *Chemosphere* 16: 1539-1553.

Brown D and Laboureur P, 1983. The degradation of dyestuffs: Part I - Primary biodegradation under anaerobic conditions. *Chemosphere* 12: 397-404.

Brown JP, 1981. Reduction of polymeric azo and nitro dyes by intestinal bacteria. *Applied and Environmental Microbiology* 41: 1283-1286.

Brown MA and DeVito SC, 1993. Predicting azo dye toxicity. *Critical Reviews in Environmental Science and Technology* 23: 249-324.

Cao JS, Wei LP, Huang QG, Wang LS and Han SK, 1999. Reducing degradation of azo dye by zero-valent iron in aqueous solution. *Chemosphere* 38: 565-571.

Carliell CM, Barclay SJ, Naidoo N, Buckley CA, Mulholland DA and Senior E, 1994. Anaerobic decolorisation of reactive dyes in conventional sewage treatment processes. *Water SA* 20: 341-344.

Carliell CM, Barclay SJ, Shaw C, Wheatley AD and Buckley CA, 1998. The effect of salts used in textile dyeing on microbial decolourisation of a reactive azo dye. *Environmental technology* 19: 1133-1137.

Cervantes FJ. 2002. Quinones as electron acceptors and redox mediators for the anaerobic biotransformation of priority pollutants. Ph.D. Wageningen University, Wageningen, The Netherlands.

Cervantes FJ, De Bok FAM, Duong-Dac T, Stams AJM, Lettinga G and Field JA, 2002. Reduction of humic substances by halorespiring, sulfate-reducing and methanogenic microorganisms. *Environmental Microbiology* 4: 51-57.

Cervantes FJ, Duong Dac T, Roest K, Akkermans ADL, Lettinga G and Field JA, 2003. Enrichment and immobilization of quinone-respiring bacteria in anaerobic granular sludge. *Water Science and Technology* 48: 9-16.

Cervantes FJ, van der Velde S, Lettinga G and Field JA, 2000. Competition between methanogenesis and quinone respiration for ecologically important substrates in anaerobic consortia. *FEMS Microbiology Ecology* 34: 161-171.

Cervantes FJ, Van der Zee FP, Lettinga G and Field JA, 2001. Enhanced decolourisation of Acid Orange 7 in a continuous UASB reactor with quinones as redox mediators. *Water Science and Technology* 44: 123-128.

Cervantes FJ, Vu-Thi-Thu L, Lettinga G and Field JA, 2004. Quinone-respiration improves dechlorination of carbon tetrachloride by anaerobic sludge. *Applied Microbiology and Biotechnology* 64: 702-711.

Chang R. 1977. Physical chemistry with applications to biological systems, New York, USA.

Chen H, Wang RF and Cerniglia CE, 2004. Molecular cloning, overexpression, purification, and characterization of an aerobic FMN-dependent azoreductase from *Enterococcus faecalis*. *Protein Expression and Purification* 34: 302-310.

Chinwekitvanich S, Tuntoolvest M and Panswad T, 2000. Anaerobic decolorization of reactive dyebath effluents by a two-stage UASB system with tapioca as a co-substrate. *Water Research* 34: 2223-2232.

Chiu PC and Reinhard M, 1996. Transformation of carbon tetrachloride by reduced vitamin B12 in aqueous cysteine solution. *Environmental Science and Technology* 30: 1882-1889.

Chivukula M and Renganathan V, 1995. Phenolic azo dye oxidation by laccase from *Pyricularia oryzae*. *Applied and Environmental Microbiology* 61: 4374-4377.

Christie R. 2001. Colour Chemistry. The Royal Society of Chemistry, Cambridge, United Kingdom.

Chung KT and Cerniglia CE, 1992. Mutagenicity of azo dyes: Structure-activity relationships. *Mutation Research* 277: 201-220.

Chung KT, Fulk GE and Egan M, 1978. Reduction of azo dyes by intestinal anaerobes. *Applied and Environmental Microbiology* 35: 558-562.

Chung KT, Murdock CA, Stevens SE, Li YS, Wei CI, Huang TS and Chou MW, 1995. Mutagenicity and toxicity studies of p-phenylenediamine and its derivatives. *Toxicology Letters* 81: 23-32.

Chung KT and Stevens SEJ, 1993. Degradation of azo dyes by environmental microorganisms and helminths. *Environmental Toxicology and Chemistry* 12: 2121-2132.

Chung KT, Stevens SEJ and Cerniglia CE, 1992. The reduction of azo dyes by the intestinal microflora. *Critical Reviews in Microbiology* 18: 175-197.

Claus H, Faber G and Koenig H, 2002. Redox-mediated decolorization of synthetic dyes by fungal laccases. *Applied Microbiology and Biotechnology* 59: 672-678.

Cooling III FB, Maloney CL, Tabinowski J and Odom JM, 1996. Inhibition of sulfate respiration by 1,8-dihydroxyanthraquinone and other anthraquinone derivatives. *Applied and Environmental Microbiology* 62: 2999-3004.

Correia VM, Stephenson T and Judd SJ, 1994. Characterisation of textile wastewaters: A review. *Environmental Technology* 15: 917-929.

De Bok FAM, Roze EHA and Stams AJM, 2002. Hydrogenases and formate dehydrogenases of *Syntrophobacter fumaroxidans*. *Antonie van Leeuwenhoek* 81: 283-291.

Delee W, O'Neill C, Hawkes FR and Pinheiro HM, 1998. Anaerobic treatment of textile effluents: a review. *Journal of chemical technology and biotechnology* 73: 323-335.

Denizli A and Piskin E, 2001. Dye-ligand affinity systems. *Journal of Biochemistry and Biophysics Methods* 49: 391-416.

DEPA. 1997. Environmental assessment of textiles, p. 220. *In* MoEa Energy (ed.). Danish Environmental Protection Agency.

Diekert G. 1991. The acetogenic bacteria, p. 517-553, The Prokaryotes. Springer-Verlag, New York, USA.

Donlon BA, Razo-Flores E, Lettinga G and Field JA, 1996. Continuous detoxification, transformation and degradation of nitrophenols in upflow anaerobic sludge blanket (UASB) reactors. *Biotechnology and Bioengineering* 51: 439-449.

Donlon BA, Razo-Flores E, Luijten M, Swarts H, Lettinga G and Field JA, 1997. Detoxification and partial mineralization of the azo dye mordant orange 1 in a continuous upflow anaerobic sludge-blanket reactor. *Applied Microbiology and Biotechnology* 47: 83-90.

Dos Santos AB. 2001. Pre-treatment of textile wastewater using the activated sludge system in batch. Master Dissertation. Federal University of Ceará, Fortaleza.

Dos Santos AB, Bisschops IAE, Cervantes FJ and Van Lier JB, 2004a. Effect of different redox mediators during thermophilic azo dye reduction by anaerobic granular sludge and comparative study between mesophilic (30ºC) and thermophilic (55ºC) treatments for decolourisation of textile wastewaters. *Chemosphere* 55: 1149-1157.

Dos Santos AB, Bisschops IAE, Cervantes FJ and Van Lier JB, 2005a. The transformation and toxicity of anthraquinone dyes during thermophilic (55ºC) and mesophilic (30ºC) anaerobic treatments. *Journal of Biotechnology In Press.*

Dos Santos AB, Bisschops IAE, Cervantes FJ and Van Lier JB, 2004b. The transformation and toxicity of anthraquinone dyes during thermophilic (55ºC) and mesophilic (30ºC) anaerobic treatments. *Journal of Biotechnology In Press.*

Dos Santos AB, Cervantes FJ and Van Lier JB, 2004c. Azo dye reduction by thermophilic anaerobic granular sludge, and the impact of the redox mediator AQDS on the reductive biochemical transformation. *Applied Microbiology and Biotechnology* 64: 62-69.

Dos Santos AB, Cervantes FJ, Yaya-Beas RE and Van Lier JB, 2003. Effect of redox mediator, AQDS, on the decolourisation of a reactive azo dye containing triazine group in a thermophilic anaerobic EGSB reactor. *Enzyme and Microbial Technology* 33: 942-951.

Dos Santos AB, Traverse J, Cervantes FJ and Van Lier JB, 2005b. Enhancing the electron transfer capacity and subsequent colour removal in bioreactors by applying thermophilic anaerobic treatment and redox mediators. *Biotechnology and Bioengineering* 89: 42-52.

Dubin P and Wright KL, 1975. Reduction of azo food dyes in cultures of *Proteus vulgaris*. *Xenobiotica* 5: 563-571.

Dunnivant FM, Schwarzenbach RP and Macalady DL, 1992. Reduction of substituted nitrobenzenes in aqueous solutions containing natural organic matter. *Environmental Science and Technology* 26: 2133-2141.

Duran N, Rosa MA, D AA and Gianfreda L, 2002. Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: A review. *Enzyme and Microbial Technology* 31: 907-931.

EPA. 1997. Profile of the textile industry. Environmental Protection Agency, Washington, USA.

Field JA and Brady J, 2003. Riboflavin as a redox mediator accelerating the reduction of the azo dye Mordant Yellow 10 by anaerobic granular sludge. *Water Science and Technology* 48: 187-193.

Field JA, Cervantes FJ, Van der Zee FP and Lettinga G, 2000. Role of quinones in the biodegradation of priority pollutants: a review. *Water Science and Technology* 42: 215-222.

Field JA, Stams AJM, Kato M and Schraa G, 1995. Enhanced biodegradation of aromatic pollutants in cocultures of anaerobic and aerobic bacterial consortia. *Antonie van Leeuwenhoek* 67: 47-77.

Fontenot EJ, Beydilli MI, Lee YH and Pavlostathis SG, 2002. Kinetics and inhibition during the decolorization of reactive anthraquinone dyes under methanogenic conditions. *Water Science and Technology* 45: 105-111.

Fontenot EJ, Lee YH, Matthews RD, Zhu G and Pavlostathis SG, 2003. Reductive decolorization of a textile reactive dyebath under methanogenic conditions. *Applied Biochemistry and Biotechnology* 109: 207-225.

Fredrickson JK, Kostandarithes HM, Li SW, Plymale AE and Daly MJ, 2000. Reduction of Fe(III), Cr(VI), U(VI), and Tc(VII) by *Deinococcus radiodurans* R1. *Applied and Environmental Microbiology* 66: 2006-2011.

Fultz M and Durst R, 1982. Mediator compounds for the electrochemical study of biological redox system: a compilation. *Analytica Chimica Acta* 140: 1-18.

Gähr F, Hermanutz F and Oppermann W, 1994. Ozonation - an important technique to comply with new German laws for textile wastewater treatment. *Water Science and Technology* 30: 255-263.

Galindo C, Jacques P and Kalt A, 2000. Photodegradation of the aminoazobenzene acid orange 52 by three advanced oxidation processes: UV/H2O2, UV/TiO2 and VIS/TiO2; Comparative mechanistic and kinetic investigations. *Journal of Photochemistry and Photobiology A: Chemistry* 130: 35-47.

Gingell R and Walker R, 1971. Mechanism of azo reduction by *Streptococcus faecalis* II. The role of soluble flavins. *Xenobiotica* 1: 231-239.

Glenn JK, Akileswaran L and Gold MH, 1986. Manganese-Ii Oxidation Is the Principal Function of the Extracellular Manganese Peroxidase from *Phanerochaete-Chrysosporium*. *Archives of Biochemistry and Biophysics* 251: 688-696.

Grzechulska J and Morawski AW, 2002. Photocatalytic decomposition of azo-dye acid black 1 in water over modified titanium dioxide. *Applied Catalysis B: Environmental* 36: 45-51.

Gujer W and Zehnder AJB, 1983. Conversion processes in anaerobic digestion. *Water Science and Technology* 15: 127-167.

Hao OJ, Kim H and Chang PC, 2000. Decolorization of wastewater. *Critical Reviews in Environmental Science and Technology* 30: 449-505.

Hassan MM and Hawkyard CJ, 2002a. Decolourisation of aqueous dyes by sequential oxidation treatment with ozone and Fenton's reagent. *Journal of Chemical Technology and Biotechnology* 77: 834-841.

Hassan MM and Hawkyard CJ, 2002b. Ozonation of aqueous dyes and dyehouse effluent in a bubble-column reactor. *Journal of Environmental Science and Health Part A Toxic Hazardous Substances and Environmental Engineering* A37: 1563-1579.

Heinfling A, Bergbauer M and Szewzyk U, 1997. Biodegradation of azo and phthalocyanine dyes by *Trametes versicolor* and *Bjerkandera adusta*. *Applied Microbiology and Biotechnology* 48: 261-66.

Ince NH and Tezcanli G, 1999. Treatability of textile dye-bath effluents by advanced oxidation: Preparation for reuse. *Water Science and Technology* 40: 183-190.

IPPC. 2003. Integrated Pollution Prevention and Control in Textile Industry.

Kao CM, Chou MS, Fang WL, Liu BW and Huang BR, 2001. Regulating colored textile wastewater by 3/31 wavelength admi methods in Taiwan. *Chemosphere* 44: 1055-1063.

Karcher S, Kornmuller A and Jekel M, 2001a. Cucurbituril for water treatment. Part I: Solubility of cucurbituril and sorption of reactive dyes. *Water Research* 35: 3309-3316.

Karcher S, Kornmuller A and Jekel M, 2001b. Screening of commercial sorbents for the removal of reactive dyes. *Dyes and Pigments* 51: 111-125.

Kashefi K, Tor Jason M, Holmes Dawn E, Van Praagh Catherine VG, Reysenbach Anna L and Lovley Derek R, 2002. *Geoglobus ahangari* gen. nov., sp. nov., a novel hyperthermophilic archaeon capable of oxidizing organic acids and growing autotrophically on hydrogen with Fe(III) serving as the sole electron acceptor. *International Journal of Systematic and Evolutionary Microbiology* 52: 719-728.

Keck A, Klein J, Kudlich M, Stolz A, Knackmuss HJ and Mattes R, 1997. Reduction of azo dyes by redox mediators originating in the naphthalenesulfonic acid degradation pathway of *Sphingomonas* sp. strain BN6. *Applied and Environmental Microbiology* 63: 3684-3690.

Keck A, Rau J, Reemtsma T, Mattes R, Stolz A and Klein J, 2002. Identification of quinoide redox mediators that are formed during the degradation of naphthalene-2-sulfonate by *Sphingomonas xenophaga* BN6. *Applied and Environmental Microbiology* 68: 4341-4349.

Knackmuss HJ, 1996. Basic knowledge and perspectives of bioelimination of xenobiotic compounds. *Journal of Biotechnology* 51: 287-295.

Kudlich M, Hetheridge MJ, Knackmuss HJ and Stolz A, 1999. Autoxidation reactions of different aromatic o-aminohydroxynaphthalenes that are formed during the anaerobic reduction of sulfonated azo dyes. *Environmental Science and Technology* 33: 896-901.

Kudlich M, Keck A, Klein J and Stolz A, 1997. Localization of the enzyme system involves in anaerobic reduction of azo dyes by *Sphingomonas* sp. strain BN6 and effect of artificial redox mediators on the rate of azo dye reduction. *Applied and Environmental Microbiology* 63: 3691-3694.

Laszlo JA, 2000. Regeneration of azo-dye-saturated cellulosic anion exchange resin by *Burholderia cepacia* anaerobic dye reduction. *Environmental Science and Technology* 34: 164-172.

Lettinga G, 1995. Anaerobic digestion and wastewater treatment systems. *Antonie van Leeuwenhoek* 67: 3-28.

Lettinga G, van Velsen A, Hobma S, de Zeeuw W and Klapwijk A, 1980. Use of the upflow sludge blanket (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment. *Biotechnology and Bioengineering* 22: 699-734.

Li K, Xu F and Eriksson KEL, 1999. Comparison of fungal laccases and redox mediators in oxidation of a nonphenolic lignin model compound. *Applied and Environmental Microbiology* 65: 2654-2660.

Libra J and Sosath F, 2003. Combination of biological and chemical processes for the treatment of textile wastewater containing reactive dyes. *Journal of Chemical Technology and Biotechnology* 78: 1149-1156.

Lie TJ, Godchaux W and Leadbetter ER, 1996. Sulfonates as terminal electron acceptors for growth of sulfite-reducing bacteria (Desulfitobacterium spp.) and sulfate-reducing bacteria: effects of inhibitors of sulfidogenesis. *Applied and Environmental Microbiology* 65: 4611-4617.

Lovley DR, Fraga JL, BluntHarris EL, Hayes LA, Phillips EJP and Coates JD, 1998. Humic substances as a mediator for microbially catalyzed metal reduction. *Acta hydrochimica et hydrobiologica* 26: 152-157.

Lovley DR, Kashefi K, Vargas M, Tor JM and Blunt-Harris EL, 2000. Reduction of humic substances and Fe(II) by hyperthermophilic microorganisms. *Chemical Geology* 169: 289-298.

Luangdilok W and Paswad T, 2000. Effect of chemical structures of reactive dyes on color removal by an anaerobic-aerobic process. *Water Science and Technology* 42: 377-382.

Madigan MT, Martinko JM and Parker J. 2003. Brock biology of microorganisms, 10th ed. Prentice-Hall, Inc., Simon & Schuster/ A Viacom Company, Upper Saddle River, New Jersey, USA.

Malpei F, Andreoni V, Daffonchio D and Rozzi A, 1998. Anaerobic digestion of print pastes: a preliminary screening of inhibition by dyes and biodegradability of thickeners. *Bioresource Technology* 63: 49-56.

Marmagne O and Coste C, 1996. Color removal from textile plant effluents. *American Dyestuff Reporter* 84: 15-21.

Mattioli D, Malpei F, Bortone G and Rozzi A. 2002. Water minimization and reuse in textile industry., p. 677pp., Water Recycling and resource recovery in industry: Analysis, technologies and implementation. IWA Publishing, Cornwall, UK.

Mazoch J, Tesarik R, Sedlacek V, Kucera I and Turanel J, 2004. Isolation and biochemical characterization of two soluble iron(III) reductases from *Paracoccus denitrificans*. *European Journal of Biochemistry* 271: 553-562.

Mccallum JEB, Madison SA, Alkan S, Depinto RL and Wahl RUR, 2000. Analytical studies on the oxidative degradation of the reactive textile dye Uniblue A. *Environmental Science and Technology* 34: 5157-5164.

Meckstroth ML, Norris BJ and Heineman WR, 1981. Mediator-titrants for thin-layer spectroelectro-chemical measurement of biocomponent $U^{o\prime}$ and n values. *Bioelectrochemistry and Bioenergetics* 8: 63-70.

Metcalf and Eddy. 2003. Wastewater engineering: treatment and reuse, 4th edition ed. McGraw-Hill, New York, USA.

Moir D, Masson S and Chu I, 2001. Structure-activity relationship study on the bioreduction of azo dyes by *Clostridium paraputrificum*. *Environmental Toxicology and Chemistry* 20: 479-484.

Moraes SG, Freire RS and Duran N, 2000. Degradation and toxicity reduction of textile effluent by combined photocatalytic and ozonation processes. *Chemosphere* 40: 369-373.

Nigam P, Banat IM, Singh D and Marchant R, 1996. Microbial process for the decolorization of textile effluent containing azo, diazo and reactive dyes. *Process Biochemistry* 31: 435-442.

O'Loughlin EJ, Burris DR and Delcomyn CA, 1999. Reductive dechlorination of trichloroethene mediated by humic-metal complexes. *Environmental science and technology* 33: 1145-1147.

O'Neill C, Hawkes FR, Hawkes DL, Lourenço ND, Pinheiro HM and Delée W, 1999. Colour in textile effluents - sources, measurement, discharge consents and simulation: a review. *Journal of Chemical Technology and Biotechnology* 74: 10009-1018.

O'Neill C, Hawkes FR, Hawkes DW, Esteves S and Wilcox SJ, 2000a. Anaerobic-aerobic biotreatment of simulated textile effluent containing varied ratios of starch and azo dye. *Water Research* 34: 2355-2361.

O'Neill C, Lopez A, Esteves S, Hawkes FR, Hawkes DL and Wilcox S, 2000b. Azo-dye degradation in an anaerobic-aerobic treatment system operating on simulated textile effluent. *Applied Microbiology and Biotechnology* 53: 249-254.

Oremland RS and Capone DG, 1988. Use of "specific" inhibitors in biogeochemistry and microbial ecology. *Advances in Microbial Ecology* 10: 285-383.

Orhon D, Babuna FG and Insel G, 2001. Characterization and modelling of denim-processing wastewaters for activated sludge. *Journal of Chemical Technology and Biotechnology* 76: 919-931.

Pagga U and Brown D, 1986. The degradation of dyestuffs: Part II. Behaviour of dyestuffs in aerobic biodegradation tests. *Chemosphere* 15: 479-491.

Paulo PL. 2002. The fate of methanol in thermophilic-anaerobic environments. Ph.D. Wageningen University, Wageningen.

Pearce CI, Lloyd JR and Guthrie JT, 2003. The removal of colour from textile wastewater using whole bacterial cells: a review. *Dyes and Pigments* 58: 179-196.

Porter JJ. 1997. Filtration and recovery of dyes from textile wastewater, Treatment of wastewaters from textile processing. Schriftenreihe Biologische Abwasserreinigung, Berlin, Germany.

Prestera T, Prochaska J and Talalay P, 1992. Inhibition of NAD(P)H: (Quinone-acceptor) oxireductase by Cibracon Blue and Related anthraquinone dyes: a structure-activity study. *Biochemistry* 31: 824-833.

Ramakrishna KR and Viraraghavan T, 1997. Dye removal using low cost adsorbents. *Water Science and Technology* 36: 189-196.

Rau J, Knackmuss HJ and Stolz A, 2002a. Effects of different quinoid redox mediators on the anaerobic reduction of azo dyes by bacteria. *Environmental Science and Technology* 36: 1497-1504.

Rau J, Maris B, Kinget R, Samyn C, Van den Mooter G and Stolz A, 2002b. Enhanced anaerobic degradation of polymeric azo compounds by *Escherichia coli* in the presence of low-molecular-weight redox mediators. *Journal of Pharmacy and Pharmacology* 54: 1471-1479.

Rau J and Stolz A, 2003. Oxygen-insensitive nitroreductases NfsA and NfsB of *Escherichia coli* function under anaerobic conditions as lawsone-dependent azo reductases. *Applied and Environmental Microbiology* 69: 3448-3455.

Razo-Flores. 1997. Biotransformation and biodegradation of N-substituted aromatics in methanogenic granular sludge. Ph.D. Wageningen University, Wageningen.

Razo-Flores E, Donlon BA, Lettinga G and Field JA, 1997. Biotransformation and biodegradation of N-substituted aromatics in methanogenic granular sludge. *FEMS Microbiology Reviews* 20: 525-538.

Robinson T, McMullan G, Marchant R and Nigam P, 2001. Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative. *Bioresource technology* 77: 247-255.

Russ R, Rau J and Stolz A, 2000. The function of cytoplasmic flavin reductases in the reduction of azo dyes by bacteria. *Applied and Environmental Microbiology* 66: 1429-1434.

Safarzadeh AA, Bolton JR and Cater SR, 1997. Ferrioxalate-mediated photodegradation of organic pollutants in contaminated water. *Water Research* 31: 787-798.

Schink B, 1997. Energetics of syntrophic cooperation in methanogenic degradation. *Microbiology and Molecular Biology Reviews* 61: 262-280.

Schink B, 2002. Synergistic interactions in the microbial world. *Antonie van Leeuwenhoek* 81: 257-261.

Scholten JCM, Conrad R and Stams AJM, 2000. Effect of 2-bromo-ethane sulfonate, molybdate and chloroform on acetate consumption by methanogenic and sulfate-reducing populations in freshwater sediment. *FEMS Microbiology Ecology* 32: 35-42.

Semdé R, Pierre D, Geuskens G, Devleeschouwer M and Moes AJ, 1998. Study of some important factors involved in azo derivative reduction by *Clostridium perfringens*. *International Journal of Pharmaceutics* 161: 45-54.

Snowden-Swan LJ. 1995. Pollution prevention in textile industries, Industrial Pollution Prevention Handbook. McGraw-Hill, New York, USA.

So CM, Cheng MY, Yu JC and Wong PK, 2002. Degradation of azo dye Procion Red MX-5B by photcatalytic oxidation. *Chemosphere* 46: 905-912.

Soares GMB, de Amorim MTP and Costa Ferreira M, 2001. Use of laccase together with redox mediators to decolourize Remazol Brilliant Blue R. *Journal of Biotechnology* 89 (2-3): 123-129.

Sober HA, Harte RA and Sober EK. 1970. Handbook of biochemistry: selected data for molecular biology. The Chemical Rubber Co., Cleveland, Ohio, USA.

Stams AJM, 1994. Metabolic interactions between anaerobic bacteria in methanogenic environments. *Antonie van Leeuwenhoek* 66: 271-294.

Stolz A, 2001. Basic and applied aspects in the microbial degradation of azo dyes. *Applied Microbiology and Biotechnology* 56: 69-80.

Talarposhti AM, Donnelly T and Anderson GK, 2001. Colour removal from a simulated dye wastewater using a two-phase anaerobic packed bed reactor. *Water Research* 35: 425-432.

Tan NCG. 2001. Integrated and sequential anaerobic/aerobic biodegradation of azo dyes. Ph.D. Wageningen University, The Netherlands, Wageningen, The Netherlands.

Tan NCG, Lettinga G and Field JA, 1999. Reduction of the azo dye Mordant Orange 1 by methanogenic granular sludge exposed to oxygen. *Bioresource Technology* 67: 35-42.

Tan NCG, Slenders P, Svitelskaya A, Lettinga G and Field JA, 2000. Degradation of azo dye Mordant Yellow 10 in a sequential anaerobic and bioaugmented aerobic bioreactor. *Water Science and Technology* 42: 337-344.

Thauer RK, 1998. Biochemistry of methanogenesis: a tribute to Marjory Stephenson. *Microbiology* 144: 2377-2406.

Thauer RK, Jungermann K and Decker K, 1977. Energy conservation in chemotrophic anaerobic bacteria. *Bacteriology Reviews* 41: 100-180.

Trüper HG and Schlegel HG, 1964. Sulphur metabolism in *Thiorhodaceae*. 1: Quantitative measurements on growing cells of *Chromatium okenii*. *Antonie van Leeuwenhoek Journal of Microbiology and Serology* 30: 225-238.

Van Bodegom PM, Scholten JCM and Stams AJM, 2004. Direct inhibition of methanogenis by ferric iron. *FEMS Microbiology Ecology* In Press.

Van der Zee F. 2002. Anaerobic azo dye reduction. PhD Thesis. Wageningen University, Wageningen, The Netherlands.

Van der Zee FP, Bisschops IAE, Blanchard VG, Bouwman RHM, Lettinga G and Field JA, 2003a. The contribution of biotic and abiotic processes during azo dye reduction in anaerobic sludge. *Water Research* 37: 3098-3109.

Van der Zee FP, Bisschops IAE, Lettinga G and Field JA, 2003b. Activated carbon as an electron acceptor and redox mediator during the anaerobic biotransformation of azo dyes. *Environmental Science and Technology* 37: 402-408.

Van der Zee FP, Bouwman RHM, Strik DPBTB, Lettinga G and Field JA, 2001a. Application of redox mediators to accelerate the transformation of reactive azo dyes in anaerobic bioreactors. *Biotechnology and Bioengineering* 75: 691-701.

Van der Zee FP, Lettinga G and Field JA, 2001b. Azo dye decolourisation by anaerobic granular sludge. *Chemosphere* 44: 1169-1176.

Van Lier JB, Grolle KCF, Frijters CTMJ, Stams AJM and Lettinga G, 1993a. Effects of acetate, propionate, and butyrate on the thermophilic anaerobic degradation of propionate by methanogenic sludge and defined cultures. *Applied and Environmental Microbiology* 59: 1003-1011.

Van Lier JB, Hulsbeek J, Stams AJM and Lettinga G, 1993b. Temperature susceptibility of thermophilic methanogenic sludge: Implications for reactor start-up and operation. *Bioresource Technology* 43: 227-235.

Van Lier JB, Rebac S, Lens P, Van BF, Oude ES, Stams AJM and Lettinga G, 1997. Anaerobic treatment of partly acidified wastewater in a two-stage expanded granular sludge bed (EGSB) system at 8ºC. *Water Science and Technology* 36: 317-324.

Van Lier JB, Sanz Martin JL and Lettinga G, 1996. Effect of temperature on the anaerobic thermophilic conversion of volatile fatty acids by dispersed and granular sludge. *Water Research* 30: 199-207.

Van Lier JB, Van der Zee FP, Tan NCG, Rebac S and Kleerebezem R, 2001. Advances in high-rate anaerobic treatment: Staging of reactor systems. *Water Science and Technology* 44: 15-25.

Vandevivere PC, Bianchi R and Verstraete W, 1998. Treatment and reuse of wastewater from the textile wet-processing industry: Review of emerging technologies. *Journal of Chemical Technology and Biotechnology* 72: 289-302.

Walker GM and Weatherley LR, 1997. A simplified predictive model for biologically activated carbon fixed beds. *Process biochemistry* 32: 327-335.

Walker R and Ryan AJ, 1971. Some molecular parameters influencing rate of reductions of azo compounds by intestinal microflora. *Xenobiotica* 1: 483-486.

Wang Y, 2000. Solar photocatalytic degradation of eight commercial dyes in TiO2 suspension. *Water Research* 34: 990-994.

Weisburger JH, 2002. Comments on the history and importance of aromatic and heterocyclic amines in public health. *Mutation Research* (506-507): 9-20.

Welham A, 2000. The theory of dyeing (and the secret of life). *Journal of the Society of Dyers and Colourists* 116: 140-143.

Willetts JRM and Ashbolt NJ, 2000. Understanding anaerobic decolourisation of textile dyes wastewater: mechanism and kinetics. *Water Science and Technology* 42: 409-416.

Yang Y, Wyatt DT and Bahorsky M, 1998. Decolorization of dyes using UV/H2O2 photochemical oxidation. *Textile Chemist and Colorist* 30: 27-35.

Yoo ES, 2002. Kinetics of chemical decolorization of the azo dye C.I. Reactive Orange 96 by sulfide. *Chemosphere* 47: 925-931.

Yoo ES, Libra J and Wiesmann U, 2000. Reduction of azo dyes by *Desulfovibrio desulfuricans*. *Water Science and Technology* 41: 15-22.

Yu J, Wang X and Yue PL, 2001. Optimal decolorization and kinetic modeling of synthetic dyes by *Pseudomonas* strains. *Water Research* 35: 3579-3586.

Zimmermann T, Gasser F, Kulla H and Leisinger T, 1984. Comparison of two bacterial azoreductases acquired during adaptation to growth on azo dyes. *Archives of Microbiology* 138: 37-43.

Zimmermann T, Kulla H and Leisinger T, 1982. Properties of purified Orange II azoreductase, the enzyme initiating azo dye degradation by *Pseudomonas* KF46. *European Journal of Biochemistry* 129: 197-203.

Zollinger H. 1987. Color chemistry - Syntheses, properties and applications of organic dyes pigments. VCH, New York, NY.

List of Abbreviations

Dyes used as model compounds

AO7	→	Acid orange 7
MY10	→	Mordant yellow 10
RR2	→	Reactive red 2
RR4	→	Reactive red 4
RO14	→	Reactive orange 14
RB5	→	Reactive blue 5
RB19	→	Reactive blue 19

Redox mediators used as model compounds

AQS	→	Anthraquinone-2-sulfonate
AH_2QS	→	Anthrahydroquinone-2-sulfonate (reduced form of AQS)
AQDS	→	Anthraquinone-2,6-disulfonate
AH_2QDS	→	Anthrahydroquinone-2,6-disulfonate (reduced form of AQDS)
BQ	→	Benzoquinone
Cyanocobalamin	→	Vitamin B12
Lawsone	→	Quinone-based compound
Riboflavin	→	Vitamin B2
$RM_{oxid.}$	→	Oxidized form of the redox mediator
$RM_{reduc.}$	→	Reduced form of the redox mediator

Others

AU	→	Absorbance unit
COD	→	Chemical oxygen demand
Ea	→	Activation energy (kJ/mol)
E_0'	→	Standard redox potential (pH 7)
FAD	→	Flavin adenine dinucleotide
FMN	→	Flavin adenine mononucleotide
EGSB	→	Expanded anaerobic sludge blanket
HRT	→	Hydraulic retention time
IC_{50}	→	Concentration exerting 50% reduction in the methanogenic activity
k_0	→	Zero-order reaction constant (mM/day)
k_1	→	First-order reaction constant (day^{-1})
OLR	→	Organic loading rate
NAD	→	Nicotinamide adenine dinucleotide
NADH	→	Reduced form of NAD
NAD(P)	→	Nicotinamide adenine dinucleotide phosphate
NAD(P)H	→	Reduced form of NAD(P)
R	→	Universal gas constant (8.314 J K^{-1} mol^{-1})
VFA	→	Volatile fatty acids
VSS	→	Volatile suspended solids
UASB	→	Upflow anaerobic sludge blanket

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Turn me loose from your hands Let me fly to distant lands Over green fields, trees and mountains Flowers and forest fountains Home along the lanes of the skyway… (Skyline Pigeon / Elton John)

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André

...Fly away skyline pigeon fly. Towards the dreams you've left so very far behind (Skyline Pigeon / Elton John)

About the Author

André Bezerra dos Santos was born in Fortaleza, Ceará, Brazil on March 23th, 1976. In 1998, he obtained the Bachelor degree in Civil Engineering at Federal University of Ceará, with *Magna cum Laude*. In 1999, he was selected on first place for the M.Sc. program in Environmental Engineering at Federal University of Ceará. In 2001, he obtained the M.Sc. degree with dissertation topic "Pre-treatment of textile wastewater using activated sludge system in batch mode". In September of 2001, he started the Ph.D. research at the Department of Environmental Technology of Wageningen University.

Email: andsantos@hotmail.com

Publications

1. Dos Santos AB and Santaella ST (2002). Remoção de DQO de água residuária de indústria têxtil empregando o processo de lodos ativados em batelada. Revista Engenharia Sanitária e Ambiental **7(3)**: 151-155.
2. Dos Santos AB, Cervantes FJ, Beas REY and van Lier JB (2003) Effect of redox mediator, AQDS, on the decolourisation of a reactive azo dye containing triazine group in a thermophilic anaerobic EGSB reactor. Enzyme and Microbial Technology **33(7):** 942-951.
3. Dos Santos AB, Cervantes FJ and van Lier JB (2004). Azo dye reduction by thermophilic anaerobic granular sludge, and the impact of the redox mediator AQDS on the reductive biochemical transformation. Applied Microbiology and Biotechnology **64:** 62-69.
4. Dos Santos AB, Bisschops IAE, Cervantes FJ and van Lier JB (2004). Effect of different redox mediators during thermophilic azo dye reduction by anaerobic granular sludge and comparative study between mesophilic (30°C) and thermophilic (55°C) treatments for decolourisation of textile wastewaters. Chemosphere **55:** 1149-1157.
5. Dos Santos AB, Bisschops IAE, Cervantes FJ and van Lier JB (2005a). The transformation and toxicity of anthraquinone dyes during thermophilic (55ºC) and mesophilic (30ºC) anaerobic treatments. Journal of Biotechnology. *In Press.*
6. Dos Santos AB, Traverse J, Cervantes FJ and van Lier JB (2005b). Enhancing the electron transfer capacity and subsequent colour removal in bioreactors by applying thermophilic anaerobic treatment and redox mediators. Biotechnology and Bioengineering **89(1):** 42-52.
7. Dos Santos AB, Cervantes FJ and van Lier JB. Current technologies for decolourisation of textile wastewaters: a critical review with a biotechnological perspective. Bioresource Technology. Submitted.
8. Dos Santos AB, Cervantes FJ, Madrid MP, de Bok FAM, Stams AJM and van Lier JB. The contribution of acidogenic bacteria and methanogenic archaea to azo dye reduction by thermophilic anaerobic consortium. Enzyme and Microbial Technology. Submitted.
9. Dos Santos AB, Madrid MP, Stams AJM, van Lier JB and Cervantes FJ. Azo dye reduction by mesophilic and thermophilic anaerobic consortia. Biotechnology Progress. Submitted.

Conferences

1. Dos Santos AB, Traverse J, Cervantes FJ and van Lier JB (2004) Thermophilic treatment by anaerobic granular sludge as an effective approach to accelerate the electron transfer and improve decolourisation rates in bioreactors. *In: Proceedings of the 10th World Congress on Anaerobic Digestion (AD10-2004)*. Montreal, Canada.
2. Dos Santos AB, Cervantes FJ and van Lier JB (2004) The effect of steric factors rather than the standard redox potential of the mediator in azo dye reduction by anaerobic granular sludge. *In: Proceedings of the 2nd Leading Edge Conference on Water and Wastewater Treatment Technologies - IWA*. Prague, Czech Republic.
3. Dos Santos AB, Bisschops IAE, Cervantes FJ and van Lier JB (2004) The effect of a quinone-based redox mediator on the decolourisation of textile wastewaters: a comparative study between mesophilic (30ºC) and thermophilic (55ºC) anaerobic treatments by granular sludge. *In: Proceedings of the European Symposium on Environmental Biotechnology.* Oostende, Belgium.
4. Dos Santos AB, Cervantes FJ and van Lier JB (2004). Toxicity of anthraquinone dye-containing wastewaters during anaerobic decolourisation. *In: Proceedings of the Young Researchers Conference – IWA.* Wageningen, The Netherlands.
5. Dos Santos AB, Cervantes FJ and van Lier JB (2003) Azo dye reduction by thermophilic anaerobic granular sludge and the impact of redox mediator on the reductive biochemical transformation. *In: Proceedings of the 11th European Congress on Biotechnology*. Basel, Switzerland.
6. Dos Santos AB, Cervantes FJ, Beas REY and van Lier JB (2003) Colour removal of Reactive Red 2 using thermophilic anaerobic EGSB bioreactors, and the impact of the redox mediator AQDS, on the decolourisation rates. *In Proceedings of the IWA Biofilm Congress.* Cape Town, South Africa.
7. Cervantes FJ Dos Santos AB, Madrid MP, Stams AJM and van Lier JB (2004) Reductive decolourisation of azo dyes by mesophilic and thermophilic methanogenic consortia. *In: Proceedings of the 10th World Congress on Anaerobic Digestion (AD10-2004)*. Montreal, Canada.
8. Bisschops IAE, Dos Santos AB and Spanjers H (2004) Waste sizing liquid as co-substrate for anaerobic textile wastewater decolourisation. *In: Proceedings of the 10th World Congress on Anaerobic Digestion (AD10-2004)*. Montreal, Canada.
9. Paulo PL, Dos Santos AB, Ide CN and Lettinga G (2004) Phosphate inhibition on thermophilic acetoclastic methanogens: a warning. *In: Proceedings of the 10th World Congress on Anaerobic Digestion (AD10-2004)*. Montreal, Canada.
10. Yaya-Beas RE, Dos Santos AB and van Lier JB (2003) Reducción Anaerobia de Colorantes Azo y el Impacto de los Mediadores Redox a 55°C. *In: Congreso Bolivariano de Ingeniería Sanitaria y Ambiental: Ambiente Y Desarrollo, Impulsadores Del Progreso*. Ecuador.

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Cover: An illustration of a granular sludge treating dye-coloured wastewaters.