Anaerobic azo dye reduction

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Azo dyes, aromatic moieties linked together by azo (-N=N-) chromophores, represent the largest class of dyes used in textile-processing and other industries. The release of these compounds into the environment is undesirable, not only because of their colour, but also because many azo dyes and their breakdown products are toxic and/or mutagenic to life. To remove azo dyes from wastewater, a biological treatment strategy based on anaerobic reduction of the azo dyes, followed by aerobic transformation of the formed aromatic amines, holds promise. However, the first stage of the process, anaerobic azo dye reduction, proceeds relatively slow. Therefore, this thesis research aimed at optimising anaerobic azo dye reduction, by studying the reaction mechanism and by consequently applying the obtained insights.

In this thesis it is shown that non-adapted anaerobic granular sludge has the capacity to non-specifically reduce azo dyes. As there was no correlation between a dye's reduction rate and its molecular characteristics (i.e. its size and its number of sulphonate groups and other polar substituents), it is unlikely that the mechanism of azo dye reduction involves cell wall penetration. Moreover, the presence of bacteria is not a prerequisite: azo dyes can also be reduced by sulphide in a purely chemical reaction. As dye containing wastewater usually contains sulphate and other sulphur species that will be biologically reduced to sulphide during treatment in anaerobic bioreactors, azo dye reduction will be a combination of biotic and abiotic processes. However, it was demonstrated that under normal conditions in high-rate anaerobic bioreactors (high sludge content, moderate sulphide levels), chemical azo dye reduction by sulphide hardly contributes to the overall reaction. Anaerobic azo dye reduction is therefore mainly a biological process, either a direct enzymatically catalysed reaction involving non-specific enzymes or a reaction with enzymatically reduced electron carriers. Azo dye reduction by sludge that had not earlier been exposed to dyes was found to relate to the oxidation of endogenous substrate and, especially, to the oxidation of hydrogen when present in bulk concentrations. Enrichment was required for the utilisation of electrons from volatile fatty acids for dye reduction.

Examination of the reduction of twenty chemically distinct azo dyes by anaerobic granular sludge revealed a large variation in the reaction rates. Especially reactive azo dyes with triazyl reactive groups were slowly reduced. For these common occurring reactive dyes, long contact times may be necessary to reach a satisfying extent of decolourisation. Consequently, they pose a serious problem for applying high-rate anaerobic treatment as the first stage in the biological degradation of azo dyes. However, this problem can be overcome by using redox mediators, compounds that speed up the reaction rate by shuttling electrons from the biological oxidation of primary electron donors or from bulk electron donors to the electron-accepting azo dyes.

It was observed that one of the constituent aromatic amines of the azo dye Acid Orange 7 had an autocatalytic effect on the dye's reduction, probably by acting as a redox mediator. Other compounds, e.g. the artificial redox mediator anthraquinone-2,6-disulphonate (AQDS), a compound that is known

to catalyse the reductive transfer of several pollutants, and the commonly occurring flavin enzyme cofactor riboflavin, were found to be extremely powerful catalysts, capable of raising the pseudo first-order reaction rate constants by orders of magnitude. Moreover, a large stimulatory effect was found for autoclaved sludge, presumably due to the release of internal electron carriers, e.g. enzyme cofactors like riboflavin, during autoclaving.

AQDS was successfully applied to improve the continuous reduction of Reactive Red 2 (a reactive azo dye with a triazyl reactive group) in a lab-scale anaerobic bioreactor that was operated under moderate hydraulic loading conditions. Without AQDS, the reactor's dye removal efficiency was very low, which gave rise to severe dye toxicity towards the biological activity. Addition of catalytic concentrations of AQDS to the reactor influent caused an immediate increase of the dye removal efficiency and recovery of the methane production. Eventually, almost complete RR2 colour removal could be reached.

Though effective AQDS dosage levels are low, continuous dosing has disadvantages with respect to the costs and the discharge of this biologically recalcitrant compound. Therefore, the feasibility of activated carbon (AC), which is known to contain quinone groups at its surface, to act alternatively as an insoluble/immobilised redox mediator was explored. Incorporation of AC in the sludge of lab-scale anaerobic bioreactors that treated Reactive Red 2 in synthetic wastewater containing volatile fatty acid as primary electron donor resulted in enhanced continuous dye reduction as compared to the control reactors without AC. The effect of AC was in large excess of its dye adsorption capacity. In addition, it was shown that bacteria could utilise AC as terminal electron acceptor in the oxidation of acetate. Moreover, AC catalysis of chemical azo dye reduction by sulphide was demonstrated. These results clearly suggest that AC accepts electrons from the microbial oxidation of organic acids and transfers the electrons to azo dyes, thereby accelerating their biological reduction.

The research presented in this thesis makes clear that the reduction of azo dyes can be optimised by utilising redox mediators, i.e. either by continuous dosing of soluble quinones or by incorporation of AC in the sludge blanket. The potential of using redox mediators is probably not limited to enhancing azo dye reduction but may be extrapolated to other non-specific reductive (bio)transformations, e.g. reduction of halogenated or nitroaromatic compounds. The potential of using redox mediators is furthermore probably not limited to wastewater treatment but may also apply to bioremediation of soils polluted with e.g. polychlorinated solvents or nitroaromatic pesticides.

Abstract

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1.1 Dyes, history

Ever since the beginning of humankind, people have been using colorants for painting and dyeing of their surroundings, their skins and their clothes. Until the middle of the 19th century, all colorants applied were from natural origin. Inorganic pigments such as soot, manganese oxide, hematite and ochre have been utilised within living memory. Palaeolithic rock paintings, such as the 30,000 year old drawings that were recently discovered in the Chauvet caves in France, provide ancient testimony of their application [54]. Organic natural colorants have also a timeless history of application, especially as textile dyes. These dyes are all aromatic compounds, originating usually from plants (e.g. the red dye alizarin from madder and indigo (Figure 1.1) from woad) but also from insects (e.g. the scarlet dye kermes from the shield-louse *Kermes vermilio*), fungi and lichens.

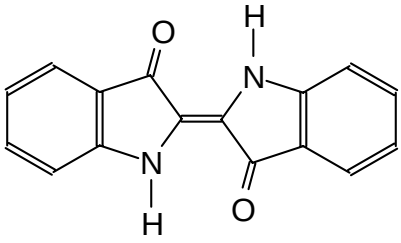


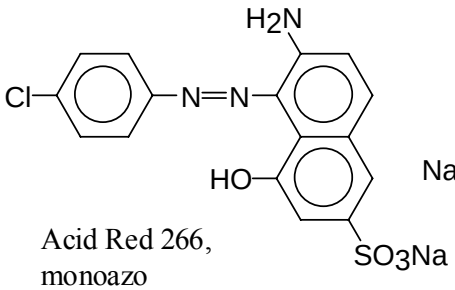
Figure 1.1 Indigo

Synthetic dye manufacturing started in 1856, when the English chemist W.H. Perkin, in an attempt to synthesise quinine, obtained instead a bluish substance with excellent dyeing properties that later became known as aniline purple, Tyrian purple or mauveine. Perkin, 18 years old, patented his invention and set up a production line. This concept of research and development was soon to be followed by others and new dyes began to appear on the market, a process that was strongly stimulated by Kékulé's discovery of the molecular structure of benzene in 1865. In the beginning of the 20th century, synthetic dyestuffs had almost completely supplanted natural dyes [344].

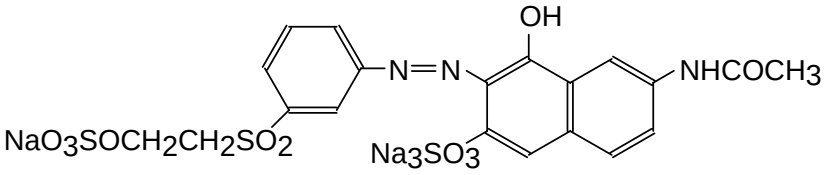
1.2 Dye classification

All aromatic compounds absorb electromagnetic energy but only those that absorb light with wavelengths in the visible range (~350-700 nm) are coloured. Dyes contain *chromophores*, delocalised electron systems with conjugated double bonds, and *auxochromes*, electron-withdrawing or electron-donating substituents that cause or intensify the colour of the chromophore by altering the overall energy of the electron system. Usual chromophores are -C=C-, -C=N-, -C=O, -N=N-, $-NO_2$ and quinoid rings, usual auxochromes are $-NH_3$, -COOH, $-SO_3H$ and -OH.

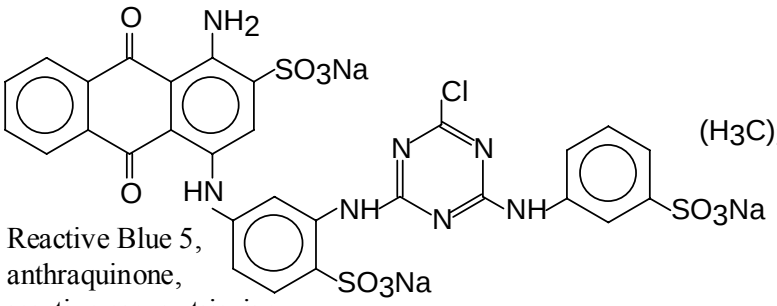
Based on chemical structure or chromophore, 20-30 different groups of dyes can be discerned. Azo (monoazo, disazo, triazo, polyazo), anthraquinone, phthalocyanine and triarylmethane dyes are quantitatively the most important groups. Other groups are diarylmethane, indigoid, azine, oxazine, thiazine, xanthene, nitro, nitroso, methine, thiazole, indamine, indophenol, lactone, aminoketone and hydroxyketone dyes and dyes of undetermined structure (stilbene and sulphur dyes). Figure 1.2 shows the structure formulas of several different dyes.



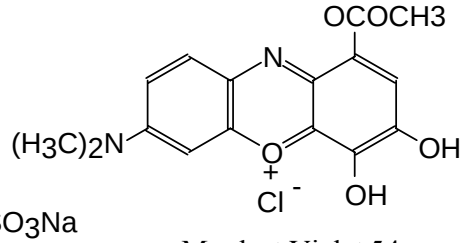
Acid Red 266,
monoazo



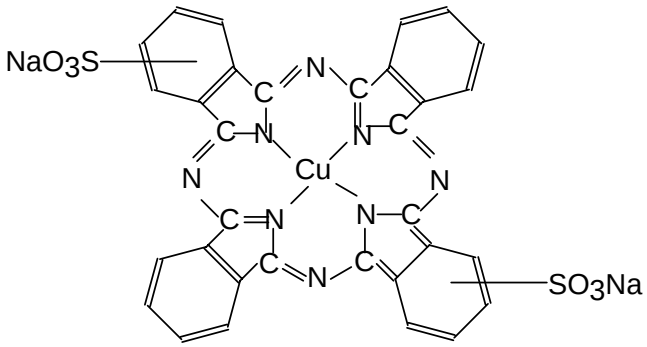
Reactive Orange 7, monoazo
reactive group: vinyl sulphone



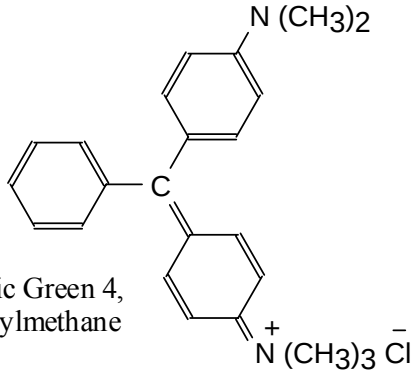
Reactive Blue 5,
anthraquinone,
reactive group: triazine



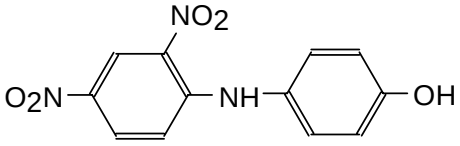
Mordant Violet 54,
oxazine



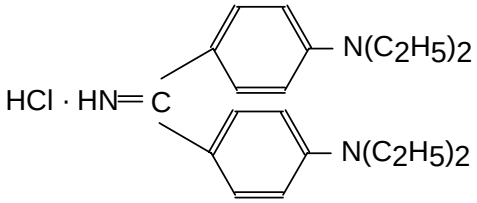
Direct Blue 86,
phthalocyanine



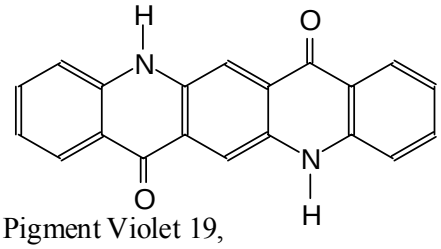
Basic Green 4,
triarylmethane



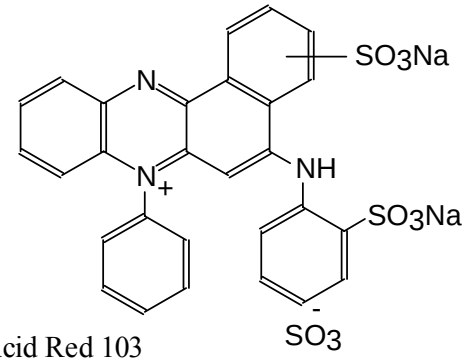
Disperse Yellow 1,
nitro



Basic Yellow 3,
diphenylmethane



Pigment Violet 19,
quinacridone (acridine)



Acid Red 103
methine (aposafranine, rosinduline)

Figure 1.2 Structure formulas of several dyes

The vast array of commercial colorants is classified in terms of colour, structure and application method in the Colour Index (C.I.) which is edited since 1924 (and revised every three months) by the Society of Dyers and Colourists and the American Association of Textile Chemists and Colorists. The Colour Index (3rd Edition, issue 2) lists about 28,000 commercial dye names, representing ~10,500 different dyes, 45,000 of which are currently produced. Each different dye is given a C.I. generic name determined by its application characteristics and its colour. The Colour Index discerns 15 different application classes:

1.2.1 Acid dyes

The largest class of dyes in the Colour index is referred to as *Acid dyes* (~2300 different acid dyes listed, ~40% of them are in current production). Acid dyes are anionic compounds that are mainly used for dyeing nitrogen-containing fabrics like wool, polyamide, silk and modified acryl. They bind to the cationic NH_4^+-ions of those fibres. Most acid dyes are azo (yellow to red, or a broader range colours in case of metal complex azo dyes), anthraquinone or triarylmethane (blue and green) compounds. The adjective 'acid' refers to the pH in acid dye dyebaths rather than to the presence of acid groups (sulphonate, carboxyl) in the molecular structure of these dyes.

1.2.2 Reactive dyes

Reactive dyes are dyes with reactive groups that form covalent bonds with OH-, NH-, or SH-groups in fibres (cotton, wool, silk, nylon). The reactive group is often a heterocyclic aromatic ring substituted with chloride or fluoride, e.g. dichlorotriazine. Another common reactive group is vinyl sulphone (as in Reactive Orange 7, see Figure 1.2). The use of reactive dyes has increased ever since their introduction in 1956, especially in industrialised countries. In the Colour Index, the reactive dyes form the second largest dye class with respect to the amount of active entries: about 600 of the ~1050

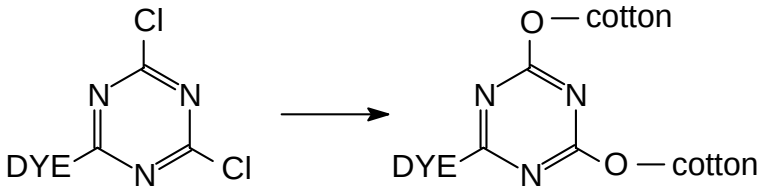


Figure 1.3 Principle of cotton dying with a triazyl reactive dye

different reactive dyes listed are in current production. During dying with reactive dyes (Figure 1.3), hydrolysis (i.e. inactivation) of the reactive groups is an undesired side reaction that lowers the degree of fixation. In spite of the addition of high quantities of salt and ureum (up to respectively 60 and 200 g/l) to raise the degree of fixation, it is estimated that 10 to 50% will not react with the fabric and remain –hydrolysed– in the water phase. The problem of coloured effluents is therefore mainly identified with the use of reactive dyes. Most (~80%) reactive dyes are azo or metal complex azo compounds but also anthraquinone and phthalocyanine reactive dyes are applied, especially for green and blue.

1.2.3 (Metal complex dyes)

Among acid and reactive dyes, many *Metal complex dyes* can be found (not listed as a separate category in the Colour Index). These are strong complexes of one metal atom (usually chromium, copper, cobalt or nickel) and one or two dye molecules, respectively 1:1 and 1:2 metal complex dyes. Metal complex dyes are usually azo compounds. About 1/6 of all azo dyes listed in the Colour Index are metal complexes [37] but also phthalocyanine metal complex dyes are applied.

1.2.4 Direct dyes

Direct dyes are relatively large molecules with high affinity for especially cellulose fibres. Van der Waals forces make them bind to the fibre. Direct dyes are mostly azo dyes with more than one azo bond or phthalocyanine, stilbene or oxazine compounds. In the Colour Index, the direct dyes form the second largest dye class with respect to the amount of different dyes: About 1600 direct dyes are listed but only ~30% of them are in current production.

1.2.5 Basic dyes

Basic dyes are cationic compounds that are used for dyeing acid-group containing fibres, usually synthetic fibres like modified polyacryl. They bind to the acid groups of the fibres. Most basic dyes are diarylmethane, triarylmethane, anthraquinone or azo compounds. Basic dyes represent ~5% of all dyes listed in the Colour Index.

1.2.6 Mordant dyes

Mordant dyes are fixed to fabric by the addition of a mordant, a chemical that combines with the dye and the fibre. Though mordant dyeing is probably one of the oldest ways of dyeing, the use of mordant dyes is gradually decreasing: only ~23% of the ~600 different mordant dyes listed in the Colour Index are in current production. They are used with wool, leather, silk, paper and modified cellulose fibres. Most mordant dyes are azo, oxazine or triarylmethane compounds. The mordants are usually dichromates or chromium complexes.

1.2.7 Disperse dyes

Disperse dyes are scarcely soluble dyes that penetrate synthetic fibres (cellulose acetate, polyester, polyamide, acryl, etc.). This diffusion requires swelling of the fibre, either due to high temperatures (>120 °C) or with the help of chemical softeners. Dying takes place in dyebaths with fine disperse solutions of these dyes. Disperse dyes form the third largest group of dyes in the Colour Index: about 1400 different compounds are listed, of which ~40% is currently produced. They are usually small azo or nitro compounds (yellow to red), anthraquinones (blue and green) or metal complex azo compounds (all colours).

1.2.8 Pigment dyes

Pigment dyes (i.e. organic pigments) represent a small but increasing fraction of the pigments, the most widely applied group of colorants. About 25% of all commercial dye names listed in the Colour Index are pigment dyes but these ~6900 product names stand for less than 800 different dyes. These insoluble, non-ionic compounds or insoluble salts retain their crystalline or particulate structure throughout their application. Pigment dyeing is achieved from a dispersed aqueous solution and therefore requires the use of dispersing agents. Pigments are usually used together with thickeners in print pastes for printing diverse fabrics. Most pigment dyes are azo compounds (yellow, orange, and red) or metal complex phthalocyanines (blue and green). Also anthraquinone and quinacridone pigment dyes are applied.

1.2.9 Vat dyes

Vat dyes are water-insoluble dyes that are particularly and widely used for dyeing cellulose fibres. The dyeing method is based on the solubility of vat dyes in their reduced (leuco) form. Reduced with sodium dithionite, the soluble leuco vat dyes impregnate the fabric. Next, oxidation is applied to bring back the dye in its insoluble form. Almost all vat dyes are anthraquinones or indigoids. Indigo itself is a very old example of a vat dye, with about 5000 years of application history. 'Vat' refers to the vats that were used for the reduction of indigo plants through fermentation.

1.2.10 Anionic dyes and ingrain dyes

Azoic dyes and *Ingrain dyes* (naphthol dyes) are the insoluble products of a reaction between a coupling component (usually naphthols, phenols or acetoacetylamides; listed in the Colour Index as C.I. azoic coupling components) and a diazotised aromatic amine (listed in the Colour Index as C.I. azoic diazo components). This reaction is carried out on the fibre. All naphthol dyes are azo compounds.

1.2.11 Sulphur dyes

Sulphur dyes are complex polymeric aromatics with heterocyclic S-containing rings. Though representing about 15% of the global dye production, sulphur dyes are not so much used in Western Europe. Dyeing with sulphur dyes involves reduction and oxidation, comparable to vat dyeing. They are mainly used for dyeing cellulose fibres.

1.2.12 Solvent dyes

Solvent dyes (lysochromes) are non-ionic dyes that are used for dyeing substrates in which they can dissolve, e.g. plastics, varnish, ink, waxes and fats. They are not often used for textile-processing but their use is increasing. Most solvent dyes are diazo compounds that underwent some molecular rearrangement. Also triarylmethane, anthraquinone and phthalocyanine solvent dyes are applied.

1.2.13 Fluorescent brighteners

Fluorescent brighteners (or bluing agents) mask the yellowish tint of natural fibres by absorbing ultraviolet light and weakly emitting visible blue. They are not dyes in the usual sense because they lack intense colour. Based on chemical structure, several different classes of fluorescent brighteners are discerned: stilbene derivatives, coumarin derivatives, pyrazolines, 1,2-ethene derivatives, naphthalimides and aromatic or heterocyclic ring structures. Many fluorescent brighteners contain triazinyl units and water-solubilising groups.

1.2.14 Other dye classes

Apart from the dye classes mentioned above, the Colour Index also lists *Food dyes* and *Natural dyes*. Food dyes are not used as textile dyes and the use of natural dyes (mainly anthraquinone, indigoid, flavenol, flavone or chroman compounds that can be used as mordant, vat, direct, acid or solvent dyes) in textile-processing operations is very limited.

1.3 Production and discharge statistics of dyes

Recent statistics on the global production and use of dyes and on the relative distribution between the different dye classes are not readily available. The most recent readily available data are from the 1993 SRI report, containing data for 1991 [245]. These data, listed in Table 1.1, show that the relative share of Western Europe is 13% of the world sale and that this share includes relatively more acid (and mordant) dyes and relatively less sulphur dyes than the world average. It is reasonable to assume that the total sale approximately equals the production and the consumption.

Table 1.1 Total sale of dyes –with the exclusion of solvent and pigment dyes– in 1991 (SRI, 1993 [245])

Dye class	**Western Europe (1,000 tonnes)**	**World (1,000 tonnes)**
acid (and mordant)	24	100
azoic	2	48
basic	8	44
direct	9	64
disperse	22	157
reactive	13	114
sulphur	3	101
vat	4	40
sum	**85**	**668**
relative share (%)	13	

The principal route by which dyes enter the environment is via wastewater [89]. To judge the relative share of the different dye classes in the wastewater of textile-processing industries, dye consumption data should be considered together with the degree of fixation of the different dye classes. These are listed in Table 1.2. From combining Tables 1.1 and 1.2 it can be estimated that approximately 75% of the dyes discharged by Western-European textile-processing industries belong to the classes of

reactive (~36%), acid (~25%) and direct (~15%) dyes, all of which are dye classes with mostly azo dyes.

Table 1.2 Estimated degree of fixation for different dye/fibre combinations [89]

Dye class	Fibre	Degree of fixation (%)	Loss to effluent (%)
acid	polyamide	80 – 95	5 – 20
basic	acrylic	95 – 100	0 – 5
direct	cellulose	70 – 95	5 – 30
disperse	polyester	90 – 100	0 – 10
metal-complex	wool	90 – 98	2 – 10
reactive	cellulose	50 – 90	10 – 50
sulphur	cellulose	60 – 90	10 – 40
vat	cellulose	80 – 95	5 – 20

As azo dyes represent the largest class of organic colorants listed in the Colour Index (60-70% of the total) and their relative share among reactive, acid and direct dyes is even higher, it can be expected that they make up the vast majority of the dyes discharged by textile-processing industries. Anthraquinone dyes are second largest class (~15% of the entries in the Colour Index), followed by triarylmethanes (~3%) and phthalocyanines (~2%).

1.4 Dyes, environmental concern

Many dyes are visible in water at concentrations as low as 1 mg l^{-1}. Textile-processing wastewaters, typically with a dye content in the range 10 – 200 mg l^{-1} [248], are therefore usually highly coloured and discharge in open waters presents an aesthetic problem. As dyes are designed to be chemically and photolytically stable, they are highly persistent in natural environments. The release of dyes may therefore present an ecotoxic hazard and introduces the potential danger of bioaccumulation that may eventually affect man by transport through the food chain.

1.4.1 Bioaccumulation

The bioaccumulation tendency of dyestuffs in fish has been comprehensively investigated in research promoted by ETAD, the Ecological and Toxicological Association of Dyes and Organic Pigments Manufacturers. The bioconcentration factors (BCF's) of 75 dyes from different application classes were determined and related to the partition coefficient n-octanol/water (K_{OW}) of each different compound. Water-soluble dyes with low K_{OW}, i.e. ionic dyes like acid, reactive and basic dyes, did not bioaccumulate (generally log BCF < 0.5). For these water-soluble dyes, log P (log K_{OW}) showed a linear relationship with log BCF so it was expected that dyestuffs with higher K_{OW} would bioaccumulate. However, water-insoluble organic pigments with extremely high partition coefficients did not bioaccumulate probably due to their extremely low water and fat solubilities and also the BCF values for disperse dyes, i.e. scarcely soluble compounds with a moderately lipophilic nature, were

much lower than expected. In all cases, log BCF < 2, which indicates that none of the dyes tested showed any substantial bioaccumulation [7-10].

1.4.2 Toxicity of dyestuffs

Dyestuff toxicity has been investigated in numerous researches. These toxicity (i.e. mortality, genotoxicity, mutagenicity and carcinogenicity) studies diverge from tests with aquatic organisms (fish, algae, bacteria, etc.) to tests with mammals. Furthermore, research has been carried out to effects of dyestuffs and dye containing effluents on the activity of both aerobic and anaerobic bacteria in wastewater treatment systems.

The acute toxicity of dyestuffs is generally low. Algal growth (photosynthesis), tested with respectively 56 and 46 commercial dyestuffs, was generally not inhibited at dye concentrations below 1 mg/l. The most acutely toxic dyes for algae are –cationic– basic dyes [117, 188]. Fish mortality tests showed that 2% out of 3000 commercial dyestuffs tested had LC_{50} values below 1 mg/l. The most acutely toxic dyes for fish are basic dyes, especially those with a triphenylmethane structure. Fish also seem to be relatively sensitive to many acid dyes [65]. Mortality tests with rats showed that only 1% out of 4461 commercial dyestuffs tested had LD_{50} values below 250 mg/kg body weight [65]. Therefore, the chance of human mortality due to acute dyestuff toxicity is probably very low. However, acute sensitisation reactions by humans to dyestuffs often occurs. Especially some disperse dyestuffs have been found to cause allergic reactions, i.e. eczema or contact dermititis [309].

Chronic effects of dyestuffs, especially of azo dyes, have been studied for several decades. Researchers were traditionally mostly focused on the effects of food colorants, usually azo compounds. Furthermore, also the effects of occupational exposure to dyestuffs of human workers in dye manufacturing and dye utilising industries have received attention. Azo dyes in purified form are seldom directly mutagenic or carcinogenic, except for some azo dyes with free amino groups [39]. However, reduction of azo dyes, i.e. cleavage of the dye's azo linkage(s), leads to formation of aromatic amines and several aromatic amines are known mutagens and carcinogens. In mammals, metabolic activation (= reduction) of azo dyes is mainly due to bacterial activity in the anaerobic parts of the lower gastrointestinal tract. Various other organs, especially the liver and the kidneys, can, however, also reduce azo dyes.

After azo dye reduction in the intestinal tract, the released aromatic amines are absorbed by the intestine and excreted in the urine. The acute toxic hazard of aromatic amines is carcinogenesis, especially bladder cancer. The carcinogenicity mechanism probably includes the formation of acyloxy amines through N-hydroxylation and N-acetylation of the aromatic amines followed by O-acylation. These acyloxy amines can be converted to nitremium and carbonium ions that bind to DNA and RNA, which induces mutations and tumour formation [39].

The mutagenic activity of aromatic amines is strongly related to molecular structure. In 1975 and in 1982, the International Agency for Research on Cancer (IARC) summarised the literature on suspected azo dyes, mainly amino-substituted azo dyes, fat-soluble azo dyes and benzidine azo dyes, but also a few sulphonated azo dyes [135, 136]. Most of the dyes on the IARC list were taken out of production [39].

In Germany, concern about the hazard of dyes resulted per July 1995 in prohibition of dyes and products containing dyes that yield any of 20 specified aromatic amines listed in Table 1.3 (Lebensmittel- und Bedarfgegenständegesetz (LMBG) § 5, Sect. 1, No. 6). In 1998, the same restrictions became valid in The Netherlands (Warenwetbesluit Azo-Kleurstoffen, 1998 Staatsblad number 339) and Austria (Verordnung der Bundesministerin für Frauenangelegenheiten und Verbraucherschutz über das Verbot der Verwendung bestimmter Azofarbstoffe und Azopigmente bei Gebrauchsgegenständen (Azofarbstoffverordnung), 1998 Bundesgesetzblatt II, number 241, page 1235, 29 July 1998).

Table 1.3 Specific amines forbidden in Germany, The Netherlands and Austria

compound	**synonym**
aromatic amines with two benzene rings (benzidines/toluidines/dianilines):	
benzidine	4,4'-diaminobiphenyl
4,4'-thiodianiline	di(4-aminophenyl)sulphide
3,3'-dichlorobenzidine	---
o-dianisidine	3,3'-dimethoxybenzidine
4-aminobiphenyl	biphenyl-4-ylamine
3,3'-dimethylbenzidine	4,4'-bi-toluidine
o-toluidine	2-methylbenzenamine
4-chloro-o-toluidine	4-chloro-2-methyl-benzamine
5-nitro-o-toluidine	2-methyl-5-nitro-benzeneamine
6-methoxy-m-toluidine	2-methoxy-5-methyl-benzamine
4,4'-bi-toluidine	3,3'-dimethylbenzidine
4,4'-methylenedi-o-toluidine	3,3'-dimethyl-4,4'-diamino-diphenylmethane
4-o-tolylazo-o-toluidine	4-amino-2',3-dimethylazobenzene
4,4'-methylenebis(2-chloroaniline)	---
4,4'-methylenedianiline	4,4-diaminodiphenylmethane
4,4'-oxydianiline	di (4-aminophenyl) ether
aromatic amines with one benzene ring:	
4-chloroaniline	4-chloro-benzenamine
2,4,5-trimethylaniline	2,4,5-trimethylbenzenamine
4-aminoazobenzene	4-(phenylazo)-benzenamine
o-anisidine	2-methoxy-benzeneamine
4-methoxy-m-phenylenediamine	4-methoxy-1,3-benzenediamine
4-methyl-m-phenylenediamine	4-methyl-1,3-benzenediamine
aromatic amines with a naphthalene structure:	
2-naphthylamine	---

Generally stated, genotoxicity is associated with all aromatic amines with benzidine moieties, as well as with some aromatic amines with toluene, aniline and naphthalene moieties. The toxicity of aromatic amines depends strongly on the spatial structure of the molecule or –in other words– the location of the amino-group(s). For instance, whereas there is strong evidence that 2-naphthylamine is a carcinogen, 1-naphthylamine is much less toxic [47]. The toxicity of aromatic amines depends furthermore on the nature and location of other substituents. As an example, the substitution with nitro, methyl or methoxy groups or halogen atoms may increase the toxicity, whereas substitution with

carboxyl or sulphonate groups generally lowers the toxicity [59]. As most soluble commercial azo dyestuffs contain one or more sulphonate groups, insight in the potential danger of sulphonated aromatic amines is particularly important. In an extensive review of literature data on genotoxicity and carcinogenicity of sulphonated aromatic amines, it was concluded that sulphonated aromatic amines, in contrast to some of their unsulphonated analogues, have generally no or very low genotoxic and tumorigenic potential [148].

1.5 Dye removal techniques

Various physical, chemical and biological pre treatment, main treatment and post treatment techniques can be employed to remove colour from dye containing wastewaters [70, 116, 120, 147, 281, 308, 329, 330]. Physico-chemical techniques include membrane filtration, coagulation/flocculation, precipitation, flotation, adsorption, ion exchange, ion pair extraction, ultrasonic mineralisation, electrolysis, advanced oxidation (chlorination, bleaching, ozonation, Fenton oxidation and photocatalytic oxidation) and chemical reduction. Biological techniques include bacterial and fungal biosorption and biodegradation in aerobic, anaerobic, anoxic or combined anaerobic/aerobic treatment processes.

Several factors determine the technical and economic feasibility of each single dye removal technique:

- dye type
- wastewater composition
- dose and costs of required chemicals
- operation costs (energy and material)
- environmental fate and handling costs of generated waste products

In general, each technique has its limitations. The use of one individual process may often not be sufficient to achieve complete decolourisation. Dye removal strategies consist therefore mostly of a combination of different techniques. The most important dye removal techniques are briefly discussed in sections 1.5.1 – 1.5.6.

1.5.1 Membrane filtration

Nanofiltration and reverse osmosis, using membranes with a molecular weight cut-off (MWCO) below ~10,000 Dalton, can be applied as main or post treatment processes for separation of salts and larger molecules including dyes from dyebath effluents and bulk textile-processing wastewaters. Filtration with bigger membranes, i.e. ultrafiltration and microfiltration, is generally not suitable as the membrane pore size is too large to prevent dye molecules passing through [70] but it can be successful as pre treatment for further nanofiltration or reverse osmosis [283]. Membrane filtration is a quick method with low spatial requirement. Another advantage is that the permeate, as well as some of the concentrated compounds, including non-reactive dyes, can be reused [40, 72]. This reuse, however, applies mostly only for smaller waste flows [331]. The disadvantages of membrane techniques are flux decline and membrane fouling, necessitating frequent cleaning and regular replacement of the modules. Another important drawback is that the generated concentrate must be processed further, for

instance by ozonation [346]. The capital costs of membrane filtration are therefore generally rather high [70, 120, 330]. Filtration techniques for the treatment of textile wastewaters are especially widely applied in South Africa [40, 326].

1.5.2 Coagulation/flocculation

Coagulation/flocculation is often applied in the treatment of textile-processing wastewater, either to partly remove Chemical Oxygen Demand (COD) and colour from the raw wastewater before further treatment [5, 186, 238], to polish the final effluents of biologically or otherwise treated wastewater [70, 201, 290] or even as the main treatment process [325]. The principle of the process is the addition of a coagulant followed by a generally rapid chemical association between the coagulant and the pollutants. The thus formed coagulates or flocs subsequently precipitate or are to be removed from the water phase by flotation.

Various inorganic coagulants are used, mostly lime, magnesium, iron and aluminium salts. Inorganic compounds are, however, generally not very suitable to remove highly soluble (= sulphonated) dyes from solution [123, 308] unless rather large quantities are dosed [120]. Coagulation/flocculation with inorganic chemicals generates considerable volumes of useless or even toxic sludge that must be incinerated or handled otherwise. This presents a serious drawback of the process [308].

Recently developed organic polymers have been proven highly effective as dye coagulants, even for coagulation of reactive dyes, while the sludge production associated with polymer dosing is relatively low [62, 158]. Most of the polymers used for colour removal are, however, cationic and may be toxic to aquatic life at very low concentrations (less than 1 mg/l) and in biological wastewater treatment plants, some cationic polymers have been found to inhibit the nitrification process [64].

1.5.3 Sorption and ion exchange

Activated carbon or other materials can be used to remove dyes from wastewater, either by adsorption (anionic dyes) or by combined adsorption and ion exchange (cationic dyes). Sorption techniques yield waste sludge, i.e. dye-saturated material, that should be disposed off or regenerated. As there are non-ionic, anionic and cationic dyes, most adsorbents do not remove all different dye types. Activated carbon is capable of adsorbing many different dyes with high adsorption capacity [159, 166, 183, 277] but it is expensive and the costs of regeneration are high because desorption of the dye molecules is not easily achieved [70, 209]. Various other (mostly low-cost) adsorbents have therefore been investigated as an alternative to activated carbon. Those adsorbents include:

- non-modified cellulose (plant) biomass, e.g. corn/maize cobs [91, 231, 240], maize stalks [215], wheat straw [240], linseed straw [28], rice husks [209, 215, 236], wood chips [128, 240, 267], sawdust [2, 28, 182, 215], bark [209, 218, 219], coirpith [227], banana pith [228], bagasse pith [4, 210, 211, 208, 231], palm fruit bunch particles [234, 232, 233], peat moss [127, 215, 236], peat [28, 128, 266, 277], linseed cake [189], sugar beet pulp [28], sugar industry mud [28], cotton waste [209] and cellulose [110];
- modified cellulose biomass, e.g. carbonised coirpith [227], carbonised coconut-tree sawdust [149], chemically modified sunflower stalks [300], polyamide-epichlorohydrin-cellulose [134, 133, 132],

carbamoyl-cellulose [355], quaternised-cellulose [104-106, 169, 170], quaternised-lignocellulose [11]; sugarcane bagasse derived anion exchange resin [168];

- bacterial biomass, e.g. *Aeromonas* [130], actinomycetes [360], activated sludge [252, 299]; dried and powdered biogas waste slurry [226, 225];
- fungal biomass [31, 100, 217, 221, 240, 265, 313];
- yeast biomass [78];
- chitin, a material that can be found in e.g. shells, insect shields and fungal cell walls [28, 110, 191, 206, 302]; chitosan, deacylated chitin [146, 302]; cross-linked chitosan fibres [352-354];
- soil material, e.g. sand [215], silica [3], natural clay [231], bentonite clay [28, 277], diatomite clay [182], montmorillonite clay [28], vermiculite clay [56, 215], fuller's earth [166], synthetic clay [67, 166];
- wood charcoal [202], bone charcoal [28], barbecue charcoal [215], magnetic charcoal [287];
- activated bauxite [166], activated alumina [182];
- other materials, e.g. pressed sludge cake (pulp mill waste), pyrolysed tire, leather hide powder, dealginated seaweed, coal dust [28], chrome sludge [171], steel plant slag, fly ash [277] and hair [209].

Some of these materials show high dye removal capacities, comparable or –especially in the case of disperse dyes [277] –even higher than activated carbon. This depends strongly on the dye class. Many of the materials listed, e.g. rice husks, bark, cotton waste and hair, have a high capacity for binding (cationic) basic dyes but hardly remove dyes from other classes [209]. Acid and reactive dyes are generally the most difficult to remove: some materials, e.g. bentonite clay, bind several dye types except acid dyes [209, 277] whereas Fuller's earth, an adsorbent capable of binding dyes from many classes including acid dyes [207] fails to bind reactive dyes [166]. Chitin and chitosan have extremely high acid and reactive dye binding capacity [206].

Based on adsorption capacity for two basic dyes and one acid dye, it was calculated that the use of natural clay, bagasse pith and maize cob would require only about 2-10% of the costs of activated carbon, even though the adsorption capacity of these low-cost materials was considerably lower than that of activated carbon [231]. To evaluate the feasibility of a potential dye adsorbent, not only its costs and its dye-binding capacity should be considered, but also its adsorption kinetics, its regeneration properties and its requirements and limitations with respect to environmental conditions like pH, temperature and salt concentration. In a review of the literature on the removal of acid dyes by using dead plant and animal matter, it was concluded that cross-linked chitosan and quaternised lignocellulose were the best materials with respect to adsorption or ion exchange capacity, adsorption kinetics and costs. Most non-modified biological materials had a low adsorption capacity, adsorbents with a high adsorption capacity like chitin, chitosan and polyamide-epichlorohydrin-cellulose had the drawback of very slow kinetics, and quaternised cellulose was too expensive [167].

Despite the large number of publications on dye adsorption, full-scale application is limited to combinations, e.g. combined adsorption and biodegradation in activated carbon amended activated sludge systems [71, 203, 335, 334] or anaerobic bioreactors [160], or combined sorption and coagulation by a synthetic clay slurry [166].

1.5.4 Electrolysis

Electrolysis is based on applying an electric current through to the wastewater to be treated by using electrodes. The anode is a sacrificial metal (usually iron) electrode that withdraws electrons from the electrode material, which results in the release of Fe(II)-ions to the bulk solution and precipitation of $Fe(OH)_2$ at the electrode surface. Moreover, water and chloride ions are oxidised, resulting in the formation of O_2, O_3 and Cl_2. The cathode is a hydrogen electrode that produces H_2 gas from water.
Organic compounds like dyes react through a combination of electrochemical oxidation, electrochemical reduction, electrocoagulation and electroflotation reactions:

- at the anode sorption onto precipitated iron, direct electrochemical oxidation forming oxidised radicals and oxidation by the produced O_3 and Cl_2 gases;
- at the cathode electrochemical reduction forming reduced radicals and
- in the bulk solution chemical reduction or coagulation by the released Fe(II) ions, followed (in case of coagulation) by flotation by bubbles of the produced H_2 gas.

In several studies, electrochemical methods have been successfully applied to achieve decolourisation of dye solutions and dye containing wastewaters [1, 83, 140, 184, 186, 204, 235, 243, 333, 345]. However, the process is expensive due to large energy requirements [70] and the limited lifetime of the electrodes [330]. Furthermore, as radical reactions are involved, uncontrolled formation of unwanted breakdown products may occur [70, 120, 186]. Another possible drawback is foaming [330].

1.5.5 Advanced oxidation processes

Advanced oxidation can be defined as oxidation by compounds with an oxidation potential (E_0) higher than that of oxygen (1.23 V), i.e. hydrogen peroxide (E_0 = 1.78 V), ozone (E_0 = 2.07 V) and the hydroxyl radical (E_0 = 2.28 V). Hydrogen peroxide alone is, however, usually not powerful enough [305]. Advanced oxidation processes (AOPs) are therefore mostly based on the generation of highly reactive radical species (especially the hydroxyl radical HO•) that can react with a wide range of compounds, also with compounds that are otherwise difficult to degrade, e.g. dye molecules. The four AOPs that have been most widely studied are ozonation, UV/H_2O_2, Fenton's reagent (Fe^{2+}/H_2O_2) and UV/TiO_2 [13].
In the *ozonation* process, hydroxyl radicals are formed when O_3 decomposes in water:

$$H_2O + O_3 \rightarrow HO_3^+ + OH^- \rightarrow 2\ HO_2$$
$$\downarrow +\ 2\ O_3 \rightarrow HO\bullet + 2\ O_2 \qquad [1.1]$$

Though ozone itself is a strong oxidant, hydroxyl radicals are even more reactive. Decomposition of ozone requires high pH (>10). Ozone treatment of organic molecules proceeds therefore faster in alkaline solutions than at neutral or acidic pH where ozone is the main oxidant [13, 57, 284].
Ozone rapidly decolourises water-soluble dyes but non-soluble dyes (vat dyes and disperse dyes) react much slower [201]. Textile-processing wastewater furthermore usually contains many refractory constituents other than dyes (e.g. surfactants) that will react with ozone, thereby increasing the ozone demand [102]. It is advised, therefore, to pre-treat the wastewater before ozonation is applied [330]. For example, in Leek, England, ozonation is used as the final stage (after biological treatment and

filtration) for treating textile-processing wastewater at full-scale [63]. This concept is, however, not logical as ozonation seldom leads to complete oxidation. Instead, ozone converts the organic compounds into smaller (usually biodegradable) molecules like dicaroboxylic acids and aldehydes [258, 261]. The reduction of COD is therefore low, while some of the ozonation products (especially the aldehydes) are highly toxic. It is better, therefore, to treat the effluent of the ozonation stage, logically by using inexpensive biological methods [178, 179, 181, 261, 339].

Fenton oxidation is based on the generation of hydroxyl radicals from Fenton's reagent (Fe^{2+}/H_2O_2) when ferrous iron is oxidised by hydrogen peroxide:

$$Fe^{2+} + H_2O_2 \rightarrow Fe(OH)^{2+} + HO\bullet \qquad [1.2]$$

Also higher oxidised iron species like $[Fe(OH)_2(H_2O)_5]^{2+}$ may be formed and it may even be possible that these species are the main oxidants in Fenton oxidation processes [13]. In addition, re-reduction of ferric iron (redox cycling) can take place, thereby enabling iron to act as a catalyst in the generation of radicals:

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2\bullet + H^+ \qquad [1.3]$$

However, reaction (1.3) proceeds much slower than reaction (1.2), unless at very high temperatures [229] or when the reaction is catalysed by UV-light [13, 19, 137, 244]. In the latter case, both Fenton's reagent and Fenton-like reagent (Fe^{3+}/H_2O_2) can be used. Another enhanced Fenton-like process uses H_2O_2 in combination with iron powder. The oxidation reaction here is the conventional dark Fenton's process but adsorption of dyes to the iron powder increases its effectiveness [322].

Fenton or Fenton-like oxidation can decolourise a wide range of dyes [19, 93, 138, 139, 151, 165, 187, 185, 256, 270, 290, 305, 347]. In comparison to ozonation, the process is relatively cheap and results generally in a larger COD reduction, although post-treatment (by for instance activated sludge) may still be required. A drawback for application of Fenton or Fenton-like oxidation for the treatment of –the usually highly alkaline- textile-processing wastewaters is that the process requires low pH (2 – 5). At higher pH, large volumes of waste sludge are generated by the precipitation of ferric iron salts and the process loses effectiveness as H_2O_2 is catalytically decomposed to oxygen [13]. Fenton or Fenton-like oxidation will furthermore be negatively affected by the presence of radical scavengers and strong chelating agents in the wastewater.

Photocatalytic oxidation processes (UV/H_2O_2, UV/TiO_2; UV/Fenton's reagent; UV/O_3 and other) are all based on the formation of free radicals due to UV irradiation. Typically, as UV light does not penetrate sufficiently in highly coloured waste streams, application of photocatalytic processes is limited to the post-treatment stage [330].

When UV is used in combination with hydrogen peroxide, hydroxyl radicals are formed according to the following (simplified) reaction:

$$H_2O_2 + h\nu \rightarrow 2\ HO\bullet \qquad [1.4]$$

Drawbacks of the UV/H_2O_2 process are the relatively high costs and the occasional lack of effectiveness [120, 330]. Faster, cheaper and more effective photocatalytic processes receive therefore increasing attention, especially those based on catalysis by solid semiconductor materials, mostly TiO_2 particles. When this material is irradiated with photons of less than 385 nm, the band gap energy is

exceeded and an electron is promoted from the valence band to the conduction band. The resultant electron-hole pair has a lifetime in the space-charge region that enables its participation in chemical reactions [114]. In general, oxygen is used to scavenge the conduction band electron to produce a superoxide anion radical ($O_2•^-$), while adsorbed water molecules are oxidised to hydroxyl radicals:

$$O_2 + H_2O \xrightarrow{h\nu/TiO_2} O_2•^- + HO• \qquad [1.5]$$

With TiO_2 catalysed UV treatment, a wide range of dyes can be oxidised. The dyes are generally not only decolourised but also highly mineralised [22, 76, 114, 115, 131, 176, 177, 230, 258, 268, 269, 279, 280, 284, 320, 321, 323, 332, 359, 361].

1.5.6 Biological techniques

Biological dye removal techniques are based on microbial biotransformation of dyes. As dyes are designed to be stable and long-lasting colorants, they are usually not easily biodegraded. Nevertheless, many researches have demonstrated partial or complete biodegradation of dyes by pure and mixed cultures of bacteria (section 1.5.6.1), fungi (section 1.5.6.2) and algae (section 1.5.6.3)

1.5.6.1 Bacterial biodegradation

For a general evaluation of dye biodegradability, the dyes' chemical structures, rather than their application classes, should be considered. Investigations to bacterial dye biotransformation have so far mainly been focused to the most abundant chemical class, that of the azo dyes. The electron-withdrawing nature of the azo linkages obstructs the susceptibility of azo dye molecules to oxidative reactions [94]. Therefore, azo dyes generally resist aerobic bacterial biodegradation [103, 143, 251, 252, 299]. Only bacteria with specialised azo dye reducing enzymes (section 1.6.1.1) were found to degrade azo dyes under fully aerobic conditions. In contrast, breakdown of azo linkages by reduction under anaerobic conditions is much less specific (section 1.6.1.1). This anaerobic reduction implies decolourisation as the azo dyes are converted to -usually colourless but potentially harmful (section 1.4.2)- aromatic amines. Aromatic amines are generally not further degraded under anaerobic conditions. Anaerobic treatment must therefore be considered merely as the first stage of the complete degradation of azo dyes. The second stage involves conversion of the produced aromatic amines (section 1.6.2). For several aromatic amines, this can be achieved by biodegradation under aerobic conditions. Combined anaerobic and aerobic bacterial biodegradation of azo dyes, as well as its applications in wastewater treatment processes, will further be discussed in section 1.6.3.

Bacterial biodegradation of non-azo dyes has received little attention so far:

Anthraquinone dyes. Anthraquinone dyes may possibly be aerobically degraded analogous to anthraquinone [214] or anthraquinone-2-sulphonate [295]. At least it has been demonstrated that three bacterial strains could grow with the anthraquinone dye Acid Blue 277:1 as sole source of energy [336]. Under anaerobic conditions, the transformation of anthraquinone dyes is presumably limited to reduction of quinone to hydroquinone, a reaction that reverses once the molecule is again exposed to oxygen [99, 257, 295]. Some anthraquinone dyes have been observed to be removed from the water phase by

formation of an 'insoluble pigment' under anaerobic conditions [36]. This is in line with the observation that electrochemical reduction of an anthraquinone dye increased its adsorptive properties [80].

Triphenylmethane dyes. Aerobic decolourisation of triphenylmethane dyes has been demonstrated repeatedly [15, 288, 291, 349, 350] but it has also been stated that these dyes resist degradation in activated sludge systems [139]. Under anaerobic conditions, the transformation of triphenylmethane dyes is presumably limited to reversible reactions like the reduction of malachite green (Basic Green 4) to leucomalachite green [124].

Phthalocyanine dyes. Phtalocyanine dyes are probably not biodegradable. Reversible reduction and decolourisation occurs under anaerobic conditions [26, 239].

It should be noticed that the brief overview in this section did not include dye degradation by *Streptomyces* and other actinomycetes, i.e. bacteria that produce extracellular oxidative enzymes like white-rot fungi (section 1.5.6.2). Those extracellular oxidative enzymes are relatively non-specific enzymes catalysing the oxidation of a variety of dyes [17, 212, 360].

1.5.6.2 Fungal biodegradation

Lignin-degrading fungi, white-rot fungi, can degrade a wide range of aromatics. This property is mainly due to the relatively non-specific activity of their lignolytic enzymes, such as lignin peroxidase, manganese peroxidase and laccase. The reactions catalysed by these extracellular enzymes are oxidation reactions, e.g. lignin peroxidase catalyses the oxidation of non-phenolic aromatics, whereas manganese peroxidase and laccase catalyse the oxidation of phenolic compounds [212].

The degradation of dyes by white-rot fungi was first reported in 1983 [112] and has since then been the subject of many research papers. An exhaustive review of these papers was recently published [100]. Virtually all dyes from all chemically distinct groups are prone to fungal oxidation but there are large differences between fungal species with respect to their catalysing power and dye selectivity. A clear relationship between dye structure and fungal dye biodegradability has not been established so far [100].

Fungal degradation of aromatic structures is a secondary metabolic event that starts when nutrients (C, N and S) become limiting [154]. Therefore, while the enzymes are optimally expressed under starving conditions, supplementation of energy substrates and nutrients are necessary for propagation of the cultures. Other important factors for cultivation of white-rot fungi and expression of lignolytic activity are the availability of enzyme cofactors and the pH of the environment.

Although stable operation of continuous fungal bioreactors for the treatment of synthetic dye solutions has been achieved [216, 253, 358], application of white-rot fungi for the removal of dyes from textile wastewater faces many problems. As wastewater is not the natural environment of white-rot fungi, the enzyme production may be unreliable [281] and the biomass growth and retention in bioreactors will be a matter of concern [310]. As treatment of large water volumes may be difficult, extraction and concentration of dyes prior to fungal treatment, may be necessary [240]. Furthermore, the low optimum pH for lignin peroxidase (4.5 – 5) requires extensive acidification of the usually highly alkaline textile wastewater and causes inhibition of other useful microorganisms like bacteria [315]. Moreover, other wastewater constituents, especially aromatics, may interfere with fungal dye degradation [310].

1.5.6.3 Algal biodegradation

Degradation of a number of azo dyes by algae has been reported in a few studies [144, 297]. The degradation pathway is thought to involve reductive cleavage of the azo linkage followed by further degradation (mineralisation) of the formed aromatic amines. Hence, algae have been demonstrated to degrade several aromatic amines, even sulphonated ones [197, 198, 304]. In open wastewater treatments systems, especially in (shallow) stabilisation ponds, algae may therefore contribute to the removal azo dyes and aromatic amines from the water phase.

1.6 Combined anaerobic – aerobic bacterial biodegradation of azo dyes

1.6.1 First stage: anaerobic azo dye reduction

Anaerobic azo dye reduction is the reductive cleavage of azo linkages, i.e. the transfer of reducing equivalents resulting in the formation of aromatic amines. As aromatic amines are generally colourless, azo dye reduction is also referred to as azo dye decolourisation. The first study on azo dye reduction was published as early as 1937, when the decolourisation of food azo dyes by lactic acid bacteria isolated from the human gut was reported [33]. Hence, as the formation of toxic aromatic amines in humans is a matter of concern, research on bacterial azo dye reduction has traditionally mostly been focused on the activity of (facultative) anaerobic bacteria from mammalian intestines [38, 58, 60, 82, 271, 337]. Later, when the removal of dyes from wastewater became a topic, also bacteria from other origins were used to investigate anaerobic azo dye reduction, e.g. pure cultures [348], mixed cultures [122], anaerobic sediments [340], digester sludge [25, 36, 44], anaerobic granular sludge [278] and activated sludge under anaerobic conditions [34]. Several review articles including many studies on bacterial azo dye reduction have been published [18, 41, 61, 60, 81, 174, 212, 310, 337]. The large number of azo dyes that can be reduced by so many different bacteria indicates that azo dye reduction is a non-specific reaction and that the capability of reducing azo dye can be considered as a universal property of anaerobically incubated bacteria.

1.6.1.1 Mechanism of azo dye reduction

The term ‘anaerobic azo dye reduction’ comprises different mechanisms (Figure 1.4). A distinction can be made between direct enzymatic azo dye reduction and indirect azo dye reduction catalysed by enzymatically (re)generated redox mediating compounds. Aside from these mechanisms, it is also possible that azo dyes are purely chemically reduced by biogenic bulk reductants like sulphide.

Direct enzymatic azo dye reduction. According to the first mechanism of biological azo dye reduction, enzymes transfer the reducing equivalents originating from the oxidation of organic substrates to the azo dyes. Enzymes that catalyse azo dye reduction may either be specialised enzymes (catalysing only the reduction of azo dyes) or non-specialised enzymes (non-specific enzymes that catalyse the reduction of a wide range of compounds, including azo dyes). Evidence for the existence of specialised azo dye reducing enzymes, so-called ‘azoreductases’, has so far only been found in studies with some aerobic and facultative aerobic bacteria that could grow with mostly simple azo compounds

as sole source of carbon and energy. These strains grew under strict aerobic conditions by using a metabolism that started with reductive cleavage of the azo linkage [163, 164]. The existence of enzymes catalysing azo dye reduction in aerobic bacteria was for the first time proven when two azoreductases from obligate aerobic bacteria were isolated and characterised [364, 362, 363]. These intracellular azoreductases showed high specificity to dye structures. Aside from these specific azoreductases, also non-specific enzymes catalysing azo dye reduction have been isolated from aerobically grown cultures of *Shigella dysenteriae* [108], *Escherichia coli* [109] and *Bacillus* sp. [314]. Where characterised, these enzymes were found to be flavoproteins [109, 108, 275].

There is no clear evidence for the existence of specific azoreductases in anaerobically grown bacteria. However, also under anaerobic conditions, non-specific enzymes may be responsible for the almost ubiquitous capacity of many strains of anaerobic, facultative anaerobic and even aerobic bacteria to reduce azo dyes. Ten bacterial strains isolated from the human intestine were found to reduce Direct Blue 15 in their culture supernatants [271]. Further research with the purified responsible enzyme from one of the strains showed that it was a flavoprotein capable of catalysing the reduction of azo dyes as well as nitroaromatics [273, 275, 272]. This observation may indicate that enzymatic anaerobic azo dye reduction is more or less a fortuitous reaction, catalysed by enzymes (e.g. hydrogenases) which are usually used for other reactions. In the research by Rafii and co-workers, the azo dye reducing enzyme was found to be located throughout the bacterial cytoplasm without showing association to membranes or other organised structures but it was secreted before acting as an 'azoreductase' *in vivo* [274]. This raises questions with respect to the mechanism, as it is unclear how the supposed extracellular enzymes gain the biochemical electron equivalents (*eg* NADH) necessary for the reduction of azo dyes [310].

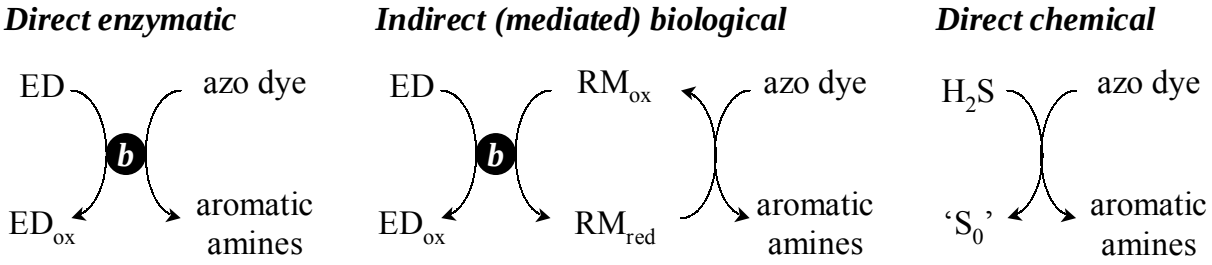


Figure 1.4 Schematic representation of the different mechanisms of anaerobic azo dye reduction
RM = redox mediator; ED = electron donor; *b* = bacteria (enzymes)

Indirect (mediated) biological azo dye reduction. According to the second mechanism of biological azo dye reduction, azo dyes are indirectly reduced by enzymatically reduced electron carriers. Early research has hypothesised that reduced flavins ($FADH_2$, $FMNH_2$, riboflavin) generated by flavin-dependent reductases can reduce azo dyes in a non-specific chemical reaction [111, 282]. Flavins were indeed often found to stimulate azo dye reduction (Table 1.4) and recent research has revealed that flavin reductases are indeed 'anaerobic azoreductases' [286]. Also other reduced enzyme cofactors capable of direct azo dye reduction have been reported, e.g. NADH [223], NADH and NADPH [125, 126] and

an NADPH-generating system [296]. Aside from enzyme cofactors, also various artificial redox mediating compounds are important stimulants of biological azo dye reduction (Table 1.4).

The redox potential (E_0') of a redox mediator (i.e. the E_0' of the couple oxidised/reduced redox mediator) for azo dye reduction should theoretically lie in between the E_0' of the primary electron donor (e.g. the E_0' of the anaerobic oxidation of a carbohydrate to CO_2) and the E_0' of the azo dye (i.e. the E_0' of the redox couple azo dye/aromatic amines). Unfortunately, reliable E0' values for the latter reaction are not available (Schwarzenbach, Tratnyek and Weber, 2001, Pers. Comm.). However, the non-retraceable value of ~100 mV, given for a general azo dye reduction reaction [118], can be considered as a rough indication. E_0' values for ordinary primary electron donors are in between –430 mV and –290 mV, the redox couples for CO_2/glucose and CO_2/acetate, respectively [199]. Consequently, it can be estimated that the E_0' value for potential azo dye reduction catalysing redox mediators will be in the range –430 to –100 mV. This corresponds with the range of E_0' values for the redox mediators listed in Table 1.4.

Table 1.4 Redox mediators for azo dye reduction by bacteria or cell free extracts

Redox mediator	E_0' [a]	References
methyl viologen	-440	58
benzyl viologen	-360	30, 38, 58, 161, 296
Riboflavin	-208	271, 282, 293, 296, 338
FAD	-219	58, 271, 282, 293, 338
FMN	-219	58, 111, 161, 271, 282, 293, 338
Phenosaphranin	-252	58
Menadione	-203	58
Neutral Red	-325	58
Janus Green B	-225	58
AQS[b]	-218	161
AQDS[b]	-184	49, 161
2-hydroxy-1,4-naphthoquinone	-139	161

[a] data E_0' from references [38, 119, 303]

[b] AQS: anthraquinone-2-sulphonate; AQDS: anthraquinone-2,6-disulphonate

*Regeneration of redox mediators***.** Several bacterial enzymes have been found able to regenerate redox mediating enzyme cofactors and artificial electron carriers by reduction. For example, a periplasmic hydrogenase of *Desulfovibrio vulgaris* was shown to reduce several quinone compounds as exogenous electron acceptors coupled to hydrogen oxidation [324]. An NADH:ubiquinone oxidoreductase situated in the membrane of *Sphingomonas sp.* BN6 could reduce AQS [161]. Enzymes may not be directly needed to regenerate some quinone electron carriers as non-enzymatic reduction of *p*-benzoquinones by NADH or by an NADH analogue (9,10 – dyhydro – 10 – methylacridine) has been reported [32, 222]. Recently, it was furthermore reported that the reduction of AQDS by *Shewanella putrefaciens* proceeds via excretion of unidentified quinones for extracellular electron transfer and it was suggested that the biological reduction of insoluble metal oxides might also involve a similar mechanism [237].

Chemical azo dye reduction. Textile manufacturers are aware that addition of reducing agents to dye-containing effluents leads to considerable decolourisation [113]. Hence, azo dye can be reduced by chemical reductants like dithionite [77, 342, 357] and zerovalent iron [224]. Moreover, chemical azo dye

reduction by biogenic reductants like sulphide, is possible as well [351]. Dye-containing wastewaters usually contain moderate to high sulphate concentrations. Sulphate is often an additive of dyebaths or it is formed by the oxidation of more reduced sulphur species used in dyeing processes, such as sulphide, hydrosulphite and dithionite. Sulphate also results from neutralisation of alkaline dye effluents with sulphuric acid. Sulphide is therefore a relevant compound, as it will be generated by sulphate reducing bacteria during treatment of these wastewaters in anaerobic bioreactors.

1.6.1.2 Location of the reaction

The chemical reaction between the dye and the electron carrier, as well as the enzymatic reduction of the electron carrier, can occur both intracellularly and extracellularly. Cofactors like $FADH_2$, $FMNH_2$, NADH and NADPH, as well as the enzymes reducing these cofactors are located in the cytoplasm [286]. Lysis of cells would release cofactors in the extracellular environment. Hence, it has been reported several times that cell extracts or starving or lysed cells show higher azo dye reduction rates than intact or resting cells [87, 213, 282, 286, 337, 348]. However, for intact cells, a membrane transport system would be a prerequisite for the reduction of azo dyes by these cofactors. This presents a serious obstacle, especially for dyes containing (highly polar) sulphonate groups. In addition, also FAD and FMN cannot readily cross cell walls. In contrast, riboflavin is able to move across cell membranes.

Moreover, the lack of a clear relationship between a dye's structure (size, molecular weight, degree of sulphonation) and its reduction rate [25, 36, 38, 44] suggests that intracellular azo dye reduction mechanisms are not likely to play an important role. In a study to the anaerobic reduction of amaranth by whole cells, cell extracts and cell membranes of *Sphingomonas* sp. strain BN6, enzymatic azo dye reduction activity was found to be located in the cytoplasm (a soluble FAD-dependent enzyme) as well as in the membrane fraction (presumably and NADH; ubiquinone oxidoreductase) but it was suggested that that azo dye reduction by whole cells is mainly related to the latter [161]. Most probably, anaerobic biological azo dye reduction occurs outside the cells, catalysed directly by periplasmic enzymes or indirectly, in a reaction with reduced electron carriers that are regenerated by these periplasmic enzymes.

1.6.2 Second stage: aerobic oxidation of aromatic amines

Various (substituted) amino-benzene, amino-naphthalene and amino-benzidine compounds have been found aerobically biodegradable [16, 35, 90]. The conversion of these compounds generally requires enrichment of specialised aerobes. Pseudomonads have often been found to be the responsible microorganisms [157, 190, 285]. In some cases, biodegradation was only achieved in nitrogen-free medium [157]. Especially sulphonated aromatic amines are difficult to degrade. This low biodegradability is due to the hydrophilic nature of the sulphonate group, which obstructs membrane transport. Generally, biodegradation of sulphonated aromatic amines has only been demonstrated for relatively simple sulphonated aminobenzene and aminonaphthalene compounds [317].

Another transformation that aromatic amines may undergo when being exposed to oxygen is autoxidation. Especially aromatic amines with ortho substituted hydroxy groups are susceptible to autoxidation [162]. Many aromatic amines, e.g. substituted anilines, aminobenzidines and naphthylamines, have been found to oxidise, initially to oligomers and eventually to dark-coloured

polymers with low solubility that are easily removed from the water phase [95, 155]. However, autoxidation does not always imply a high degree of polymerisation. For example, in a study on the autoxidation pathway of three sulphonated aromatic amines from azo dyes, only one of the compounds investigated (1-amino-2-hydroxynaphthalene-6-sulphonate) underwent dimerisation, not further. The two other compounds reacted differently. One of these aromatic amines (1-amino-2-hydroxynaphthalene-3,6-disulphonate) was quickly deaminated to form its corresponding naphthoquinone, which was further oxidised involving ring opening. The other aromatic amine (1,2,7-triamino-8-hydroxynaphthale-3,6-disulphonate) only underwent deamination of one of its amino groups, yielding a stable, dark-coloured naphthoquinone imidine as the autoxidation product. When those aromatic amines were aerobically incubated with activated sludge, the formed autoxidation products underwent further transformations. The amines disappeared but complete mineralisation was not achieved. The complete decolourisation of the autoxidation product of the third aromatic amine was probably a biological conversion [162].

1.6.3 Combined anaerobic-aerobic treatment of azo dyes in (semi-)continuous bioreactors

The prerequisite of (reductive) fission of the azo linkage in azo dye molecules prior to (oxidative) further degradation, makes a process in which anaerobic and aerobic conditions are combined the most logical concept for the biological removal of azo dyes [95, 156, 365]. Two different approaches can be discerned: sequential treatment in separate reactors (Table 1.5a) and integrated treatment in a single reactor (Tables 1.5bc). The integrated approach is based on temporal separation of the anaerobic and the aerobic phase, like in sequencing batch reactors (SBR: Table 1.5b) or on the principle that diffusion of oxygen in microbial biofilms is usually limited to 10-100 μm [173] so that anaerobic and aerobic conditions coexist in a single environment (Table 1.5c).

Colour removal. The removal of colour achieved in the anaerobic stages of the studies listed in Tables 1.5abc was generally high: mostly higher than 70% and in several cases almost 100%. Colour removal efficiencies differed between dyes: when the removal of different azo dyes was tested under similar conditions, different colour removal efficiencies were achieved [98, 193, 276, 298]. Only one of the azo dyes studied was not removed at all [21].

Time requirement. The reaction time is an important factor in the anaerobic removal of azo dyes: decreasing the hydraulic retention time of the anaerobic stage was found to result in lower colour removal efficiencies [6, 298]. It should be noted that the hydraulic retention times applied in many of the cited laboratory studies is relatively high in comparison with the average hydraulic retention time of high-rate anaerobic bioreactors. Apparently, anaerobic azo dye reduction is a rather slow reaction.

Biomass concentration. The biomass concentration plays also a role in the anaerobic removal of azo dyes: lowering the biomass and the solid retention time of a sequencing batch reactor resulted in a lower colour removal efficiency [192].

Other factors important for anaerobic azo dye reduction. The different conditions applied in the studies make it difficult to assess which other factors are determining in the anaerobic transformation of azo dyes. As azo dye reduction requires a primary electron donor, the concentration and type of the

organic substrate, as well as the presence of chemical reductants, may be important. Many different organic substrates were used, which suggests that the type of electron donor is probably not determining. However, complete omission of an organic electron donor has been found to restrict, but not to completely suppress, the anaerobic colour removal efficiency [73].

Anaerobic formation of aromatic amines. The studies that paid attention to aromatic amines all reported evidence for the formation of aromatic amines, indicating azo dye reduction. The recovery percentages ranged between <1% to almost 100%. This wide range may partly be explained by the difficulties encountered in analysing these often chemically unstable compounds. Two studies report further anaerobic mineralisation of the aromatic amine 5-amino salicylate [150, 318], which is in line with the results from a previous study describing the anaerobic mineralisation of the azo compound azodisalicylate [278].

Aerobic fate of aromatic amines. The fate of aromatic amines in the aerobic stage cannot be conclusively determined. Partial or complete removal of many aromatic amines can be suspected from the decrease or disappearance of the -sometimes unidentified- peaks in HPLC-chromatograms [98, 121, 141, 150, 192, 250, 292, 307, 318], from the disappearance of aromatic amines as detected with a diazotisation-based method [276], as well as from the decrease of UV absorbance [73]. Moreover, a large decrease of toxicity to aerobic bacterial activity was measured between the effluent of the anaerobic stage and the effluent of the anaerobic stage [250, 307]. However, some aromatic amines may not be removed. Especially cleavage products from the reactive azo dyes Reactive Black 5 and Reactive Violet 5 were often reported not to be removed aerobically (observations based on HPLC measurements [192, 193, 196, 254] or DOC measurements [306]).

Factors important for aromatic amine removal. The different conditions applied in the studies make it difficult to assess which factors are determining in the aerobic transformation of aromatic amines. As aerobic biodegradation of aromatic amines requires specified microorganisms, the type of biomass may play a role: at least in one laboratory reactor study it was found that the degradation of an aromatic amine, sulphanilic acid, could only be achieved after bioaugmentation with a proper bacterial culture [318].

Autoxidation. Autoxidation of aromatic amines during aerobic treatment, as suggested by an increase of colour, has only been observed in a few studies [150, 307, 319]. In the contrary, a slight decrease of the colour was much more often observed [6, 21, 142, 160, 196, 250, 255, 254, 276, 292, 306]. Since many of the azo dyes treated in these studies yield aromatic amines that are expected to autoxidise (section 1.6.2), the latter observation suggests general removal of these compounds or their autoxidation products from the water phase.

Reactor configuration. Comparison of the results of the cited studies does not allow giving judgement on which type of combined anaerobic-aerobic treatment system suits best to remove azo dyes from wastewater. However, the sequential or 'spatially-staged' approach has better perspectives for full-scale applications than the integrated approach, as the requirement of a well-balanced control of the supply of oxygen and electron-donating co-substrate may present a drawback [319, 316].

To summarise, combined anaerobic-aerobic biological treatment holds promise as a method to remove azo dyes from wastewater. However, it can be concluded that there are two possible bottlenecks: (i) anaerobic azo dye reduction is a time-consuming process, reflected by the requirement of long reaction times and that (ii) the fate of aromatic amines during aerobic treatment is not conclusively elucidated. This thesis reports the research that was done to solve the first possible bottleneck.

1.7 Research objective and thesis outline

The objective of this dissertation is to optimise the first stage of the complete biodegradation of azo dyes, anaerobic azo dye reduction. The main focus of the study was unravelling the reaction mechanism and applying the obtained insights. Possible technological translations of the insights were explored in laboratory bioreactor experiments.

The first part of the thesis (Chapters 2 – 4) presents the research that was done to explore the mechanism of anaerobic azo dye reduction. In Chapter 2, a survey of the reduction of large number of azo dyes by anaerobic granular sludge is presented. Chapter 3 goes further on the kinetics of the reaction and the catalysing role of redox mediators. Chapter 4 deals with revealing relative importance of biotic and abiotic azo dye transformations in anaerobic sludge. In the second part of the thesis (Chapters 5 and 6), the application of redox mediators is further investigated. Chapter 5 describes the first reactor study where a redox mediator was applied to accelerate azo dye reduction. This concept is extended in Chapter 6, where a simple way to immobilise the catalyst within the anaerobic is demonstrated. Chapter 7, finally, is the summary and general discussion of the preceding chapters.

Table 1.5a Anaerobic-aerobic treatment of dye-containing wastewater : Spatial and temporal separation of the anaerobic and aerobic phases

system	dyes/wastewater	performance, anaerobic	performance, aerobic	reference
anaerobic upflow fixed bed (36 h) → agitated tank (36 h); both systems inoculated with a mixture of 4 pseudomonads isolated from dyeing effluent contaminated soils	Acid Orange 10 (monoazo), Acid Black 1, Direct Red 2 and Direct Red 28 (all disazo) at different conc. (10 – 200 mg/l) in nitrogen-free mineral medium with 20 mM glucose	complete anaerobic decolourisation at lower dye conc.: (< 50 mg/l of the acid dyes; <100 mg/l of the direct dyes); ~25-50 recovery aromatic amines (measured with a diazotisation-based method)	(suggestion of) complete removal aromatic amines (as measured with a diazotisation-based method)	276
UASB (24 h) → activated sludge unit (16 h) → settler (3 h); anaerobic reactor inoculated with granular sludge from paper-pulp processing wwtp; aerobic reactor inoculated with sludge from municipal wwtp	Reactive Red 141 (disazo, 2x monochlorotriazinyl) at 450 mg/l in simulated textile-processing wastewater (mainly mineral medium with starch + acetate, 3.3 g COD/l); simulated sewage added to aerobic unit	64% anaerobic decolourisation; some evidence of aromatic amine formation (HPLC measurements)	11% further decolourisation; suggestion of partial removal aromatic amines (HPLC measurements)	250
UASB (24 h) → activated sludge unit (16 h) → settler (3 h); anaerobic reactor inoculated with granular sludge from paper-pulp processing wwtp; aerobic reactor inoculated with sludge from municipal wwtp	Reactive Red 141 (disazo, 2x monochlorotriazinyl) at 150-750 mg/l in simulated textile-processing wastewater (mainly mineral medium starch + acetate; different concentrations)	max. 77% decolourisation	fate aromatic amines not examined	249
I: UASB (24-48 h) → activated sludge unit + settler (HRT not mentioned); anaerobic reactor inoculated with granular sludge from paper-pulp processing wwtp	Reactive Red 141 (disazo, 2x monochlorotriazinyl) at 150-750 mg/l in simulated textile-processing wastewater (mainly mineral medium starch + acetate)	max. 78% decolourisation	fate aromatic amines not examined	247
II: ITD (inclined tubular digester; 34 – 84 h) → activated sludge unit + settler; anaerobic reactor inoculated with digester sludge (municipal sewage)		max. 62% decolourisation		
anaerobic fluidised bed reactor (1 – 24 h) → Swisher activated sludge unit	Acid Orange 7, Acid Orange 8, Acid Orange 10, Acid Red 14 (all monoazo; 5 – 40 mg/l) in simulated municipal wastewater (165-185 mg COD/l)	20 – 90% decolourisation, depending on dye, dye concentration and HRT	no further decolourisation; fate aromatic amines not examined	298

Table 1.5a (continued)

system	dyes/wastewater	performance, anaerobic	performance, aerobic	reference
anaerobic fluidised bed reactor (31 h) → Swisher activated sludge unit (3.1 h)	Acid Orange 10, Acid Red 14 and Acid Red 18 (all monoazo; 10 mg/l) in simulated municipal wastewater (175 mgCOD/l)	~90% decolourisation (red dyes); ~62% decolourisation (orange dye); <1% recovery of aromatic amines in anaerobic effluent (HPLC-MS analysis) suggesting extensive anaerobic degradation	possibly further removal (partly) of the very small quantity of the aromatic amines retrieved in the anaerobic effluent	98
anaerobic rotating disc bioreactor (15 h) → 2 aerobic rotating disk bioreactors (7.5 h)	Reactive Violet 5 (monoazo, Cu-complex, vinylsulphonyl; 650 and 1300 mg/l) in mineral medium with acetate and yeast extract	650 mg/l → 90% decolourisation; 1300 mg/l → 95% decolourisation; ~75% recovery of aromatic amines (HPLC measurements)	colour increase, presumably due to autoxidation; almost complete removal of aromatic amines but probably by polymerisation (autoxidation) rather than by biomineralisation	307
anaerobic rotating disc bioreactor (15 h) → 2 aerobic rotating disk bioreactors (7.5 h)	Reactive Black 5 (monoazo, vinylsulphonyl; 600 mg/l) in mineral medium with acetate and yeast extract	~70% decolourisation	few % further decolourisation; presumably no removal of dye metabolites (hardly any DOC removal and only slight decrease of toxicity (measured with luminescence test))	306
UASB reactor (8 - 20 h)→ semi-continuous aerobic activated sludge tank (23 h)	Acid Yellow 17 (monoazo), Basic Blue 3 (oxazine), Basic Red 2 (azine) all at 40 mg/l in mineral medium with glucose (~1 g COD/l);	at HRT = 12 h: 20%, 72% and 78% for respectively the yellow, the blue and the red dye	no further decolourisation	6
UASB reactor (4 - 10 h)→ activated sludge tank (6.5 h)	wastewater from a dye manufacturing factory (mixed with simulated municipal wastewater)	70 – 80% colour removal at HRT > 6 h	10 – 20 % further decolourisation; increased BOD_5/COD ratio after anaerobic treatment may point at formation of biodegradable dye metabolites	6

Table 1.5a (continued)

system	dyes/wastewater	performance, anaerobic	performance, aerobic	reference
egalisation tank → anaerobic Rotating Biological Contactor (7-8 h) → settler → aerobic RBC (4.5–5 h) → settler en LAS en 60-85% van de kleur. Het effluent van de zuivering was nog steeds sterk gekleurd	highly coloured textile dye wastewater with PVA and LAS as the main COD sources (total COD 780 mg/l)	anaerobic decolourisation not specifically mentioned; total system colour removal 60-85%	fate aromatic amines not examined	356
suspended sludge UASB reactor (6-10 h) → activated sludge system (6 h)	same wastewater as [356]	90–95 % anaerobic decolourisation	slightly further decolourisation (up to 96%); fate aromatic amines not examined	142
anaerobic filter (6 h) → aerobic filter 1 (7.7 h) → aerobic filter 2 (8.6 h)	Acid Yellow 17 (monoazo, 25 mg/l) Basic Red 22 (monoazo, 200 mg/l) in mineral medium with starch or glucose	>99% removal of the basic dye but no decolourisation of the acid dye	further decolourisation; fate aromatic amines not examined	21
EGSB reactor (0.625h x 40) → activated sludge system (0.25 h x 40)	Mordant Yellow 10 (monoazo, 100-200 mg/l) in synthetic wastewater with ethanol (1-2 gCOD/l)	almost complete decolourisation; almost complete recovery of one of the dye metabolites (sulphanilic acid); partial anaerobic degradation of the other dye metabolite (5-amino salicylic acid)	almost complete mineralisation of 5-amino salicylic acid; after bioaugmentation with proper bacteria also complete mineralisation of sulphanilic acid (HPLC measurements, sulphate measurements)	318
anaerobic biofilter (discontinue, variable cycle time) → aerobic biofilter (discontinue, 12-24 h per cycle)	Disperse Blue 79 (monoazo, 25-150 mg/l) in mineral medium with or without glucose or acetate	98% decolourisation is possible; ~40% aromatic amine recovery (measured by spectrophotometry)	in 24 h, 65% removal of aromatic amines (measured by spectrophotometry)	73
upflow anaerobic fluidised bed reactor → aerobic fixed film – fixed bed reactor (total system HRT 96 h)	Reactive Red 198 (monoazo, vinylsulphonyl; 5 g/l) in medium with starch, wax and acetate	97% anaerobic decolourisation	slightly further decolourisation (to 99%); evidence for removal of aromatic amines (HPLC measurements)	292

Table 1.5a (continued)

system	dyes/wastewater	performance, anaerobic	performance, aerobic	reference
UASB reactor with granular activated carbon (24-48 h) → semi-continuous activated sludge reactor	highly red coloured textile wastewater	70-90% decolourisation	further decolourisation to almost 100%; no attention paid to fate aromatic amines	160
anaerobic-aerobic hybrid reactor (UASB with aerated upper part; 1-100 h)	Direct Yellow 26 (disazo) at 300 mg/l in mineral medium with 820 mg COD/l ethanol	almost complete decolourisation + evidence for partial anaerobic mineralisation of some of the dye metabolites	further mineralisation aromatic amines; increase of colour due to autoxidation	150

Table 1.5b Anaerobic-aerobic treatment of dye-containing wastewater II. temporal separation of anaerobic and aerobic phase

system	dyes/wastewater	performance, anaerobic	performance, aerobic	reference
SBR (24h cycle: 0.83 h fill → 13 h mixing → 8 h aeration → 1 h settling → 0.92 h draw → 0.25 h idle; SRT 10 or 15 days)	Reactive Violet 5 (monoazo, vinylsulphonyl; 60-100 mg/l) in synthetic wastewater with starch derivative (750 mgCOD/l)	decolourisation almost exclusively during mixed (anaerobic) phase: ~90% colour removal at SRT=15 days with 2.0 gVSS/l; only ~30% colour removal at SRT=10 days with 1.2 gVSS/l; HPLC chromatography suggests aromatic amine formation	Fate metabolites (HPLC analysis): benzene amine probably converted aerobically, naphthalene amine probably not degraded	192
SBR (24h cycle: 0.83 fill → 9-13h mixing → 8-12h aeration → 0.92h draw → 0.25 h idle; SRT 10, 15 or 20 days)	Reactive Violet 5 (monoazo, vinylsulphonyl; 60-100 mg/l) and Reactive Black 5 (disazo, vinylsulphonyl) in synthetic wastewater with starch derivative	Reactive Violet 5: results comparable to [192]; Reactive Black 5: less decolourisation	Fate metabolites Reactive Violet 5 (HPLC analysis): no degradation Fate metabolites Reactive Black 5 not evaluated	193
SBR (18 h anoxic/anaerobic → 5h aerobic → 0.85 settle → 0.15 h draw)	Reactive Black 5 (disazo, vinylsulphonyl), Reactive Blue 19 (anthraquinone, vinylsulphonyl), Reactive Blue 5 (anthraquinone, monochlorotriazinyl), Reactive Blue 198 (oxazine) at 20 or 100 mg/l in mineral medium with glucose and acetate	at 20 mg dye/l 63, 64 and 66%; at 100 mg dye/l 58, 32 and 41% decolourisation for resp. Reactive Black 5, Reactive Blue 19 and Reactive Blue 5; decolourisation oxazine dye could not be quantified; anthraquinone dyes: adsorption to the sludge; azo dye probably reduced to aromatic amines (HPLC measurements)	slightly further decolourisation; aromatic amines from Reactive Black 5 are probably not removed (HPLC measurements)	196, 254
SBR (18 h anoxic/anaerobic → 5h aerobic → 0.85 h settle → 0.15 h draw)	Reactive Black 5 (disazo, vinylsulphonyl) at 10 mg/l in mineral medium with glucose and acetate	72% decolourisation; colour shift spectrum (spectrophotometry measurements) suggests azo linkage cleavage	1.6% further decolourisation; fate aromatic amines not examined	255

Table 1.5c Anaerobic-aerobic treatment of dye-containing wastewater III. integrated systems (based on biofilms with limited oxygen penetration)

system	dyes/wastewater	performance	reference
EGSB reactor with oxygenation of recycled effluent	Mordant Yellow 10 and 4-phenylazophenol (monoazo)	Mordant Yellow 10: similar overall results as in [318]; 4-phenylazophenol: complete reduction → complete mineralisation of one metabolite (aniline) and autoxidation of the other metabolite (4-aminophenol)	319, 316
Rotating drum biofilm reactor (HRT variable <3h); different air/COD ratios applied	Acid Orange 7 (monoazo, 5 mg/l) in synthetic wastewater	at low air high COD flux, aromatic amine formation (HPLC measurements) → anaerobic azo dye reduction at high air low COD flux, complete mineralisation → aerobic biodegradation	121
Rotating drum biofilm reactor (HRT 2h); different air/COD ratios applied	Acid Orange 8, Acid Orange 10 and Acid Red 14 (monoazo dyes; different conc.) in synthetic wastewater	as Harmer and Bishop (1992). However, at high air low COD flux only aerobic degradation of Acid Orange 8.	141

2

Azo dye decolourisation by anaerobic granular sludge

Abstract The decolourisation of 20 selected azo dyes by granular sludge from an upward-flow anaerobic sludge bed (UASB) reactor was assayed. Complete reduction was found for all azo dyes tested, generally yielding colourless products. The reactions followed first-order kinetics and reaction rates varied greatly between dyes: half-life times ranged from 1 hour to about 100 hours. The slowest reaction rates were found with reactive dyes with a triazine reactive group. There was no correlation between a dye's half-life time and its molecular weight, indicating that cell penetration was probably not an important factor. Since granular sludge contains sulphide, eight dyes were also monitored for direct chemical decolourisation by sulphide. All of these dyes were reduced chemically albeit at slower rates than in the presence of sludge at comparable sulphide levels. Increasing sulphide concentrations, even when present in huge excess, stimulated the azo dye reduction rate.
The results indicate that granular sludge can decolourise a broad spectrum of azo dye structures due to non-specific extracellular reactions. Reducing agents (e.g. sulphide) in sludge may play an important role. The presence of anaerobic biomass is probably beneficial for maintaining the pools of these reduced compounds.

Van der Zee, F.P., Lettinga, G. and Field, J.A. (2001) Chemosphere 44:1169-1176

2.1 Introduction

One of the main problems associated with the treatment of textile wastewater is the removal of dyes. Most (60-70%) of the more than 10,000 dyes applied in textile-processing industries are azo compounds, i.e. molecules with one or more azo (N=N) bridges linking substituted aromatic structures [46]. Discharge of azo dyes is undesirable, not only for aesthetic reasons, but also because many azo dyes and their breakdown products are toxic toward aquatic life [61], and mutagenic for humans [59].
Azo dyes are persistent to biodegradation under aerobic conditions [103, 143, 251, 252, 299], but they undergo reductive fission of the azo linkage relatively easily under anaerobic conditions [36, 38, 44, 278]. Although the phenomenon of anaerobic azo dye reduction is unanimously accepted, some aspects of the reaction mechanism remain to be clarified. Different observations have been reported on the involvement of enzymes, the location of the reaction, and its kinetic order.
High rate anaerobic treatment systems have been considered for the treatment of azo dyes in textile industry wastewater [6, 86, 98, 298]. However, due to the wide variety of dyes used in the industry, a broad capability of the biomass in these reactor systems to reduce different dye structures needs to be ascertained. The goal of this research was to evaluate the feasibility of granular sludge in upward-flow anaerobic sludge bed (UASB) reactors to reduce 20 different types of azo dyes. Since sludge granules contain high concentrations of chemically reactive sulphide both the biological and chemical activity of the sludge towards azo dye reduction was considered.

2.2 Materials and methods

The biological dye decolourisation assays were conducted in 120 ml serum bottles containing 50 ml of medium and an overlying headspace composed of N_2/CO_2 (80%/20%) which was sealed with a butyl rubber stopper. The primary electron donating substrate of the medium was composed of 2 g l^{-1} chemical oxygen demand (COD) of a NaOH-neutralised volatile fatty acids (VFA) mixture containing acetate, propionate and butyrate in a COD based ratio of 1:10:10. The basal nutrients of the medium were composed of 2.8 g l^{-1} NH_4Cl, 0.057 g l^{-1} $CaCl_2$, 2.5 g l^{-1} KH_2PO_4, 1 g l^{-1} $MgSO_4 \cdot 7H_2O$ and the medium was buffered at a pH of 7.3 ± 0.2 with $NaHCO_3$ (5 g l^{-1}). Non-adapted anaerobic granular sludge was added to the medium at a concentration of 1.5 g l^{-1} volatile suspended solids (VSS). The medium was flushed with the N_2/CO_2 (80%/20%) and pre-incubated with the sludge for 2 to 3 days. The background level of sulphide in the medium was 0.7 ± 0.02 mM. The selected dye was added to a final concentration of approximately 0.3 mM (100-300 mg l^{-1}) with a syringe from a concentrated stock solution. The serum bottles were incubated at 30 °C in a rotary shaker at 50 rpm. At selected

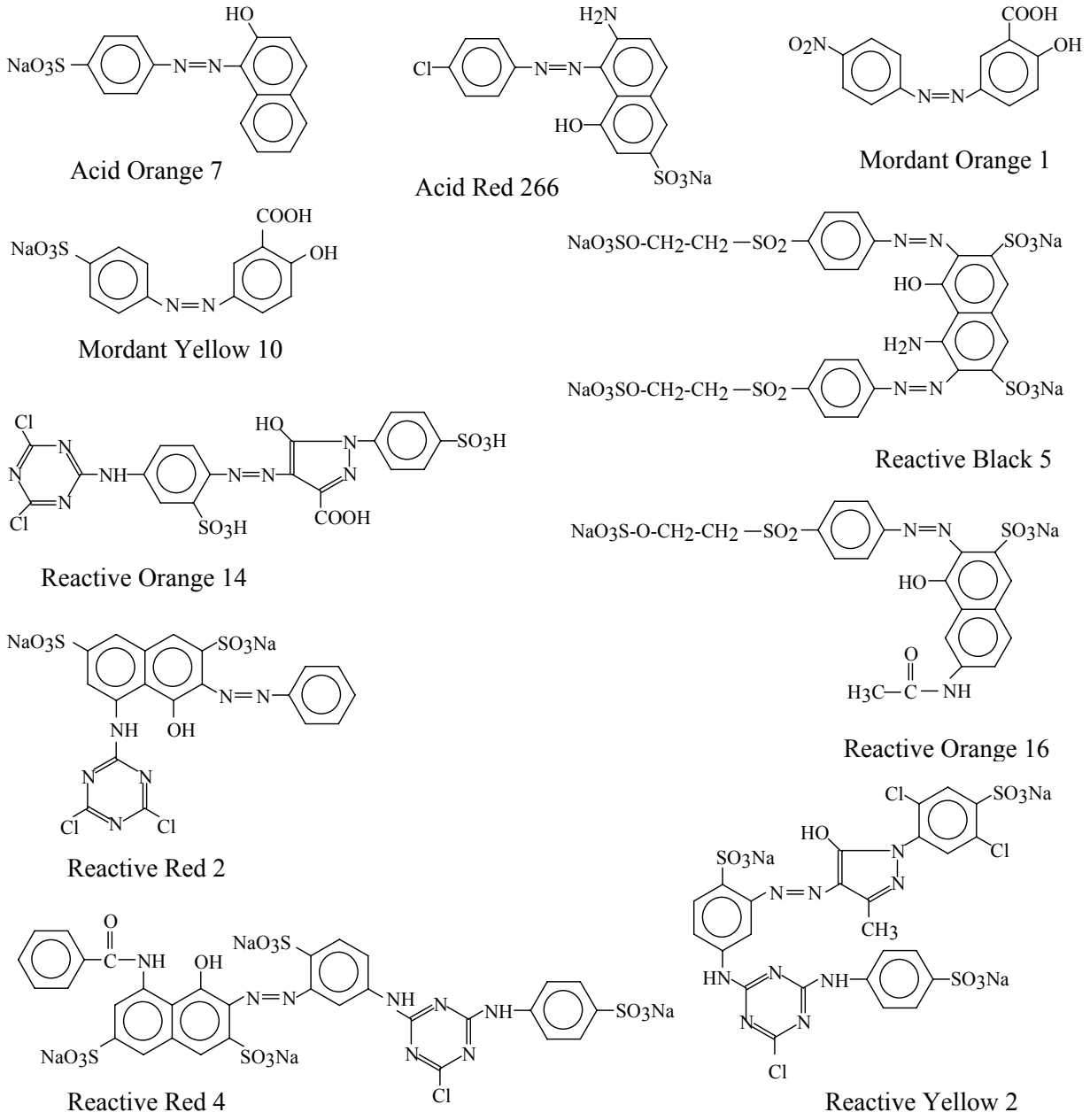


Figure 2.1a Structure formulas of the acid, mordant and reactive dyes used in this study

intervals, colour was measured spectrophotometrically at the dye's wavelength of maximum absorbance (λ_{max}). For this purpose, samples were centrifuged after dilution to less than 1 absorbance unit (AU) in a phosphate buffer (10.86 g l^{-1} $NaH_2PO_4 \cdot 2H_2O$; 5.38 g l^{-1} $Na_2HPO_4 \cdot H_2O$) that contained ascorbic acid (200 mg l^{-1}) to effectively prevent autoxidation. The background light absorbance of the control medium in the buffer was less than 0.5% of the absorbance due to dye containing medium in the buffer and could therefore be neglected. The chemical decolourisation assays were conducted identically as the biological assays with the exception that the granular sludge and VFA were excluded from the medium and sulphide was added to final concentrations ranging from 1 to 70 mM. The colour was measured as light absorbance at selected time intervals at each dye's λ_{max} as described previously for the biological assay. To assess autoxidation of the aromatic amines formed during dye reduction,

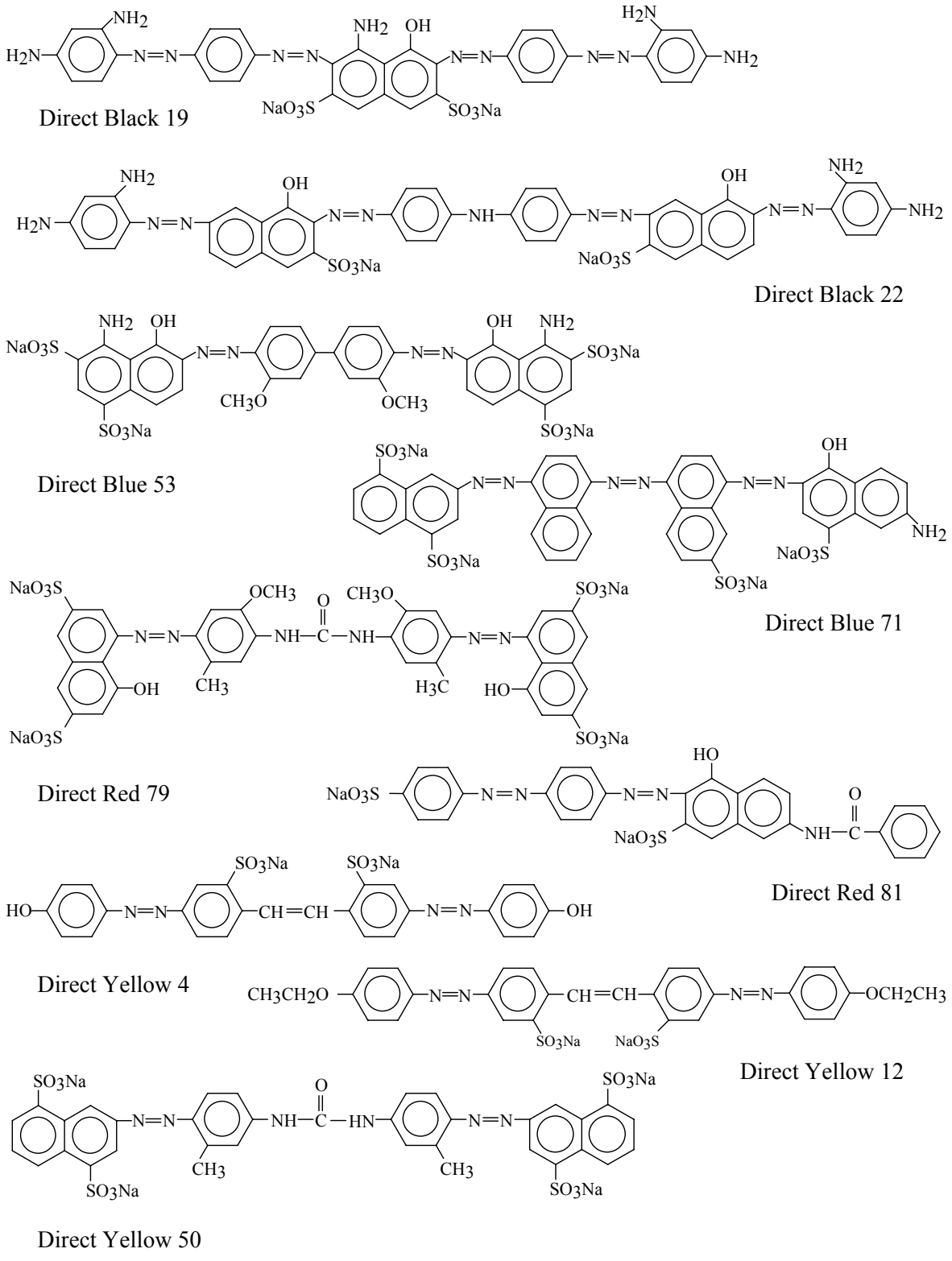


Figure 2.1b Structure formula of the direct dyes used in this study

samples of completely decolourised dye solutions were brought into 1.5 ml microcentrifuge tubes which were left open to the air for respectively 5-10 minutes, 1 hour and 1 day prior to dilution in phosphate buffer without ascorbic acid. After centrifugation, the 200-800 nm colour spectrums were scanned and compared with scans of original dye solutions in phosphate buffer.

The dyes were purchased from either Aldrich (Gillingham, England), Acros (Geel, Belgium), Sigma (Bornem, Belgium), Sigma-Aldrich (Steinheim, Germany), Crompton & Knowles (Tertre Belgium) or Ciba-Geigy (Basel, Switzerland) and were used without any further purification. As far as available, the purities of the dyes according to the manufacturer are mentioned in Table 2.1 and the structure formulas are shown in Figures 1a and 1b (Colour Index generic names are used). For Acid Yellow 137, Acid Yellow 159 and Basic Red 23, the structure formulas are not known.
Anaerobic granular sludge came from an alcohol distillery (NEDALCO, Bergen op Zoom, The Netherlands)

2.3 Results

2.3.1 Biological azo dye reduction

The decolourisation of 20 azo dyes by anaerobic granular sludge was measured as the decrease of visible light absorbance at the previously assessed wavelength of maximum absorbance (λ_{max}). As summarised in Table 2.1, all azo dyes studied were decolourised. The reactions proceeded without lag phase. The decolourisation was complete or nearly complete (>95% decrease of absorbance at λ_{max}) for most of the dyes. Important exceptions were Direct Yellow 12 and Reactive Yellow 2. Direct Yellow 12 formed a new absorption peak with a maximum at 336 nm, close to λ_{max}, resulting in relatively high (~14%) residual absorbance at the λ_{max}. Reactive Yellow 2 had an exceptionally slow rate of decolourisation, which was not yet complete after 342 days of incubation.
In most cases, the reaction products were colourless. Two exceptions were Reactive Red 2 and Reactive Red 4, in which a shift from red to yellow was observed. The decolourisation of the azo dyes

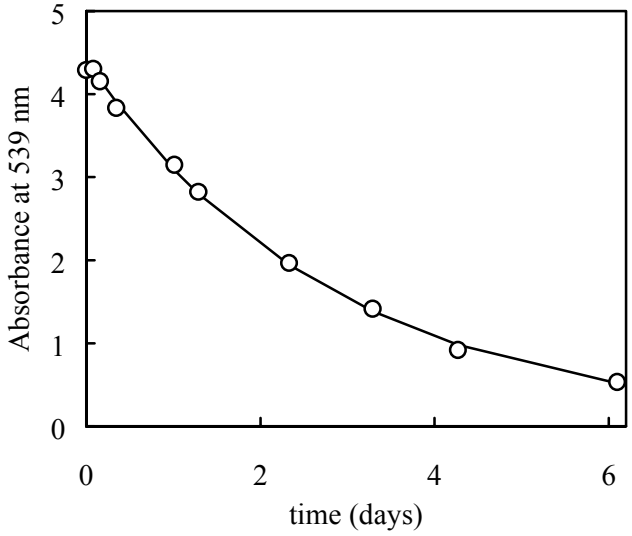


Figure 2.2 Decolourisation of Reactive Red 2 in the presence of anaerobic granular sludge.
Experimental data (open circles) and first-order fit (curve)

in all cases proceeded without a lag phase. In the cases of monoazo dyes, the reaction followed first-order kinetics as shown for the example of Reactive Red 2 in Figure 2.2. In contrast, dyes with more than one azo linkage displayed multiphase kinetics.

The first-order rate constants (k), resulting from fitting equation [2.1] to the whole curve (monoazo dyes) or to the first part of the curve (disazo and polyazo dyes) are listed in Table 2.1.

$$A_t = A_0 \cdot e^{-kt} \qquad [2.1]$$

with: A_t = light absorbance at λ_{max} at a given time (t)

A_0 = light absorbance at λ_{max} at time 0

k = first order rate constant

t = time

Table 2.1 Overall results of azo dye decolourisation by anaerobic granular sludge

Dye	Purity (%)	λ_{max} (nm)	Decolourisation $\%_{max}$	k [d] (d^{-1})	
Acid Orange 7	98	484	99	1.49	± 0.07
Acid Red 266	NA[a]	492	95	0.20	± 0.07
Acid Yellow 137	NA	456	95	0.35	
Acid Yellow 159	NA	362	97	0.72	
Basic Red 23	NA	526	99	10	
Direct Black 19	NA	675	99 [b]	3	± 1 [e]
Direct Black 22	NA	484	99 [b]	NM [f]	
Direct Blue 53	85	608	99	0.24	
Direct Blue 71	50	579	100	0.61	± 0.04
Direct Red 79	NA	510	97	16.6	± 1.6
Direct Red 81	50	509	99	7.8	± 0.3
Direct Yellow 4	70	402	95	1.03	± 0.05
Direct Yellow 12	65	401	86	1.17	± 0.07
Direct Yellow 50	60	402	99	2.0	± 0.3
Mordant Orange 1	80	373	97	1.74	± 0.07
Mordant Yellow 10	85	355	95	1.86	± 0.05
Reactive Black 5	55	595	99	5.0	± 0.9
Reactive Orange 14	NA	433	98	0.17	± 0.01
Reactive Orange 16	50	492	97	2.1	± 0.4
Reactive Red 2	50	539	100 [c]	0.31	± 0.03
Reactive Red 4	50	521	99 [c]	0.45	± 0.02
Reactive Yellow 2	50	405	73	0.01	

[a] NA = information not available;
[b] dye does not dissolve well: the decolourisation of the water phase is possibly a combination of reduction, adsorption and precipitation;
[c] reaction products are yellow;
[d] k-values (first-order rate constants) were obtained from fitting equation [2.1] to the complete decolourisation curve (monoazo dyes) or to the first part of the decolourisation curve (disazo and polyazo dyes); for experiments which were replicated standard deviations are mentioned behind the ±-sign;
[e] dye does not dissolve well: very rough estimation k;
[f] dye does not dissolve well: rate could not be measured.

Under the applied conditions, k-values varied greatly between different dyes yielding half-life times between 1 and 100 hours. No correlation between k and molecular weight could be observed. For instance, the large dye Direct Red 79 (M_W = 1049 g mol^{-1}) decolourised much faster than the small

dye Mordant Orange 1 (M_W = 287 g mol^{-1}). However, the four dyes containing triazine as a reactive group (Reactive Orange 14, Reactive Red 2, Reactive Red 4 and Reactive Yellow 2) were among the dyes which were reduced at the slowest rates.

2.3.2 Chemical azo dye reduction

Since anaerobic granular sludge contains inorganic reducing agents like sulphide, the direct chemical azo dye reduction by sulphide was investigated. All eight dyes tested (Acid Orange 7, Direct Black 19, Direct Red 81, Direct Yellow 4, Direct Yellow 50, Mordant Orange 1, Mordant Yellow 10 and Reactive Red 2) were found to be reduced by sulphide. In contrast to what was found for biological azo dye reduction, the decolourisation curves of most of these dyes deviated slightly from the first-order kinetics. As a result of catalysis by azo dye reduction reaction products (Chapter 3), a lag phase was observed immediately after dye addition. Thereafter dye decolourisation accelerated in time before assuming a typical first order kinetics (Figure 2.3). This effect was especially evident at low dye or sulphide concentrations. Due to this deviation, the k values obtained with data fitted with the first order kinetics were considered as pseudo first-order rate constants.

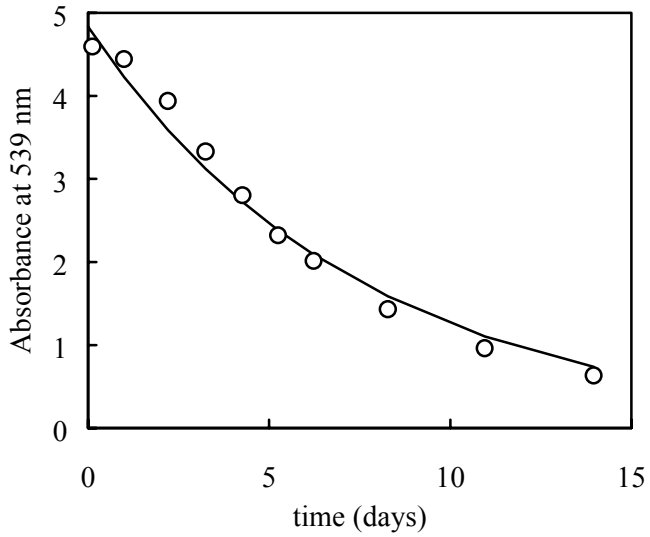


Figure 2.3 Decolourisation of Reactive Red 2 by sulphide (initial sulphide concentration 2.8 mM)
Experimental data (open circles) and first-order fit (curve)

At comparable sulphide concentrations, azo dye reduction rates were stimulated by the presence of sludge. For example, the pseudo first-order rate constant (k) for the reduction of Reactive Red 2 by 1.3 mM of sulphide was 0.06 d^{-1}, which was considerably lower than the k in the presence of anaerobic granular sludge (1.5 g VSS l^{-1}) of 0.3 d^{-1} at an initial sulphide concentration of 0.8 mM.

The pseudo first order rate constants of dye reduction rates (k-values) increased with increasing sulphide concentration (Figure 2.4). Up to a sulphide concentration of 0 to 60-70 mM, a more or less linear relationship between k and sulphide concentration was observed for Reactive Red 2 and C.I. Acid Orange 7 (a slowly decolourising and a moderately slow decolourising dye, respectively). In

contrast, for the fast decolourising Direct Red 81, the increase of k declined at high sulphide concentrations.

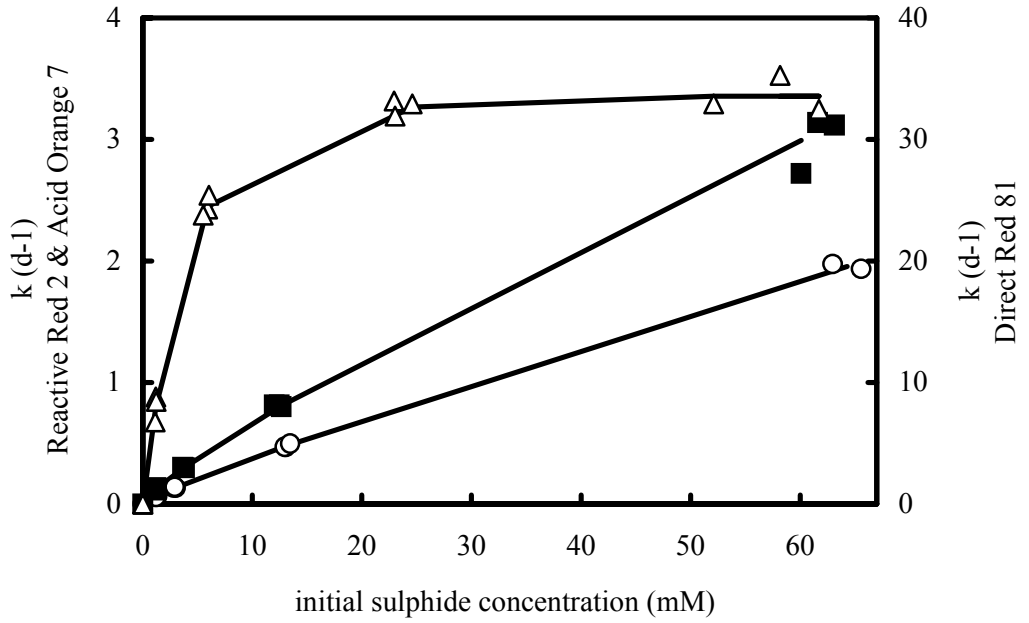


Figure 2.4 Effect of sulphide on the chemical decolourisation of Reactive Red 2 (open circles), Acid Orange 7 (full squares) and Direct Red 81 (open triangles)

2.3.3 Autoxidation

The products of anaerobic azo cleavage are aromatic amines, which have been reported to undergo autoxidation reactions when they are exposed to oxygen [162, 242]. Therefore, samples from decolourised dye solutions of the biological assays were exposed to air to investigate this phenomenon qualitatively. With the exceptions of Direct Yellow 12, Direct Yellow 50 and Reactive Yellow 2, all decolourised solutions of azo dyes formed autoxidised coloured products upon exposure to oxygen. Generally, the autoxidation reactions proceeded quickly with colour development only after a few minutes of exposure to air, which was also evident from a largely altered UV-VIS spectrum. However, prolonged exposure to air generally did not result in further changes of the UV-VIS spectrum. Only in two cases (Mordant Orange 1 and Acid Orange 7) did autoxidation lead to the formation of clearly visible flocs, which could be separated by centrifugation.

2.4 Discussion

The results of this study indicate that granular sludge from high rate anaerobic bioreactors can reduce and decolourise a broad spectrum of azo dye structures without any apparent lag-phase. The rate of decolourisation was not dependent on the molecular weight of the dye indicating that cell permeation was probably not an important issue in the reductive mechanism. This observation combined with the non-specificity and lack of any lag-phase points to a non-enzymatic extracellular reaction mechanism

involving reduced compounds. The mechanism is supported by the observation that sulphide, which is abundantly present in sludge, can directly cause the chemical reduction of azo dyes. Furthermore, data in the literature also suggest the involvement of reduced compounds causing direct chemical reduction of azo bonds, such as zero valent iron [43, 343] as well as reduced biochemical cofactors, including reduced flavins [111] and NADH [223]. Since azo dyes could be decolourised by sulphide, biological activity is not a prerequisite for azo dye reduction. As sulphide is inevitably present in anaerobic sludge environments, chemical azo dye reduction will contribute to the overall decolourisation process under 'living' anaerobic conditions. Nevertheless, at comparable sulphide concentrations, azo dye reduction proceeds faster in the presence of sludge. The exact nature of the presence of sludge and living organisms on contributing to an accelerated dye decolourisation rate is not fully known. An important plausible role of 'living bacteria' in the sludge could be the regeneration of reducing agents, such as sulphide, ferrous iron and reduced biochemical cofactors. Also organic matter in the sludge may contain humic substances which are known to accelerate reductive processes by redox mediation. The chemical reduction of particle bound 4-aminoazobenzene by zero valent iron was accelerated by the quinone, juglone [343]. Also the reduction of amaranth (an azo dye) by bacteria was accelerated by the presence of another quinone, anthraquinone sulphonate [161].

The course of the decolourisation process approximates first-order kinetics with respect to the dye concentration. First-order kinetics with respect to the dye concentration have also been reported by [44, 341, 340, 348] whereas other researchers found zero-order kinetics [38, 87, 121]. A probable explanation for these contradictory observations is that the rate-limiting step in the reduction of azo dyes may differ between the different experimental conditions studied. In pure cultures for instance, the production of reducing equivalents, a zero-order process [87], is far more likely to be rate-determining than in anaerobic sludge environments where reducing equivalents are abundantly present. In the latter case it can be assumed that the transfer, rather than the production, of reducing equivalents is rate-determining, which is supported by the observation that increasing sulphide concentrations speeded up the azo dye reduction rate even up to very high concentrations.

The ability of granular sludge to reduce a broad spectrum of dyes holds promise for the application of high rate anaerobic systems as a feasible first stage in the complete removal of azo dyes from wastewater. However, the kinetic data predict that reactive dyes with a triazine reactive group are slowly reduced. Long residence times would be necessary to reach a satisfying extent of decolourisation. However, this problem may be overcome, as the results presented here reveal shortage of reducing equivalents and literature data indicate that redox mediators can be used to accelerate the transfer of reducing equivalents.

During aerobic post treatment of anaerobically treated azo dye containing wastewater there will be competition between biodegradation and autoxidation of aromatic amines. The autoxidation of aromatic amines in a subsequent aerobic post treatment step may be problematic, not only because the formed products are coloured but also because some of these compounds, e.g. azoxy compounds, may cause toxicity [95]. It may as well be possible, however, that autoxidation leads to the formation of large, bulky, non-toxic, 'humic' polymers that can easily be separated from the water phase.

3

The role of (auto)catalysis in the mechanism of anaerobic azo dye reduction

Abstract Azo dyes are non-specifically reduced under anaerobic conditions, but the slow rates at which many dyes react may present a serious problem for the application of anaerobic technology as a first stage in the complete biodegradation of these compounds. Therefore, it is significant to explore the mechanism of anaerobic azo dye reduction, especially with respect to its kinetics. With that purpose, decolourisation of the monoazo dye C.I. Acid Orange 7 (AO7) was studied in batch experiments. Experiments indicated that chemical reduction by sulphide is partially responsible for the anaerobic conversions of AO7. Mathematical evaluation of the experimental results pointed out that autocatalysis played an important role in the chemical reduction of AO7. Further tests made clear that 1−amino−2−naphthol was the dye's constituent aromatic amines that accelerated the reduction process, possibly by mediating the transfer of reducing equivalents. The impact of redox mediation by quinones was further evaluated by testing the catalysing effects of anthraquinone-2,6-disulphonic acid (AQDS) and of autoclaved sludge. AQDS appeared to be an extremely powerful catalyst, capable of increasing the first-order chemical reduction rate constants by a factor 10 to 100. Also autoclaved sludge, possibly because of mediation by sludge organic matter, accomplished accelerated azo dye reduction rates. Azo dye reduction in living sulphidogenic anaerobic sludge environments is 3 times more rapid than the chemically catalysed reaction with sulphide. The exact role of the biological activity remains to be clarified.

Van der Zee, F.P., Lettinga, G. and Field, J.A. (2000) Water Sci. Technol. 42:301-308

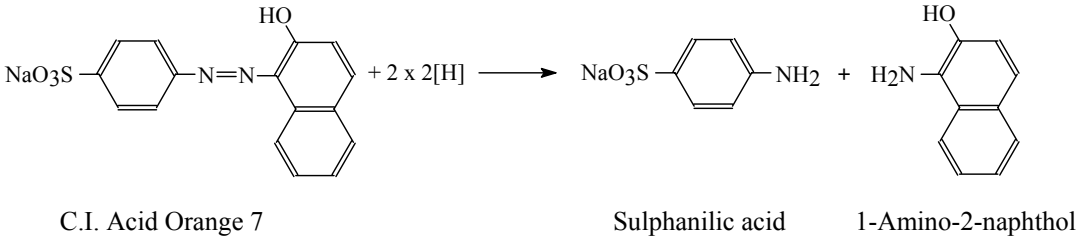
3.1 Introduction

Removal of dyes is a major concern when treating textile-processing wastewater. The vast majority (60-70%) of the more than 10,000 dyes applied in textile-processing industries are azo compounds, i.e. molecules with one or several azo (N=N) bridges linking substituted aromatic structures [46]. Their discharge is undesirable, not only for aesthetic reasons, but also because many azo dyes and their breakdown products have been proven toxic to aquatic life [61] and mutagenic to humans [59]. Azo dyes are generally persistent under aerobic conditions [251, 299]. However, under anaerobic conditions, they undergo relatively easy reductive fission, yielding aromatic amines. The latter compounds, in turn, generally require aerobic conditions for their degradation [35].

The process of anaerobic azo dye reduction has been intensively studied [25, 36, 44, 46, 111, 278, 337]. Most researchers agree that anaerobic azo dye reduction is a non-specific and presumably extracellular process, in which reducing equivalents from either biological or chemical source are transferred to the dye. Both zero-order [38, 87] and first-order [25, 44, 45, 341, 340, 348] kinetics have been reported for the dye concentration.

Preliminary research in our laboratory, corresponding to the screening of the anaerobic decolourisation of 20 azo dyes (Chapter 2), revealed that all azo dyes studied decolourised in the presence of VFA fed granular sludge. Interestingly, they also decolourised chemically, by a reaction with sulphide. The results strongly indicated that the transfer, rather than the production of reducing equivalents was rate-limiting in the reduction of azo dyes. The decolourisation process followed first-order kinetics, with half-life times varying greatly between dyes. The kinetic data predicted that for many azo dyes, long contact times are necessary to reach a satisfying extent (>90%) of decolourisation, which may represent a serious problem for applying high-rate anaerobic treatment as the first stage in the biological degradation of azo dyes. However, there is evidence to overcome this problem, as recent research revealed that redox mediating compounds, mostly quinones, can speed up azo dye reduction rates by shuttling reducing equivalents from the electron donor to the azo dye [23, 152, 161].

In this study, the mechanism of anaerobic azo dye reduction was investigated by studying the decolourisation of AO7 (Figure 3.1) under different circumstances, with focus on reaction kinetics and catalysis.



C.I. Acid Orange 7 Sulphanilic acid 1-Amino-2-naphthol

Figure 3.1 The reaction studied in this report: the reduction of the monoazo dye Acid Orange 7

3.2 Materials and methods

For 'biological' decolourisation experiments, 117-ml glass serum vials were filled with 50 ml basal medium containing (mg l^{-1}): $NaHCO_3$ (5000), NH_4Cl (280), $CaCl_2$ (5.7), KH_2PO_4 (250), $MgSO_4 \cdot 7H_2O$ (100), H_3BO_3 (0.05), $FeCl_2 \cdot 4H_2O$ (2), $ZnCl_2$ (0.05), $MnCl_2 \cdot 4H_2O$ (0.5), $CuCl_2 \cdot 2H_2O$ (0.04), $(NH_4)_6Mo_7O_{24} \cdot 5H_2O$ (0.05), $CoCl_2 \cdot 6H_2O$ (1), $NiCl_2 \cdot 6H_2O$ (1) and $Na_2SeO_3 \cdot 5H_2O$ (0.16). Non-adapted granular sludge from a distillery wastewater treatment plant (Nedalco, Bergen op Zoom, The Netherlands) was added to the vials at Volatile Suspended Solids (VSS) concentrations of 0.2-10 g l^{-1}. The vials were sealed with butyl rubber stoppers and the gas headspace was flushed for 5 minutes with 70%:30% N_2/CO_2. Cosubstrate (2 g COD l^{-1} of a 1:10:10 mixture of acetate, propionate and butyrate) was added by syringe from a neutralised concentrated stock solution. After a 3-days pre-incubation period, again the headspace of the vials was flushed and 2 g COD l^{-1} of cosubstrate was added. For the experiments with autoclaved sludge, similarly prepared batches were, after 3 days pre-incubation and flushing, cooked for 90 minutes in a pressure cooker. For the other chemical decolourisation experiments, 117-ml glass serum vials were filled with 50 ml of a 5 g l^{-1} $NaHCO_3$ solution, sealed with butyl rubber stoppers and flushed for 5 minutes with 70%:30% N_2/CO_2. Sulphide was added by syringe from a partly neutralised (1 M HCl per M Na_2S) stock solution. Sulphanilic acid and anthraquinone-2,6-disulphonic acid were added from neutralised stock solutions immediately after sulphide injection, whereas 1-amino-2-naphthol was added as a powder at the start of the preparation procedure. All batches were prepared in triplicate, except those used for the experiment with 1-amino-2-naphthol (1 vial per concentration).

To start the experiments, the azo dye AO7 was injected into the vials from a neutralised concentrated stock solution to obtain a final concentration of 0.25-0.3 mM. All vials were incubated at 30 °C in a rotary shaker at 50 rpm. In all experiments, the pH of the liquid phase was 7.2 ± 0.2 during the whole incubation period.

At intervals, colour was measured spectrophotometrically with a Spectronics 60 spectrophotometer (Milton Ray Analytical Products Division, Belgium) at the dye's wavelength of maximum absorbance (484 nm). Liquid phase samples (0.75 ml) were centrifuged and diluted up to an absorbance of less than 1 in a phosphate buffer (10.86 g l^{-1} $NaH_2PO_4 \cdot 2H_2O$; 5.38 g l^{-1} $Na_2HPO_4 \cdot H_2O$). The buffer contained freshly added ascorbic acid (~200 mg l^{-1}) to prevent autoxidation. Without dye, light absorbance of medium and buffer was less than 0.5% of the absorbance right after dye addition and could therefore be neglected. Sulphide was measured before dye addition according the colorimetrical method described by Trüper and Schlegel [328]. Sulphanilic acid (SA) was measured by High Performance Liquid Chromatography. The chromatograph was equipped with two reverse phase C18 columns (200 x 3 mm, Chromosphere C18, Chrompack) at 20 °C. The carrier liquid, a 0.5% acetic acid solution at pH 5.9, was pumped at a flow rate of 300 µl min^{-1}. SA was detected spectrophotometrically, using a Spectroflow 783 UV detector (Kratos Analytical) at 248 nm.

C.I. Acid Orange 7 (Orange II, dye content 98%) was purchased from Aldrich Chemical Company Ltd., Gillingham, England. Sulphanilic acid (99%, A.C.S. reagent) was purchased from Sigma-Aldrich

Chemie GmbH, Steinheim, Germany. Anthraquinone-2,6-disulphonic acid, disodium salt and 1-amino-2-naphthol hydrochloride (technical grade, 90%) were purchased from Aldrich Chemical Company Inc., Milwaukee, USA.

3.3 Results and discussion

The colour-versus-time plots for the decolourisation of AO7 in an anaerobic sludge environment and in a reaction with sulphide (Figure 3.2) represent typical examples of respectively 'biological' and chemical monoazo dye reduction under anaerobic conditions. Decolourisation, representing the reduction of AO7, was followed spectrophotometrically. HPLC analysis confirmed the formation of sulphanilic acid, one of the cleavage products. The molar recovery of this aromatic amine was 76 ± 8% and 99 ± 10% for biological and chemical reducing systems, respectively. Large time-scale differences between both conditions were observed: comparison between Figures 2a and 2b clearly demonstrates that the decolourisation process proceeds much faster in the presence than in the absence of granular sludge, even though the initial total sulphide concentration was about three times higher in the latter case.

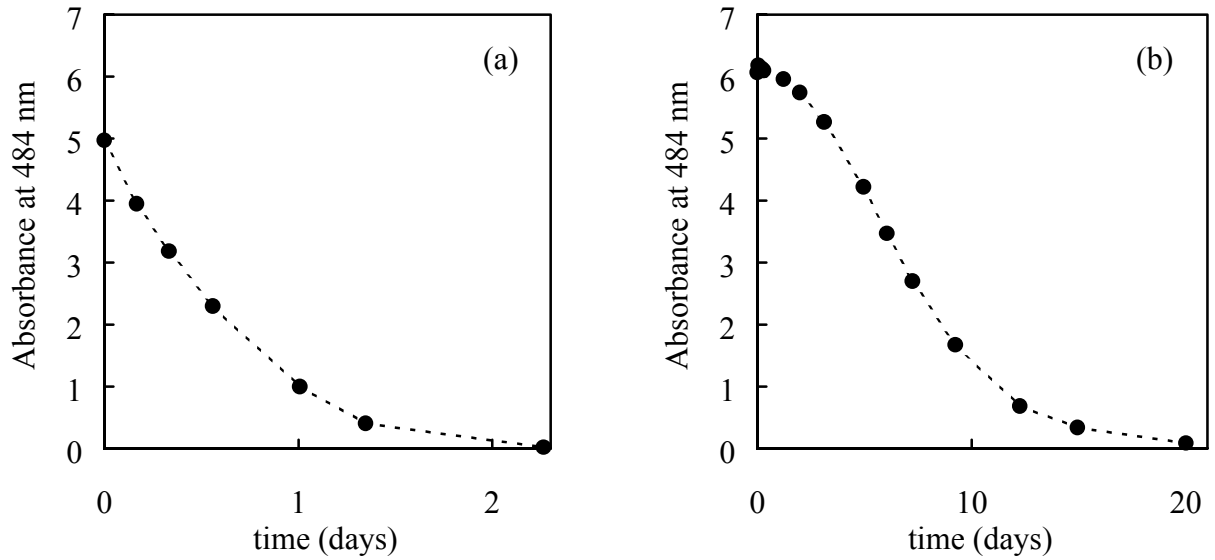


Figure 3.2 Decolourisation of AO7 (0.25-0.3 mM):
(a) in the presence of anaerobic granular sludge (3 g VSS · l^{-1}) at an initial sulphide concentration of 0.3 mM;
(b) in the absence of granular sludge at an initial sulphide concentration of 1 mM.

First-order kinetics (equation [3.1]) can be used to describe the decolourisation process. For AO7, as well as for other monoazo dyes studied (data not shown), the biological decolourisation curve fitted equation [3.1] rather well. However, when chemical azo dye reduction was followed in time, many of the azo dyes studied decolourised with a deviation from first-order kinetics. As illustrated in Figure 3.2b, a kind of lag phase could be observed: the reaction rates were initially slow but accelerated in time according to the extent to which the dye was reduced. Based on these observations, it was hypothesised that products of azo dye reduction may increase the rate of the reduction process, i.e. the

reaction has an autocatalytic nature. Mathematically, this phenomenon can be described by expanding equation [3.1] with a second part that expresses the contribution of autocatalysis as a function of the reduced dye concentration (equation [3.2]). During the course of the reduction process, equations [3.3] and [3.4] describe respectively the concentration of directly reduced dye and the concentration of dye reduced via autocatalysis can now be calculated.

$$\frac{dA}{dt} = -k \cdot A_t \quad \Rightarrow \quad A_t = A_0 \cdot e^{-kt} \qquad [3.1]$$

$$\frac{dA}{dt} = -k_1 \cdot A_t - k_2 \cdot A_t \cdot (A_0 - A_t) \Rightarrow A_t = \frac{A_0 \cdot (k_2 \cdot A_0 + k_1)}{k_1 \cdot e^{(k_2 \cdot t \cdot A_0 + k_1 \cdot t)} + k_2 \cdot A_0} \qquad [3.2]$$

$$N_{1,t} = \frac{k_1}{k_2} \cdot \left[(k_2 \cdot A_0 + k_1) \cdot t - \ln\left(\frac{A_0}{A_t}\right) \right] \qquad [3.3]$$

$$N_{2,t} = (1 - k_1 \cdot t) \cdot A_0 - A_t - \frac{{k_1}^2}{k_2} - \frac{k_1}{k_2} \ln\left(\frac{A_t}{A_0}\right) \qquad [3.4]$$

where: k = first-order rate constant (d^{-1});

A_t = Absorbance at time t; A_0 = Absorbance at the start of the experiment;

k_1 = first-order rate constant for the direct chemical reaction (d^{-1});

k_2 = second-order rate constant for the autocatalytic reaction ($d^{-1} \cdot$ absorbance unit^{-1});

$N_{1,t}$ = concentration of directly reduced dye at time t;

$N_{2,t}$ = concentration of dye reduced via autocatalysis at time t.

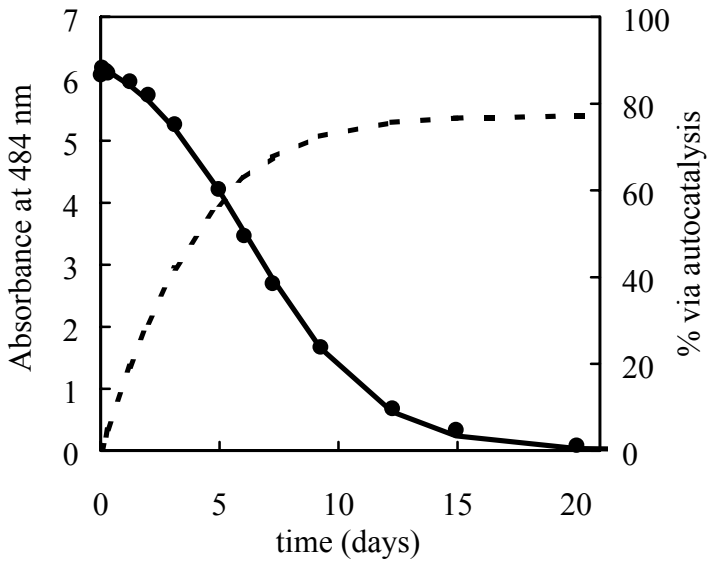


Figure 3.3 Chemical decolourisation of AO7 by sulphide (initial concentrations respectively 0.3 and 1.0 mM):
experimental data (full circles); fit equation 2 (full line); relative contribution of autocatalysis, calculated from equations 3 and 4 as $N_2/(N_1+N_2)\bullet 100\%$ (dashed line)

When applying equation [3.2] to describe the chemical decolourisation data, the curves fitted perfectly (Figure 3.3), thereby supporting the hypothesis of autocatalytic azo dye reduction. From the ratio $N_2/(N_1+N_2)$ depicted in Figure 3.2 it is also clear that, under the experimental conditions applied, autocatalysis contributed to a large extent (up to ~80%) to the complete reduction of AO7. In this example, 14 days are required to reach 95% decolourisation. Without autocatalysis (k_2=0) this would have taken more than 90 days.

As quinones have been reported to act as mediators in the transfer of reducing equivalents to azo dyes [23, 161], it may be assumed that autocatalysis will occur if quinone compounds are products of the azo dye reduction process. This applies for many dyes: Reduction of AO7, for instance, yields sulphanilic acid and 1-amino-2-naphthol. The latter compound, by being in equilibrium with its amino quinone (Figure 3.4), may possess redox mediating properties and thus induces autocatalysis during azo dye reduction.

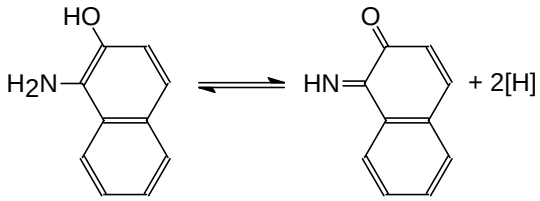


Figure 3.4 Equilibrium between 1-amino-2-naphthol and its amino quinone

In order to test this hypothesis, the effect of the aromatic amine constituents of AO7 on its chemical decolourisation was tested. The results (Figure 3.5) show that the reduction rate was indeed considerably increased by 1-amino-2-naphthol. In contrast, sulphanilic acid had no effect and also the colour of a non-reducing solution of the dye and 1.1 mM 1-amino-2-naphthol remained stable during the course of the experiment. Notwithstanding the impurities, the poor solubility and the instability of

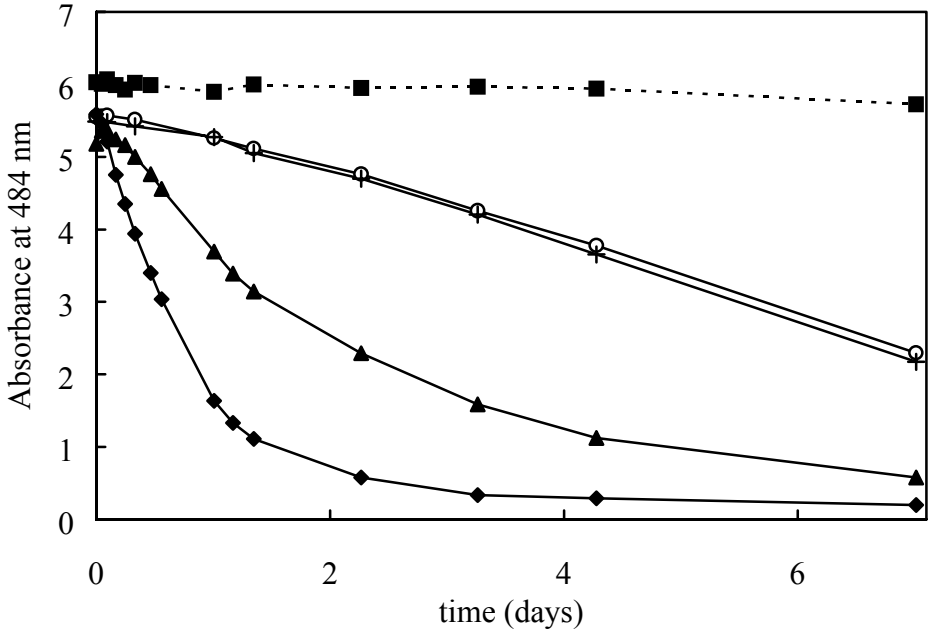


Figure 3.5 Decolourisation of AO7 (0.27 mM) by sulphide (0.6 mM):
no additives (open circles); 0.1 mM 1-amino-2-naphthol (full triangles); 1.0 mM 1-amino-2-naphthol (full diamonds); 0.3 mM sulphanilic acid (plusses).
The dotted line is a control with 1.1 mM 1-amino-2-naphthol and no sulphide (full squares)

the chemical added (1-amino-2-naphthol hydrochloride powder with a purity of 90%), these results are an indication for the mediating properties of 1-amino-2-naphthol and support the dye reduction autocatalysis hypothesis.

Additional experiments revealed comparable results for the decolourisation of many other azo dyes (data not shown). As a consequence of autocatalysis, effluent recycling may be a successful method to raise the colour removal efficiency of anaerobic reactors treating azo dye containing wastewater.

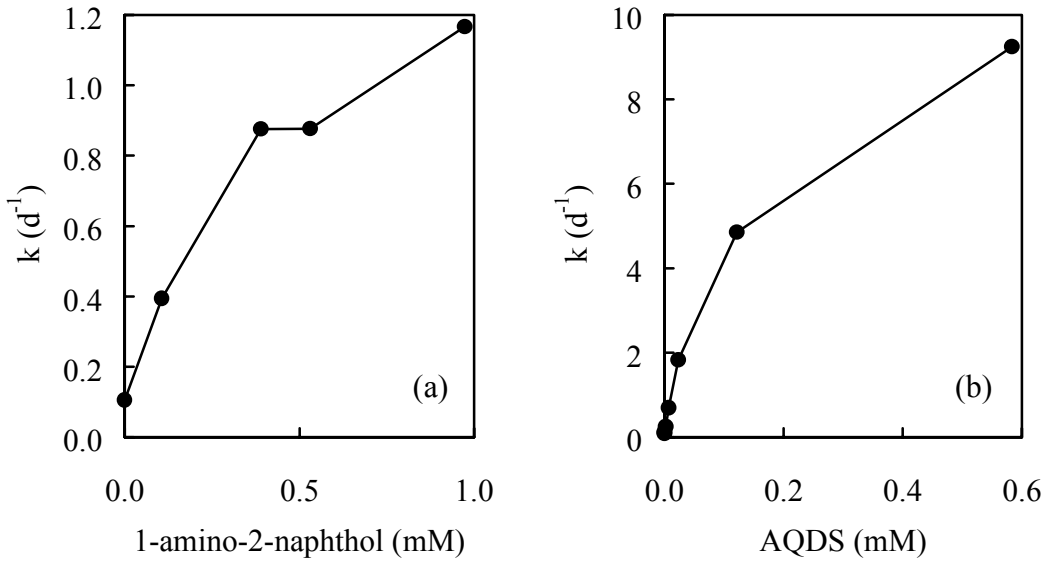


Figure 3.6 The effect of 1-amino-2-naphthol (a) and AQDS (b) to the decolourisation rate of AO7 in the presence of sulphide (initial sulphide concentration 0.6-0.7 mM; initial dye concentration 0.25-0.30 mM)

When first-order kinetics (equation [3.1]) were used to approach the overall rate constants of the AO7 decolourisation curves at different 1-amino-2-naphthol concentrations, the k-value was found to increase over 10-fold, from 0.09 d^{-1} to 1.2 d^{-1} at, respectively, 0 and 1.0 mM 1-amino-2-naphthol (Figure 3.6a). Another mediating compound is anthraquinone-2,6-sulphonic acid (AQDS). AQDS and related compounds have been reported to speed up the rate of azo dye reduction, both in a biological process [161] and in an electrochemical process [23]. In this study, the effect of different AQDS concentrations on the reduction of AO7 by sulphide was investigated. Figure 3.6b shows that the reaction rate was greatly increased by AQDS. In comparison with 1-amino-2-naphthol, AQDS is by far a better mediator, as only about 10 µM of mediator was required to reach a 10-fold increase of the reduction rate. These data, in combination with the finding that AQDS functioned similarly in the presence of sludge (e.g. Chapter 4) offer a promising method to increase the colour removal efficiencies of anaerobic reactors treating azo dye containing wastewater by introducing minor quantities (micromols) of AQDS.

As was mentioned before, azo dye reduction rates are higher in the presence of sludge than at comparable sulphide concentrations in the absence of sludge (Figure 3.2). It is obvious, therefore, that anaerobic biomass plays a role in the overall azo dye reduction process in biotic anaerobic environments. As sludge organic matter contains quinone structures, the biomass, apart from being directly involved in the azo dye reduction process by producing reducing equivalents, may as well

contribute to the process by mediating the transfer of reducing equivalents. The observation that the decolourisation rate increased with increasing concentrations of autoclaved anaerobic granular sludge (Figure 3.7) is an indication for the mediating properties of sludge organic matter. The extent to which the decolourisation rates raise when comparing living and autoclaved sludge at a certain VSS concentration can be looked at as a measure for the contribution of biological activity to the overall azo dye reduction process. The exact role of biological activity will be the subject of further investigations (e.g. Chapter 4).

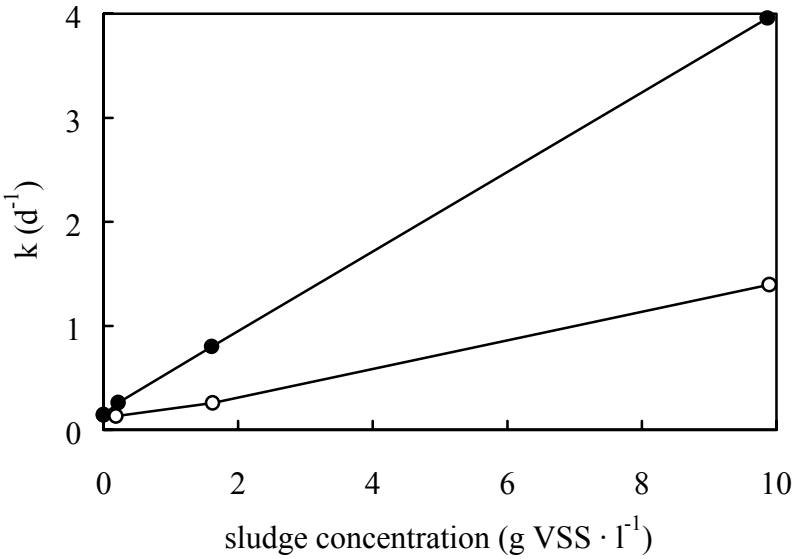


Figure 3.7 The effect of living sludge (full circles) and autoclaved sludge (open circles) to the decolourisation rate of AO7 (initial sulphide concentration 0.3-1.5 mM; initial dye concentration 0.25-0.30 mM)

4

Biotic and abiotic processes

of azo dye reduction in anaerobic sludge

Abstract Azo dye reduction results from a combination of biotic and abiotic processes during the anaerobic treatment of dye containing effluents. Biotic processes are due to enzymatic reactions whereas the chemical reaction is due to sulphide. In this research, the relative impact of the different azo dye reduction mechanisms was determined by investigating the reduction of Acid Orange 7 (AO7) and Reactive Red 2 (RR2) under different conditions. Azo dye reduction rates were compared in batch assays over a range of sulphide concentrations in the absence or presence of living or inactivated anaerobic granular sludge. Biological dye reduction followed zero order kinetics and chemical dye reduction followed 2nd order rate kinetics as a function of sulphide and dye concentration. Chemical reduction of the dyes was greatly stimulated in the presence of autoclaved sludge; whereas chemical dye reduction was not affected by living or γ-irradiated-sludge. Presumably redox mediating enzyme cofactors released by cell lysis contributed to the stimulatory effect. This hypothesis was confirmed in assays evaluating the chemical reduction of AO7 utilizing riboflavin, representative of the heat stable redox-mediating moieties of common occurring flavin enzyme cofactors. Sulphate influenced dye reduction in accordance to biogenic sulphide formation from sulphate reduction. In assays lacking sulphur compounds, dye reduction only readily occurred in the presence of living granular sludge, demonstrating the importance of enzymatic mechanisms. Both chemical and biological mechanisms of dye reduction were greatly stimulated by the addition of the redox-mediating compound, anthraquinone-disulphonate. Based on an analysis of the kinetics and demonstration in lab-scale upward-flow anaerobic sludge bed reactors, the relative importance of chemical dye reduction mechanisms in high rate anaerobic bioreactors was shown to be small due to the high biomass levels in the reactors.

Van der Zee, F.P., Bisschops, I.A.E., Blanchard, V.G., Bouwman, R.H.M., Lettinga, G. and Field, J.A. (2002) *Submitted*

4.1 Introduction

Removal of dyes is a major concern when treating textile-processing wastewater. The vast majority (60-70%) of the dyes applied in textile-processing industries are azo compounds, i.e. molecules with one or several azo (N=N) bridges linking substituted aromatic structures. Azo dyes are generally persistent under aerobic conditions [299]. However, under anaerobic conditions, they undergo reductive fission, yielding colourless aromatic amines, compounds that in turn generally require aerobic conditions for their biodegradation [35]. Anaerobic azo dye reduction as the first stage in the complete anaerobic-aerobic degradation of azo dyes has been studied intensively and most researchers agree that it is a non-specific and presumably extracellular process, in which reducing equivalents from either biological or chemical source are transferred to the dye. Azo dye reduction can result from a biological process, either as a direct enzymatic reaction or a reaction mediated by biologically regenerated enzyme cofactors or other electron carriers [310]. Moreover, azo dye reduction can result from purely chemical reactions with bulk reductants like sulphide (Chapters 2 and 3). Both biological and chemical azo dye reduction mechanisms have been shown to be greatly accelerated with the addition of redox mediating compounds like anthraquinone-sulphonate (AQS) and anthraquinone disulphonate (AQDS) [161] (Chapters 3 and 5). Azo dye reduction in anaerobic sludge environments therefore must be considered as a combination of biotic and abiotic processes. Dye-containing wastewaters, e.g. textile-processing wastewater, usually contain moderate to high sulphate concentrations. Sulphate is often an additive of dyebaths or it is formed by the oxidation of more reduced sulphur species used in dyeing processes, such as sulphide, hydrosulphite and dithionite. Sulphate also results from neutralisation of alkaline dye effluents with sulphuric acid. Sulphide is therefore a relevant compound, as it will be generated by sulphate reducing bacteria during treatment of these wastewaters in anaerobic bioreactors. The role of sulphur compounds is expected to be important. On the one hand it has been suggested that anaerobic azo dye reduction is merely due to a reaction between the dye and sulphide generated by sulphate reducing bacteria [180]. However, sulphate could have a double role. Aside from being the precursor of the bulk reductant, sulphide, it may also compete with the dye as an electron acceptor.

In this research, the relative contribution of biotic and abiotic azo dye reduction mechanisms was investigated with respect to the role of sulphur compounds. Azo dye reduction rates were compared in batch assays over a range of sulphide concentrations in the presence of living or inactivated anaerobic granular sludge. Additionally, dye decolourisation was evaluated in the presence and absence of sulphate.

4.2 Materials and methods

4.2.1 Reaction stoichiometry AO7 reduction by sulphide

The reaction stoichiometry of direct chemical azo dye reduction by sulphide was investigated in 250 ml serum bottles completely filled with liquid and buffered at pH 8.2 to minimise gaseous H_2S. AO7 was added to the assay at 18.6 h to a concentration of 0.5 mM, at which time the total-sulphide:AO7 ratios were 0.9:0.5 and 2.0:0.5. Dye concentration (based on visible absorbance) and total liquid-phase sulphide concentrations were monitored in time.

4.2.2 Reduction of AO7 in a sulphide gradient

Batch experiments at different sulphide concentrations (up to 10 mM) were performed to test the effect of sulphide on the biological and chemical reduction of 0.5 mM of the azo dye Acid Orange 7 (AO7). The tests were performed in the absence of sludge and in the presence of either living or deactivated (autoclaved or γ-irradiated) anaerobic granular sludge (1.8 g VSS l^{-1}), both in the absence and in the presence of the redox mediator AQDS (20 μM). All experiments were conducted in 117 ml glass serum vials.

Vials for the experiments with sludge were filled with 50 ml of a 60 mM $NaHCO_3$ solution in basal medium containing (mg l^{-1}) NH_4Cl (280), $CaCl_2$ (5.7), KH_2PO_4 (250), $MgSO_4 \cdot 7H_2O$ (100), H_3BO_3 (0.05), $FeCl_2 \cdot 4H_2O$ (2), $ZnCl_2$ (0.05), $MnCl_2 \cdot 4H_2O$ (0.5), $CuCl_2 \cdot 2H_2O$ (0.04), $(NH_4)_6Mo_7O_{24} \cdot 5H_2O$ (0.05), $CoCl_2 \cdot 6H_2O$ (1), $NiCl_2 \cdot 6H_2O$ (1) and $Na_2SeO_3 \cdot 5H_2O$ (0.16). Granular sludge, harvested from a lab-scale UASB-reactor treating Reactive Red 2 (RR2) in basal medium with a volatile fatty acid (VFA) mixture as the electron-donating substrate, was rinsed thoroughly with tap water and added to the vials. In the series with living sludge and autoclaved sludge in the presence of AQDS, AQDS was now added by pipette from a NaOH neutralised concentrated stock solution. Next, the vials were sealed with butyl rubber stoppers and the gas headspace was flushed for 5 minutes with oxygen-free flush gas (N_2:CO_2 70%:30%). Organic primary electron donor (a VFA mixture containing acetate, propionate and butyrate in a COD based ratio of 1:1:1) was added up to a concentration of 1 g COD l^{-1} with a syringe from a NaOH neutralised concentrated stock solution. Sulphide at different doses was added with a syringe from partly neutralised (1 M HCl per M Na_2S) stock solutions. Prior to further treatment, all vials with sludge were pre-incubated for 3 days in a rotary shaker at 30 °C. After pre-incubation, the vials of the

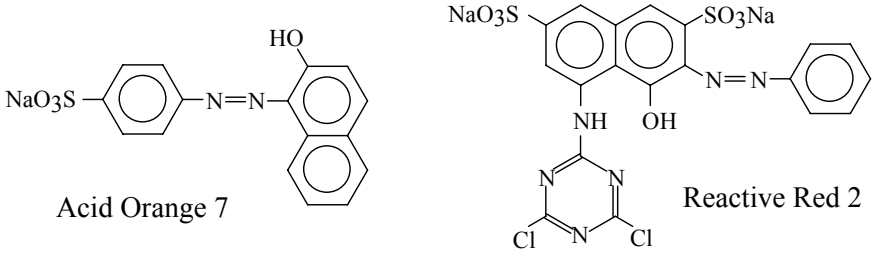


Figure 4.1 Structure formulas of the dyes used in this study

living sludge series were flushed again and a new load of VFA (up to a concentration of 3 g COD l^{-1}) was added. Next, AO7 was injected. Vials of the autoclaved sludge series were cooked for 90 minutes at 120 °C and allowed to cool down before dye injection. Vials of the γ-irradiated sludge series were irradiated at Gammaster b.v., Ede, The Netherlands (gamma irradiation with Co^{60}; at a dose of 25 kGray). After irradiation, AO7 and (for the experiment in the presence of AQDS) AQDS were added to the vials.

Vials for the experiments without sludge were filled with 50 ml of a 60 mM $NaHCO_3$ solution. AQDS was added with a pipette from a NaOH neutralised concentrated stock solution. Next, the vials were sealed with butyl rubber stoppers and the gas headspace was flushed for 5 minutes with oxygen-free flush gas (N_2:CO_2 70%:30%). Sulphide at different doses was added with a syringe from partly neutralised (1 mol HCl per mol Na_2S) stock solutions. Prior to dye injection, the vials were pre-incubated for at least 1 day to assure complete removal of oxygen.

All vials were incubated at 30 °C in a rotary shaker at 50 rpm. At selected intervals, colour was measured spectrophotometrically at 484 nm, the wavelength of maximum absorbance (λ_{max}) of AO7. Sulphide was measured at the start of the experiments.

4.2.3 Reduction of RR2 in a sulphide gradient

Prior to the experiments with AO7, similar experiments were conducted with Reactive Red 2. The initial dye concentration in these experiments was 0.2 - 0.3 mM, the sludge concentration was 2.0 g VSS l^{-1} and the sulphide concentrations ranged up to 25 mM. The main difference between the two series is that γ-irradiated sludge has only been tested in the AO7 series. The experimental procedure was for the rest the same, except that the pre-incubation period for the batch vials with sludge was only 1 instead of 3 days. At selected intervals, colour was measured spectrophotometrically at 539 nm, the λ_{max} of RR2.

Additionally, to determine whether the oxidised sulphur species formed in the reaction between RR2 and sulphide could be biologically recovered, the vials used for the assay of dye reduction in the absence of sludge were supplemented with a small amount of sludge and VFA (to concentrations of 0.2 g VSS l^{-1} and 1 g COD l^{-1}, respectively). At intervals, sulphide was measured.

4.2.4 Reduction of RR2 in a sulphate gradient

Batch experiments at different sulphate concentrations (up to 60 mM) were performed to test the effect of sulphate on the biological and chemical decolourisation of RR2 (0.25 mM) by anaerobic granular sludge (2.0 g VSS l^{-1}). The tests were performed in the absence and in the presence of the redox mediator AQDS (20 μM). Batch vials were prepared similarly to the vials with living sludge in the sulphide gradient experiment. Sulphate was added together with the dye, after a one-day pre-incubation period. Colour and sulphide were measured at intervals.

4.2.5 Riboflavin (and AQDS) as redox mediators of AO7 reduction by sulphide

A small batch experiment was performed to test whether riboflavin could stimulate chemical azo dye reduction. AQDS was tested as well, to be able to compare the mediating properties of both compounds. A control without redox mediator was incorporated. The batch vials were prepared similarly to the vials without sludge in the sulphide gradient experiment. The initial sulphide concentration in all vials was ~1.2 mM. The concentration of riboflavin and AQDS was 20 µM and AO7 was added to a concentration of 0.3 mM. Colour was measured at intervals. The experiment was performed with triplicate vials.

4.2.6 Reactor study

Two lab-scale UASB reactors (wet volume 0.25 l) with anaerobic granular sludge (35 g VSS l^{-1}) were fed with pre-hydrolysed RR2 (100 mg/l ≈ 0.054 mM) and a neutralised VFA mixture (1.5 g COD l^{-1} at a 1:1:1 COD based rate of acetate, propionate and butyrate) in basal medium. RR2 was previously hydrolysed (i.e. the chloro groups were replaced by hydroxyl groups) to prevent dye toxicity (Chapter 5). For that purpose, the dye was heated in a Na_2CO_3 solution; a treatment that does not affect the dye's reduction rates [25]. The hydraulic retention times of both reactors were kept constant at 5-5.5 h. Effluent was recycled at a 1:1 influent:effluent flow ratio. After a 14-days period (Period I) in which both reactors were operated identically, Na_2SO_4 was added to the influent of one of the reactors (R_S): 0.7 mM in Period II (days 14 – 25) and 3.5 mM in Period III (days 25 – 42). The other reactor (R_0) was the control reactor that did not receive any sulphate during the entire operational period. The reactor's dye decolourisation and VFA removal efficiencies were monitored on a daily to sub-daily basis.

4.2.7 Analysis

AO7 and RR2 colour was measured spectrophotometrically with a Spectronics 60 spectrophotometer (Milton Ray Analytical Products Division, Belgium) at the dyes' wavelengths of maximum absorbance (484 nm and 539 nm, respectively). The estimated molar extinction coefficients at these wavelengths are $22.9 \cdot 10^3$ and $38 \cdot 10^3$ cm^{-1} M^{-1} for AO7 and RR2 respectively. Liquid phase samples (0.75 ml) were centrifuged (2 minutes at 10,000 rpm) and diluted up to an absorbance of less than 1 in a 0.1 M phosphate buffer. The buffer contained freshly added ascorbic acid (200 mg l^{-1}) to prevent autoxidation. Without dye, light absorbance of medium and buffer was less than 1% of the absorbance right after dye addition and could therefore be neglected.

Sulphide was determined colorimetrically after reaction with *N,N*-dimethyl-*p*-phenylenediamine oxalate according the method described by Trüper and Schlegel [328]. VFA (Volatile Fatty Acids) were determined by gas chromatography. The chromatograph (Hewlett Packard 5890) was equipped with a 2m x 2mm glass column packed with Supelcoport (100-120 mesh) coated with 10% Fluorad FC 431. The temperatures of the column, injection port and flame ionisation unit were respectively 130, 200 and 280 °C. The carrier gas was nitrogen saturated with formic acid (40 ml per minute). Samples were centrifuged (3 minutes at 10,000 rpm) and diluted 1:1 in a 3% formic acid solution. The pH was determined with a Kinck 511 pH meter (Berlin, Germany) and a Schott Geräte N32A double electrode

(Hofheim, Germany). Volatile Suspended Solids (VSS) were determined according to standard methods[12].

4.3 Results

4.3.1 Reaction stoichiometry dye reduction by sulphide

The reaction stoichiometry of direct chemical azo dye reduction by sulphide was investigated at two different molar total-sulphide:AO7 ratios, 1.8:1 and 4:1(Figure 4.2A and 2B, respectively). Figure 4.2 shows the simultaneous decline of the azo dye and sulphide concentrations. In Figure 4.2A, sulphide was limiting and the azo dye reduction stopped when sulphide was exhausted. In Figure 4.2B, AO7 was limiting and the decline in sulphide concentration ceased when AO7 was completely reduced. In the absence of sulphide, no reaction occurred with the dye. In the dye-free controls, there was only a small loss of sulphide.

The stoichiometry of the reaction, calculated at both total-sulphide:AO7 ratios and corrected for the slight spontaneous disappearance of sulphide, was 2.16 ± 0.1 mol of sulphide per mol of dye reduced.

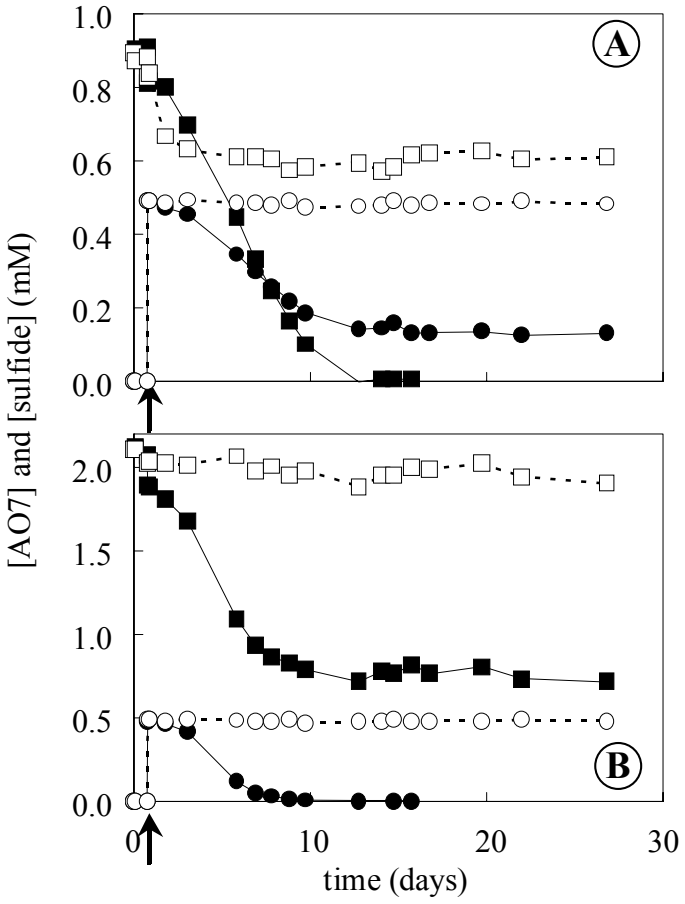


Figure 4.2 Time courses of the reactants during chemical reduction of AO7 (full circles) by sulfide (full squares). (A) Initial molar total-sulfide:AO7 ratio 0.9:0.5. (B) Initial molar total-sulfide:AO7 ratio 2.0:0.5. Open circles present the AO7 concentration in controls without sulfide. Open squares present the total-sulfide concentration in controls without dye. The arrows indicate the moment of dye addition.

4.3.2 Effect of sulphide gradient on AO7 reduction

Batch experiments at different sulphide concentrations (up to 10 mM) were performed to test the effect of sulphide on the biological and chemical reduction of AO7 (0.57 mM). The tests were performed in the absence of sludge (medium only) and in the presence of either living or deactivated (autoclaved or γ-irradiated) anaerobic granular sludge. All conditions were tested both in the absence and in the presence of 20 μM of the external redox mediator anthraquinone-2,6-disulphonate (AQDS).

4.3.2.1 Results without external redox mediator

No exogenous sulphide. Firstly, the results will be discussed in the absence of exogenous sulphide addition, where the background sulphide concentration was <0.01 mM. In this case, dye reduction did not occur in the absence of sludge, demonstrating the stability of the dye solutions in bicarbonate buffered medium. Likewise, no dye elimination occurred in the presence of autoclaved sludge, demonstrating that dye sorption or reduction by deactivated sludge biomass can be neglected. With living sludge, dye reduction was complete by day 8, which indicates that dye reduction in the absence of sulphide was a biological process. With γ-irradiated sludge, dye reduction was very slow and eventually became complete after 30 days. The activity of irradiated sludge suggests that γ-irradiation

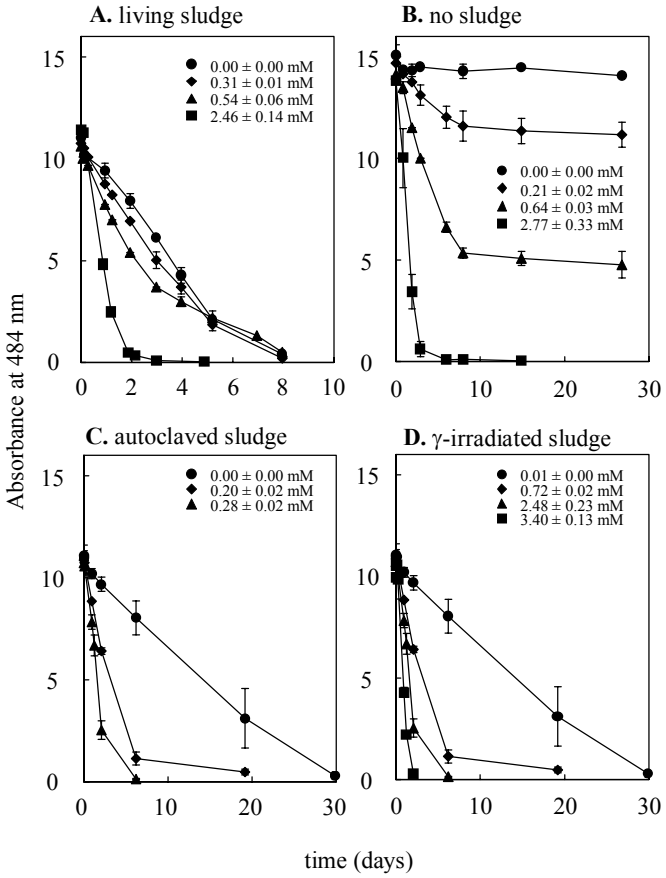


Figure 4.3 AO7 decolorization curves at different sulfide concentrations. **A.** in the presence of living sludge, **B.** in the absence of sludge (medium only), **C.** in the presence of autoclaved sludge and **D.** in the presence of γ-irradiated sludge. The concentrations listed in the legends are the initial total-sulfide concentrations. The error bars represent standard deviations.

did not completely kill all the bacteria or spores in the granular sludge. Nonetheless, no methane was formed, suggesting complete death to methanogens.

Substoichiometric sulphide concentrations. Secondly, the results will be discussed for assays where sub-stoichiometric quantities of sulphide (<1 mM) were added (Figure 4.3). In this case, dye decolourisation was incomplete in the absence of sludge or in the presence of autoclaved sludge. The extent of dye reduction increased with increasing initial sulphide concentrations (Figures 3B and 3C). The biological dye reduction by living sludge did not depend on the presence of sulphide. Up to concentrations of 0.5 mM, exogenous sulphide had no measurable impact on dye reduction by living sludge (Figure 4.3A + insert in Figure 4.4A). Only when the exogenous sulphide level was increased beyond 0.5 mM, was there a noticeable increase in the dye reduction rate due to the contribution of the chemical reduction processes in the living sludge. With γ-irradiated sludge, only one exogenous sulphide concentration was tested in the substoichiometric range. At this concentration (0.7 mM), dye reduction proceeded considerably faster than in the absence of exogenous sulphide (Figure 4.3D).

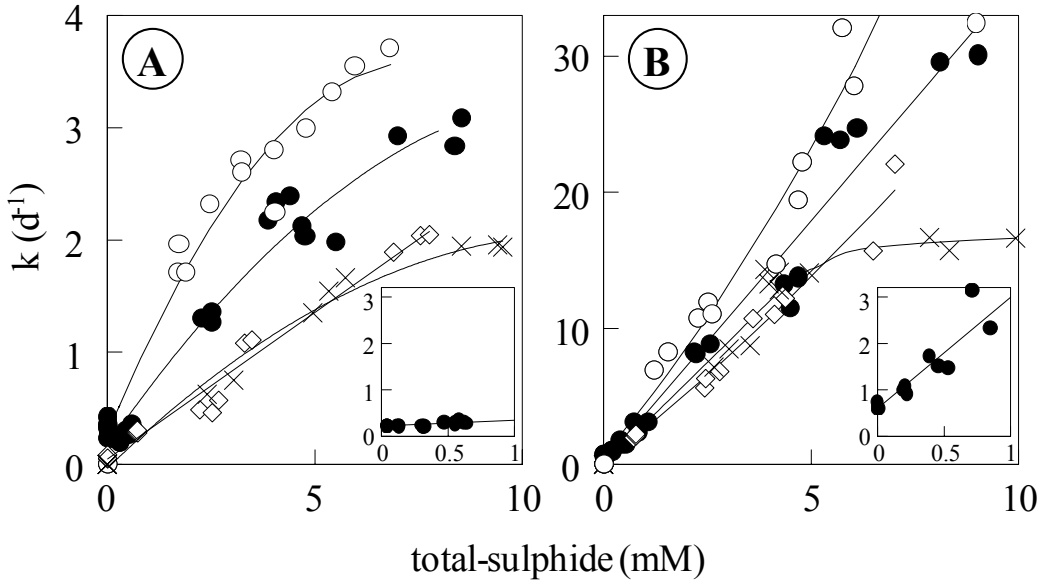


Figure 4.4 AO7 reduction rate constants at different sulphide concentrations, in the presence of living sludge (closed circles), in the absence of sludge (crosses), in the presence of autoclaved sludge (open circles) and in the presence of γ-irradiated sludge (open diamonds); in the absence of AQDS (Figures **A**) and in the presence of 20 μM AQDS (Figures **B**). The inserts show the rate constants for AO7 reduction by living sludge at low sulphide concentrations.

Excess of sulphide concentrations. Thirdly, the results will be discussed at high initial sulphide concentrations in large excess of stoichiometric amounts. In this case, complete dye reduction was achieved under all circumstances. A fairly linear relationship between the sulphide concentration and the estimated pseudo first-order reaction rate constant was observed up to 5 mM sulphide. At higher sulphide concentrations, the rate constants levelled off somewhat, especially in the case of the medium without sludge (Figure 4.4A). The reduction rates differed depending on the presence of sludge and on the quality (i.e. living, autoclaved or γ-irradiated) of the sludge. The reduction rate constants were the highest in the presence of autoclaved sludge, while the rate constants were distinctively lower for living sludge. The lowest rates were obtained with direct chemical reduction in medium without

sludge and these rates were the same as those obtained with γ-irradiated sludge. Generally, a ratio of 3:2:1:1 was observed for the estimated pseudo first-order reaction rate constants in the presence of autoclaved sludge, living sludge, γ-irradiated sludge and medium without sludge, respectively, at any given sulphide concentration in excess of stoichiometric amounts.

4.3.2.2 Results with the external mediator AQDS

Redox mediators are compounds capable of accelerating oxidation-reduction reactions by shuttling electrons between their reduced and oxidised forms. AQDS (20 μM) was chosen to determine the effect of a redox mediator on the reduction of AO7 over a gradient of sulphide concentrations. The experiment was set-up similar to that without AQDS, since dye reduction was monitored in the presence of sludge (living or deactivated) or in the absence of sludge. In addition to this experiment, experiments were conducted to verify whether AQDS could be chemically reduced by sulphide. In these experiments (data not shown), AH_2QDS formation was observed, based on spectrophotometric measurements at 450 nm. Also AQDS reduction resulted in the oxidation of sulphide as was confirmed based on the disappearance of sulphide.

The results of the sulphide gradient on AO7 reduction in the presence of AQDS (Figure 4.4B) showed that, similar to the results of the experiment without AQDS, complete azo dye reduction at sub-stoichiometric sulphide concentrations was only achieved in the assays with living sludge (and in the assays with γ-irradiated sludge after extended time periods). The reduction of AO7 by living sludge in the absence of sulphide proceeded 2.5-fold faster in the presence of AQDS, indicating acceleration of biological dye reduction by AQDS. AQDS also mediated the purely chemical reduction of AO7 by sulphide in medium without sludge. Dye reduction was always remarkably faster, even at substoichiometric sulphide concentrations with AQDS. The final level of decolourisation was more rapidly achieved. Therefore, when sulphide was included together with living sludge, the effect of the chemical reaction was noticeable. The pseudo first-order reaction rate constant increased beyond the

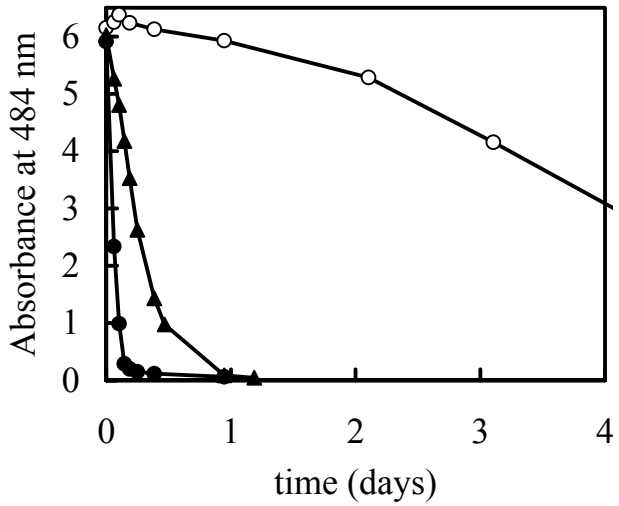


Figure 4.5 Reduction of AO7 (0.3 mM) by sulphide (1.2 mM) in the presence of 20 μM riboflavin (full circles), in the presence of 20 μM AQDS (full triangles) and in the absence of an external redox mediator (open circles).

basal biological rates proportionally with the sulphide concentration, even at very low concentrations below 0.5 mM (insert in Figure 4.4B).

At higher sulphide concentrations, all reaction rate constants increased with increasing sulphide concentrations (Figure 4.4B). The trends with respect to the presence of sludge and its inactivation were similar to those observed previously without AQDS. However, the rate constants were generally 7 to 12 times higher in the presence as compared to in the absence of AQDS.

4.3.2.3 Riboflavin as a mediator of chemical azo dye reduction

The result that autoclaved sludge stimulates chemical AO7 reduction by sulphide indicates that heat stable cofactors released by cell lysis contribute as mediators of the reaction. Riboflavin is an example of a redox active heat stable moiety present in common-occurring flavin enzyme cofactors of most cells. Therefore, riboflavin was tested as a mediator of the chemical reduction of AO7 by sulphide. A batch experiment was performed in which the reduction of AO7 by sulphide in the presence of substoichiometric concentrations of either riboflavin or AQDS was monitored in time by following dye decolourisation. The results presented in Figure 4.5 indicate that riboflavin greatly accelerates the rate of AO7 reduction and that it is a far superior redox mediator compared to AQDS.

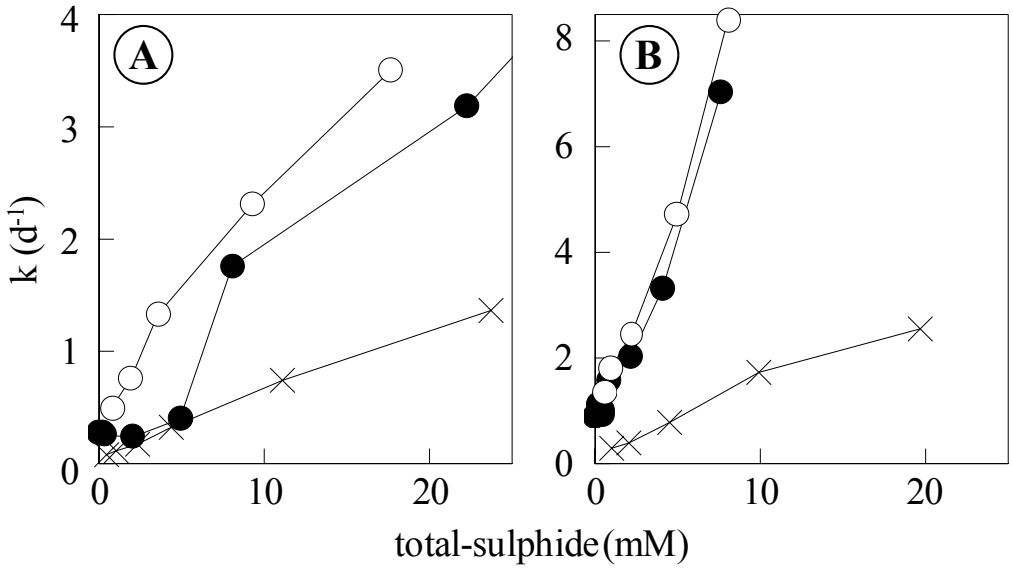


Figure 4.6 RR2 reduction rate constants at different sulfide concentrations, in the presence of living sludge (closed circles), in the absence of sludge (crosses), in the presence of autoclaved sludge (open circles, full curve) and in the presence of γ-irradiated sludge. **A.** in the absence of AQDS and **B.** in the presence of 20 μM AQDS.

4.3.3 Effect of sulphide gradient on RR2 reduction

Similar to the AO7 reduction test at different sulphide concentrations, a series of batch experiment was performed to test the effect of sulphide (up to 25 mM) on the biological and chemical reduction of the more complex azo dye, RR2 (supplied at 0.25 mM). The tests were performed in the absence of sludge and in the presence of either living or autoclaved anaerobic granular sludge. All these conditions were tested both in the absence and in the presence of 20 μM of the external redox mediator, AQDS.

The results of the experiments in a large range of sulphide concentrations are presented in Figure 4.6. The pseudo first-order rate constants of RR2 reduction were always lower than those observed for AO7 at comparable conditions. The general trends observed in the RR2 experiments were mostly similar to those of the AO7 experiments. Namely, at very low sulphide levels only biological reduction of the azo dye occurred, while in the presence of high sulphide levels, sludge greatly accelerated the rate of RR2 reduction by sulphide and autoclaved sludge provided even higher rate constants than living sludge.

In the additional experiment in which sludge and VFA were added to the vials used in the assay of chemical RR2 reduction in the absence of sludge it was observed (data not shown) that the sulphide concentrations increased up to their original value, thereby indicating that the oxidised sulphide could be biologically recovered.

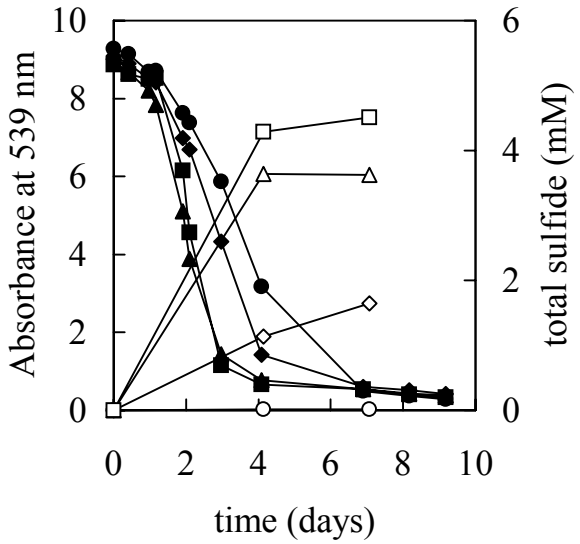


Figure 4.7 Decolourisation of 0.24 mM RR2 (closed symbols) and sulphate reduction (open symbols) by living sludge at different initial sulphate concentrations: 0.0 mM (circles), 2.0 mM (diamonds), 3.5 mM (triangles) and 30.6 mM (squares)

4.3.4 Effect of sulphate

4.3.4.1 Sulphate gradient RR2 (batch)

Similar to the RR2 reduction test at different sulphide concentrations, a series of batch experiments was performed to test the effect of sulphate (at 8 initial concentrations ranging from 0 to 58 mM) on the reduction of the azo dye RR2. The tests were performed both in the absence and in the presence of 20 μM of the external redox mediator AQDS. Examples of the time course of RR2 decolourisation and the formation of sulphide are shown in Figure 4.7. The figure illustrates that dye reduction and sulphate reduction occurred simultaneously. Biogenic sulphide contributed to chemical reduction, thereby increasing the observed rates of dye reduction. Thus dye reduction was faster in the assays with sulphate. At initial sulphate concentrations higher than 5 mM, all decolourisation rates were similar since higher sulphate levels did not correspond to increased levels of biogenic sulphide beyond

the maximum of 5 mM formed during the 5 to 7 days of incubation. The sulphate reduction was limited due to exhaustion of the electron donating VFA substrate.

4.3.4.2 Effect of sulphate on RR2 reduction in a continuous bioreactor

Since the batch experiments were conducted with a low amount of sludge (2 g VSS · l^{-1}), the effect of biogenic sulphide on anaerobic RR2 reduction was also tested in continuous bioreactors with a biomass concentration of 35 g VSS · l^{-1}. Two 250-ml lab-scale UASB reactors were maintained for 42 days. Both reactors were fed with a VFA mixture as the primary electron donor and pre-hydrolysed RR2 was added to the reactor influent at a concentration of 100 mg/l (≈ 0.057 mM). After a 14-days period (Period I) in which both reactors were operated identically, sulphate was added to the influent of one of the reactors (R_S) in periods II and III. The sulphate concentration was 0.7 mM in Period II and 3.5 mM in Period III. The other reactor (R_0) was the control reactor that did not receive any sulphate during the entire operational period.

The VFA removal efficiencies of both reactors were stable at high levels (>95%). The removal of sulphate by R_S was generally complete with the effluent sulphate concentration of 0.04 mM or less. Roughly 80-90% of the removed sulphate-S was recovered as soluble total-sulphide-S. The influent sulphate concentration of R_0 was below 0.02 mM and the sulphide levels in R_0 were below detection level (0.01 mM).

The results with respect to the removal of colour (reduction of RR2) are summarised in Table 4.1. It is clear that there is no significant difference between the two reactors. There was no obvious effect of biogenic sulphide on the reduction of RR2 in the continuous UASB-reactor.

Table 4.1 Overall results of the reactor experiment

period	#days	R_S influent-[Na_2SO_4] mM	R_S effluent-[sulphide] mM (± st.dev)	Colour removal R_S % (± st.dev.)	Colour removal R_0 % (± st.dev.)
I	14	0	0	35 ± 3	33 ± 3
II	11	0.7	0.61 ± 0.04	34 ± 3	32 ± 1
III	17	3.5	2.89 ± 0.08	36 ± 3	36 ± 2
total	42			35 ± 3	33 ± 3

4.4 Discussion

Anaerobic azo dye reduction (decolourisation) in anaerobic sludge environments is a combined process of biotic and abiotic reactions. Azo dyes can be reduced in a direct chemical reaction with bulk biogenic reducing agents (e.g. sulphide), but they can also be reduced by biological reactions, either directly as an enzymatically catalysed reaction or indirectly via reduced enzyme cofactors (Figure 4.8).

In this study, an attempt was made to assess the relative contribution of chemical versus biological azo dye reduction in a sulphide-containing anaerobic sludge environment. Azo dye reduction rates in sludge-free medium were compared to those in the presence of anaerobic granular sludge at different

sulphide concentrations. Controls with deactivated (autoclaved or γ-irradiated) sludge were used to determine the effect of sludge material and biochemicals.

Azo dyes were observed to be reduced by direct chemical reaction with sulphide as well as in a sulphide-independent biological reaction. The effect of combining living sludge with sulphide was additive, as the rate of dye reduction in those cases corresponds to the sum of the biological rate and chemical rate. If the sludge was inactivated by γ-irradiation, the rate of dye reduction was the same as the chemical rate only. This indicated that intact inactivated cells did not contribute in any way to dye reduction. However, if the sludge was inactivated by autoclaving, the rates of dye decolourisation in the presence of sulphide were remarkably higher than with living sludge. The lysis of cells caused by autoclaving is postulated to release enzyme cofactors that could function as redox mediators to accelerate the chemical reaction rates.

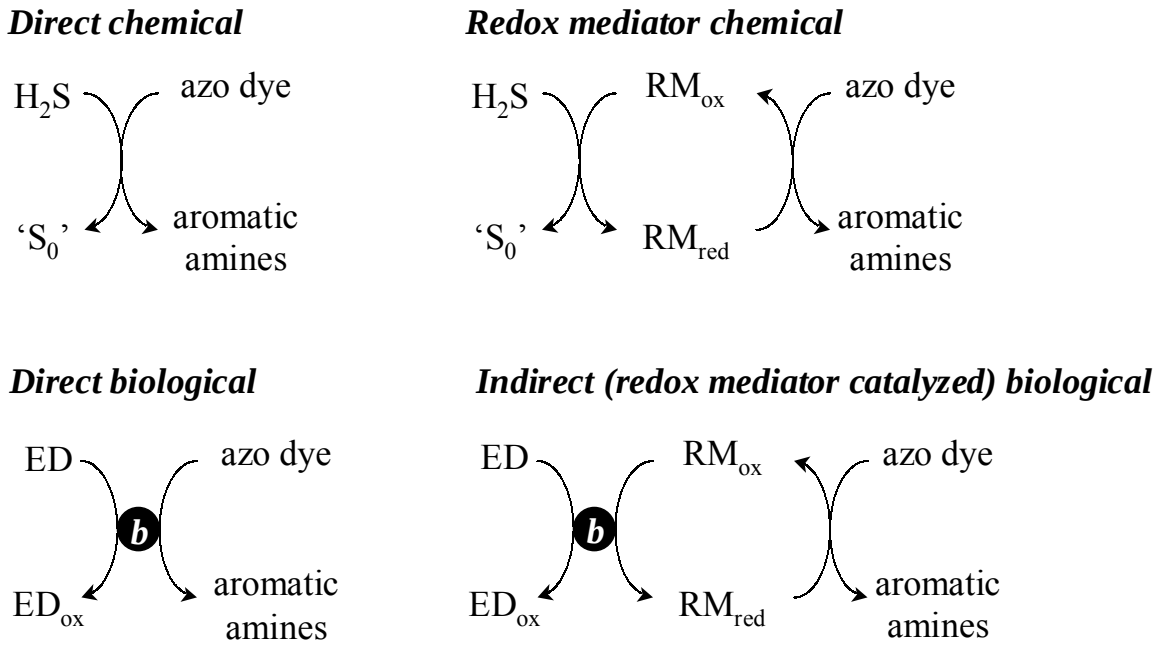


Figure 4.8 Schematic representation of the ways azo dyes can be reduced in the experimental systems used in this study
RM = redox mediator; ED = electron donor; *b* = bacteria (enzymes)

4.4.1 Chemical azo dye reduction

The two azo dyes investigated in this study, AO7 and RR2, could be chemically reduced by sulphide in medium without sludge. These results are in accordance with previous research indicating the reduction of several azo dyes by sulphide in the absence of bacteria (Chapters 2 and 3) and the reduction of a reactive azo dye by biogenic sulphide [180, 351]. Also textile manufacturers are aware that addition of reducing agents to dye-containing effluents leads to considerable decolourisation [113].

For the reduction of AO7 by sulphide (Figure 4.2), it was found that the reaction stoichiometry was 2.16 ± 0.1 mol of sulphide per mol of dye reduced. This stoichiometry indicates that the sulphur atom in sulphide (oxidation state –2) had been oxidised to an oxidation state around 0. Most probably,

sulphide is therefore oxidised to elementary sulphur (oxidation state S = 0). After reaction between the formed elementary sulphur with the remaining sulphide, polysulphide (HS_n^-) could have been formed. The average oxidation state in the more stable forms of polysulphide (HS_4^-, HS_5^- and HS_6^-) ranges between -0.25 and -0.17. According to the reaction stoichiometry of dye reduction by sulphide, 2 moles of sulphide are required per mole dye when sulphide is oxidised to elementary sulphur; whereas a maximum of 2.29 moles of sulphide are required per mole of dye when sulphide is completely oxidised to polysulphide. The chemical reduction of an azo dye by sulphide is expected to follow second-order reaction kinetics (equation [4.2]):

$$\frac{d[A]}{dt} = -k_c[A][S] \qquad [4.1]$$

with: [A] = dye concentration (mM)
[S] = total-sulphide concentration (mM)
t = time (d)
k_c = second-order reaction rate constant of the chemical reaction ($mM^{-1} \cdot d^{-1}$)

The value of k_c can be approached by assuming that the sulphide concentration at any time is related to the dye concentration according to equation [4.2]:

$$[S] = [S]_0 - a[A] \qquad [4.2]$$

with: a = molar stoichiometric ratio sulphide oxidation vs. dye reduction = 2.16 (see above)

When equation [4.2] is incorporated in equation [4.1], the solution can be plotted as follows (equation [4.3]):

$$\frac{1}{[S]_0 - a[A]_0} \cdot \ln\left[\frac{[A]_0 \cdot ([S]_0 - a([A]_0 - [A]))}{[A][S]_0}\right] = k_c t \qquad [4.3]$$

The left term of equation [4.3] can be plotted against time for each single decolourisation assay, with a, $[S]_0$ and $[A]_0$ known and [A] monitored in time. The slope of this curve represents the second-order reaction rate constant (k_c). Second-order rate kinetics was indeed found to be the case, as the k_c predicted from the data over a wide range of sulphide concentrations was found to be more or less constant. The values obtained for k_c are 0.33 ± 0.09 and 0.16 ± 0.05 $mM^{-1} \cdot d^{-1}$, for the chemical reduction of AO7 and RR2, respectively. The rate of chemical reduction of AO7 and RR2 can therefore be predicted for any given sulphide and AO7 or RR2 concentration.

In this study, the reaction rates of dye reduction are, however, discussed by comparison of the pseudo first-order reaction rate constant k_c' (in d^{-1}) at a given sulphide concentration. At high sulphide levels, $[S]_0 >> [A]_0$ and the change of the sulphide concentration can be neglected (at any time t, $[S] \approx [S]_0$). Equation [4.1] can then be written as equation [4.4], with k_c' being the product of k_c and [S] or $[S]_0$. The k_c' has a unique value at any given [S].

$$\frac{d[A]}{dt} = -k_c'[A] \Rightarrow [A] = [A]_0 \cdot e^{-k_c' t} \qquad [4.4]$$

with: k_c' = pseudo first-order reaction rate constant of the chemical reaction (d^{-1})

4.4.2 Redox mediator catalysed azo dye reduction

4.4.2.1 Redox mediation by autoclaved sludge

Figure 4.4A (AO7) and Figure 4.6A (RR2) show the accelerating effect of autoclaved sludge on the rates of chemical dye reduction. In Figure 4.5A it is furthermore shown that the presence of γ-irradiated sludge has little if any effect. When considering the difference between autoclaved sludge and γ-irradiated sludge, autoclaving disrupts the cells whereas γ-irradiation leaves the cell structure intact. The accelerating effect of autoclaved sludge on dye reduction is therefore most likely due to compounds released by cell lysis. As there is no dye reduction by autoclaved sludge in the absence of sulphide, it is clear, furthermore, that this effect is based on mediation of the transfer of reducing equivalents from the bulk electron donor (sulphide) to the electron-accepting dye.

The nature of this 'sludge redox mediator (SRM)' is not known. However, it can be expected that it has an E_0' value in between –270 mV (i.e. the E_0' value of the primary electron donor, the redox couple S_0/HS^-) and the unknown E_0' values for the redox couple azo dyes/aromatic amine. Flavin cofactors may fit this role as they are universally occurring heat-stable biochemical cofactors with E_0' values between –200 and –220 mV that are known to mediate azo dye reduction in biological systems [38, 58, 111, 161, 271, 282, 296]. The mediating role of flavins in the chemical reduction of azo dyes by sulphide has not been reported before. However, in this study, we proved that riboflavin is a powerful mediator in this reaction (Figure 4.5). It has often been observed that cell-free extracts, starving cells or lysed cells show higher azo dye reduction rates than intact or resting cells [87, 213, 282, 286, 337] and in one of these investigations, the researchers [282] established clear evidence for the leakage of a flavin cofactor, identified as riboflavin, from starving cells.

4.4.2.2 AQDS mediated chemical azo dye reduction

AQDS is an artificial external redox mediator which is known to mediate the reductive transfer of azo dyes by pure cultures of bacteria [161], by chemical reaction with sulphide (Chapter 3), and by electrochemical reactions [23]. The results presented in this study show that the reduction of AO7 and RR2 by sulphide is strongly accelerated by AQDS. According to this mechanism, first sulphide reduces AQDS to anthrahydroquinone-2,6-disulphonate, and second AH_2QDS reduces the dye. The first step of the reaction mechanism was demonstrated in this study, whereas the second step of the mechanism was demonstrated in an earlier study in which AH_2QDS (generated by biological reduction but separated from the cells) was shown to chemically reduce resin-bound AO7 [170].

An interesting aspect of the effect of AQDS (see Figures 4B and 6B) is that it does not overshadow the acceleration by autoclaved sludge. AQDS also increases dye reduction rates already mediated by SRM in autoclaved sludge. This indicates that there is transfer of electrons between SRM and AQDS.

4.4.3 Biological azo dye reduction

At near zero sulphide concentrations, complete AO7 and RR2 reduction was only achieved in the presence of living sludge. Also slow activity was observed in γ-irradiated sludge, which can be attributed to the fact that the bacteria or bacterial spores were not completely killed off by the treatment. These results clearly demonstrate that azo dye reduction in anaerobic granular sludge is due

to biological activity in the absence of chemical reducing agents. Biological azo dye reduction can be expected to follow Monod kinetics, i.e. zero-order kinetics with respect to the dye concentration if the affinity for the dye is high. The reduction of AO7 and RR2 in the absence of sulphide could indeed be described by zero-order kinetics rather than first-order kinetics, although the goodness of fit criterion does not allow a significant choice and the estimated pseudo first-order reaction constants are suitable comparative parameters. The slopes of the AO7 and RR2 decolourisation curves can therefore be used to determine the biological azo dye reduction activity of the batch assays with living sludge in the absence of sulphide: 0.072 ± 0.002 and 0.067 mM · d^{-1} for AO7 and RR2. When normalised with the sludge concentration, the values for AO7 (0.040 mmol g^{-1} VSS d^{-1}) are comparable with those calculated from previous results (Chapter 2). Those for RR2 were approximately 3 to 5-fold higher compared to experiments with a much larger pool of data (Chapter 5), thus the data derived from the previous experiment (0.0067 to 0.0117 mmol g^{-1} VSS d^{-1}) are considered more valid.

Biological azo dye reduction can be due to one of two mechanisms. The first mechanism is direct azo dye reduction by enzymes. The second mechanism is an indirect biological reaction mediated by enzymatically generated reduced electron carriers.

4.4.3.1 Direct enzymatic azo dye reduction

According to the first mechanism of biological azo dye reduction, 'azoreductases' transfer the reducing equivalents originating from the oxidation of organic substrates to the azo dyes. These enzymes are either intracellular or membrane-bound. Evidence for such a mechanism was found in studies with some aerobic and facultative aerobic bacteria that could grow with mostly simple azo compounds as sole source of carbon and energy. These strains grew under strict aerobic conditions by using a metabolism that started with reductive cleavage of the azo linkage [163]. The existence of enzymes catalysing azo dye reduction in aerobic bacteria was for the first time proven when two azoreductases from obligate aerobic bacteria were isolated and characterised [364]. These intracellular azoreductases showed high specificity to dye structures. Aside from these specific azoreductases, also non-specific enzymes catalysing azo dye reduction have been isolated from aerobically grown cultures of *Shigella dysenteriae* [108], *Escherichia coli* [109] and *Bacillus* sp. [314]. Where characterised, these enzymes were found to be flavoproteins [109, 108].

Also under anaerobic conditions, enzymes may be responsible for the almost ubiquitous capacity of many strains of anaerobic, facultative anaerobic and even aerobic bacteria to reduce azo dyes. Ten bacterial strains isolated from the human intestine were found to have azoreductase activity for Direct Blue 15 in their culture supernatants [271]. Further research with the purified azoreductase from one of the strains showed that it was a flavoprotein capable of catalysing the non-specific reduction of azo dyes as well as nitroaromatics [273].

4.4.3.2 Indirect (mediated) biological azo dye reduction

According to the second mechanism of biological azo dye reduction, azo dyes are indirectly reduced by enzymatically reduced electron carriers. Early research has hypothesised that reduced flavins ($FADH_2$, $FMNH_2$, riboflavin) generated by flavin-dependent reductases can reduce azo dyes in a non-specific chemical reaction [111, 282]. Flavins were indeed often found to stimulate azo dye reduction [58, 111,

[122, 271, 282] and recent research has revealed that flavin reductases are indeed 'anaerobic azoreductases' [286]. Also other reduced enzyme cofactors capable of direct azo dye reduction have been reported, e.g. NADH [223] and an NADPH-generating system [296].

Aside from enzyme cofactors, also various artificial redox mediating compounds are important stimulants of biological azo dye reduction, e.g. benzyl viologen [30, 38, 58, 161, 296], methyl viologen [58, 161]; AQDS [161], crystal violet, neutral red, phenosafranin, menadione, Janus Green B [58], anthraquinone-2-sulphonate (AQS) and 2-hydroxy-1,4-naphthoquinone [161]. Several bacterial enzymes have been found able to regenerate the enzyme cofactors and the artificial electron carriers by reduction. For example, a periplasmic hydrogenase of *Desulphovibrio vulgaris* was shown to reduce several quinone compounds as exogenous electron acceptors coupled to hydrogen oxidation [324]. An NADH:ubiquinone oxidoreductase situated in the membrane of *Sphingomonas sp.* BN6 could reduce AQS [161]. Enzymes may not be directly needed to regenerate some quinone electron carriers as non-enzymatic reduction of *p*-benzoquinones by NADH or by an NADH analogue (9,10 – dyhydro – 10 – methylacridine) has been reported [32, 222]. Recently, it was furthermore reported that the reduction of AQDS by *Shewanella putrefaciens* proceeds via excretion of unidentified quinones for extracellular electron transfer and it was suggested that the biological reduction of insoluble metal oxides might also involve a similar mechanism [237].

A completely different mechanism of indirect biological azo dye reduction is based on the concept of sulphur cycling, i.e. dye reduction by sulphide in combination with biological reduction of the oxidised sulphur species [180]. According to this concept, only trace quantities of sulphide are required. However, in this study low sulphide concentrations (< 0.5 mM) had no increasing effect on the rate of dye decolourisation in living sludge. Therefore, sulphur cycling is not an important mechanism here.

4.4.3.3 Location of biological azo dye reduction

The chemical reaction between the dye and the electron carrier, as well as the enzymatic reduction of the electron carrier, can occur both intracellularly and extracellularly. Cofactors like $FADH_2$, $FMNH_2$, NADH and NADPH, as well as the enzymes reducing these cofactors are located in the cytoplasm [286]. This implicates that a membrane transport system would be a prerequisite for the reduction of azo dyes by these cofactors in intact cells. However, cell wall permeation of dyes is considered a serious obstacle, especially for those containing (highly polar) sulphonate groups. In addition, also FAD and FMN cannot readily cross cell walls. In contrast, riboflavin is able to move across cell membranes. Hence, it has been reported several times that cell extracts or starving or lysed cells show higher azo dye reduction rates than intact or resting cells [87, 213, 282, 286, 337]. Moreover, the lack of a clear relationship between a dye's structure (size, molecular weight, degree of sulphonation) and its reduction rate [25, 36, 44] (Chapter 2) suggests that intracellular azo dye reduction mechanisms are not likely to play an important role. In a study to the anaerobic reduction of amaranth by whole cells, cell extracts and cell membranes of *Sphingomonas* sp. strain BN6, enzymatic azo dye reduction activity was found to be located in the cytoplasm (a soluble FAD-dependent enzyme) as well as in the membrane fraction (presumably and NADH; ubiquinone oxidoreductase) but it was suggested that that azo dye reduction by whole cells is mainly related to the latter [161]. Most probably, anaerobic biological azo dye reduction

occurs outside the cells, catalysed directly by periplasmic enzymes or indirectly, in a reaction with reduced electron carriers that are regenerated by these periplasmic enzymes.

4.4.4 Effect of sulphate

In the reduction of azo dyes, sulphate may play a double role. Apart from being the precursor of the electron donor sulphide, it may also compete with the dye as an electron acceptor. In this study, it was observed that different sulphate concentrations did not have an adverse effect on the reduction of RR2 in either the batch assays or the reactor experiment. It is therefore clear that sulphate -even when present at concentrations as high as 60 mM- does not obstruct the transfer of electron to the azo dye. The results confirm previous observations in which sulphate did not inhibit azo dye reduction by different types of sludge [46, 53, 254]. Probably, the redox potentials of the reduction of the various azo dyes involved are higher (more positive) than the redox potential of biological sulphate reduction (-220 mV). Azo dye reduction and sulphate reduction proceeded simultaneously and in batch assays with low biomass concentrations, the biogenic sulphide formed contributed to increase the overall rate of dye reduction due to its chemical reactivity.

4.4.5 Relative importance of chemical reduction in anaerobic bioreactors

During treatment of textile industry wastewater in anaerobic bioreactors, sulphide will be introduced into a system via sulphate reducing bacteria. Dye reduction will therefore result from a combination of chemical and biological reduction processes. From the discussion above, it is justified to assume that these processes are separate, i.e. (1) biological azo dye reduction does not depend on sulphide; (2) chemical azo dye reduction does not depend on either biological activity or the presence of intact sludge organic matter (cells not lysed) and (3) biological sulphur recycling can be neglected.

Based on the zero order and second order kinetic parameters estimated for the biological and chemical mechanisms of azo dye reduction (above in Discussion); respectively, the relative contribution of the chemical mechanism was estimated for RR2 under the conditions utilised for the lab scale UASB reactors; assuming 35 g VSS l^{-1} of biomass, a hydraulic retention time of 6 h, 0.06 mM of RR2 and 3.5 mM of biogenic sulphide. The kinetic analysis reveals that under those conditions only 13% of the dye removal would be due to chemical reduction, which coincides with the lack of any noticeable effect of sulphate on dye removal efficiency in the lab experiment. If the biogenic sulphide concentration were increased to 10 mM, than the contribution of the chemical reduction mechanism would be 33% of the total dye removal. Thus in anaerobic bioreactors, the biological mechanism of dye reduction is dominant due to the high biomass concentration in the reactors. The importance of chemical reduction will increase if the biomass concentration is low, or extremely high levels of biogenic sulphide occur in the reactor or if artificial redox mediating compounds are utilised.

abroad.

5

Application of redox mediators to accelerate the transformation of reactive azo dyes in anaerobic bioreactors

Abstract Azo dyes are non-specifically reduced under anaerobic conditions but the slow rates at which reactive azo dyes are converted present a serious problem for the application of anaerobic technology as a first stage in the complete biodegradation of these compounds. As quinones have been found to catalyse reductive transfers by acting as redox mediators, the application of anthraquinone-2,6-disulphonic acid (AQDS) during continuous anaerobic treatment of the reactive azo dye Reactive Red 2 (RR2) was evaluated. A mixture of volatile fatty acids was used as the electron donating primary substrate.

Batch experiments demonstrated that AQDS could increase the first-order rate constant of RR2 reductive cleavage by one order of magnitude. In the continuous experiment, treatment of RR2 containing synthetic wastewater in a lab-scale UASB reactor yielded low dye removal efficiencies (<30%). Consequently, severe toxicity problems occurred, eventually resulting in almost complete inhibition of the methanogenic activity. Addition of catalytic concentrations of AQDS (19 μM) to the reactor influent caused immediate increase of the dye removal efficiency and recovery of the biological activity. Ultimately, the RR2 removal efficiency stabilised at 88% and higher AQDS loads resulted in higher RR2 removal efficiencies (up to 98% at 155 μM AQDS).

Examination of the RR2 decolourising properties of dye-adapted reactor sludge and of non-adapted reactor seed sludge revealed that RR2 decolourisation was principally a biologically driven transfer of reducing equivalents from endogenous and added substrates to the dye. Hydrogen, added in bulk, was clearly the preferred electron donor. Bacteria that couple dye decolourisation to hydrogen oxidation were naturally present in seed sludge. However, enrichment was required for the utilisation of electrons from volatile fatty acids for dye reduction. The stimulatory effect of AQDS on RR2 decolourisation by AQDS-unadapted sludge was mainly due to assisting the electron transfer from endogenous substrates in the sludge to the dye. The stimulatory effect of AQDS on RR2 decolourisation by sludge from the AQDS-exposed reactor was in addition strongly associated with the transfer of electrons from hydrogen and acetate to the dye, probably due to enrichment of specialised AQDS-reducing bacteria.

Van der Zee, F.P., Bouwman, R.H.M., Strik, D.P.B.T.B., Lettinga, G. and Field, J.A. (2001) Biotechnol. Bioeng. 75: 691-701

5.1 Introduction

Removal of dyes is a major concern when treating textile-processing wastewater. The vast majority (60-70%) of the dyes applied in textile-processing industries are azo compounds, characterised by azo (N=N) bridges linking substituted aromatic structures [46]. It is estimated that 10 to 40% of the dyes used for textile dyeing end up in the wastewater. This fraction has increased over the last decades because of the increasing use of reactive dyes, a class of water-soluble dyes, with a relatively low degree of fixation [70, 248].

Discharge of dyes into the environment should be avoided, not only for aesthetic reasons, but also because many azo dyes and their breakdown products are toxic to aquatic life [61] and mutagenic to humans [59]. Azo dyes are generally persistent under aerobic conditions [251, 299]. However, under anaerobic conditions they undergo relatively easy reductive fission, yielding colourless aromatic amines. The reduction of azo dyes is therefore closely associated with their decolourisation. The aromatic amines released from azo dye reduction generally require aerobic conditions for their degradation [35]. The most logical treatment strategy for complete degradation of azo dyes is therefore a sequential anaerobic-aerobic approach with, for instance, an upflow anaerobic sludge blanket (UASB) reactor as the first stage.

Preliminary research in our laboratory, a screening of the anaerobic decolourisation of 20 widely varying types of azo dyes (Chapter 2), revealed that all azo dyes studied were reduced in the presence of granular sludge fed with volatile fatty acids as the electron donating primary substrate. The reaction followed first-order kinetics, with half-life times varying greatly between dyes. The reactive azo dyes with triazyl reactive groups were slowly reduced. For these common occurring reactive dyes, long contact times may be necessary to reach a satisfying extent (>90%) of decolourisation. Consequently, they pose a serious problem for applying high-rate anaerobic treatment as the first stage in the biological degradation of azo dyes. Therefore, methods to improve the rate of azo dye reduction are clearly needed.

To overcome the problem of slow azo dye reduction rates, redox mediators, i.e. compounds that speed up reaction rates by shuttling reducing equivalents between (terminal) electron donors and electron acceptors, may be helpful. Enzyme cofactors like FAD are known as effective redox mediators for azo dye reduction [101, 111, 286] and also artificial quinones can act as redox mediators: in abiotic systems, quinones accelerated chemical azo dye reduction by sulphide (Chapter 3) as well as electrochemical azo dye reduction [23] and in biological systems, quinones were shown to accelerate azo dye reduction by anaerobically incubated aerobic biomass [152, 161] as well as azo dye reduction by anaerobic granular sludge (Chapter 4).

In this study, the effects of anthraquinone-2,6-disulphonic acid (AQDS) on the continuous treatment of a synthetic wastewater containing the slowly reducible reactive azo dye Reactive Red 2 (RR2), were investigated and batch experiments were performed to further explore the mechanism of azo dye decolourisation.

5.2 Materials and methods

5.2.1 Continuous experiment

A lab-scale UASB reactor (wet volume 1.2 l) was fed with a neutralised VFA mixture (1.5 g COD l^{-1} at a 1:1:1 COD based rate of acetate, propionate and butyrate) in basal medium containing (mg l^{-1}) NH_4Cl (280), $CaCl_2$ (5.7), KH_2PO_4 (250), $MgSO_4 \cdot 7H_2O$ (100), H_3BO_3 (0.05), $FeCl_2 \cdot 4H_2O$ (2), $ZnCl_2$ (0.05), $MnCl_2 \cdot 4H_2O$ (0.5), $CuCl_2 \cdot 2H_2O$ (0.04), $(NH_4)_6Mo_7O_{24} \cdot 5H_2O$ (0.05), $CoCl_2 \cdot 6H_2O$ (1), $NiCl_2 \cdot 6H_2O$ (1) and $Na_2SeO_3 \cdot 5H_2O$ (0.16). The hydraulic retention time was kept constant at 5.9 ± 0.7 h. After a three-week start-up without dye, 200 mg l^{-1} of the textile dye RR2 (dye content ~50%) was added to the reactor influent. This influent dye concentration was kept constant, giving a dye load of 0.81 ± 0.1 g RR2 (commercial product) l^{-1} reactor volume d^{-1}. Up from day 72, AQDS was added. Four different AQDS concentration levels were applied: 19 µM (days 72–144 and 218–263); 39 µM (days 144–183); 78 µM (days 183–206) and 155 µM (days 206-218). The reactor's COD removal and dye decolourisation efficiencies were monitored regularly.

5.2.2 Batch experiments

All batch experiments were conducted in glass serum vials. The vials were filled with basal medium and anaerobic granular sludge (except for the abiotic controls). Next, the vials were sealed with butyl rubber stoppers and the gas headspace was flushed for 5 minutes with oxygen-free flush gas. Organic primary electron donors and AQDS were added with syringes from neutralised concentrated stock solutions. After a 3-days pre-incubation period (in a rotary shaker at 30 °C), again the headspace of the vials was flushed and substrate was added. RR2 was added to the vials with a syringe from a concentrated stock solution. The vials were incubated on a rotary shaker at 30 °C. The composition of the vials in each separate experiment (i.e. the type of sludge and substrate, the concentrations of sludge-VSS, substrate, nutrients, AQDS and RR2, the composition of the flush gas, as well as the liquid and headspace volumes) is listed in Table I. All experiments were performed with triplicate vials. Sulphide was measured at the start and at the end of most of the experiments.

RR2 decolourisation experiments: In batch decolourisation experiments, liquid phase samples for absorbance measurements were taken at intervals to follow the course of RR2 decolourisation and to determine the decolourisation rates. First-order decolourisation rates were calculated by fitting the decolourisation curve to equation [5.1] by using the least square method.

$$A_t = A_0 \cdot e^{-kt} \qquad [5.1]$$

with: k = first-order rate constant (d^{-1});

A_t = Absorbance at time t;

A_0 = Absorbance at t=0, immediately after dye injection;

t = incubation time (d).

The effect of AQDS on the rate of RR2 reduction was studied (AQDS gradient test) and the dependency of RR2 reduction on the type of electron donor was investigated (substrate dependency

Table 5.1 Preparation of serum vials for batch experiments

TEST		**AQDS gradient**	**Dye toxicity**		**Substrate dependency**	**AQDS reduction**	**Controls**	
			acute	*delayed*			*dead biomass*	*abiotic RR2 reduction*
sludge type[1]		S	S	S	S or R	S or R	S or R	---
[VSS]	g l^{-1}	~2.0	~2.0	~0.4	~1.0	~1.0	~1.0	---
substrate type[2]		VFA	C2	C2	---, C2, C3, C4, H_2	C2	VFA	---, VFA or H_2
$[substrate]_{t=0}$	g COD l^{-1}	~3	~3	~2.5	0 (---), ~2.5 (VFA), ~0.3 (H2)	~2.5	~0.5	0 (---), ~2.5 (VFA), ~0.3 (H2)
$[RR2]_{t=0}$[3]	mg l^{-1}	~200	~200	~400	~350	0	~350	~350
[AQDS]	µM	0 – 240	0	0	0 or 21	415 ± 5	0 or 21	0 or 21
V_{liquid}[4]	ml	45	50	50	59	59	55	59
$V_{headspace}$	ml	72	67	67	58 (--- or VFA), 253 (H_2)	58	62	58 (--- or VFA), 253 (H_2)
flush gas composition		N_2/CO_2 70%:30%	N_2/CO_2 70%:30%	N_2/CO_2 70%:30%	N_2/CO_2 70%:30% (--- or VFA), H_2/CO_2 80%:20% (H_2)	N_2 100%	N_2/CO_2 70%:30%	N_2/CO_2 70%:30% (--- or VFA), H_2/CO_2 80%:20% (H_2)
initial pressure	atm.	1	1	1	1 (--- or VFA), 1.95 (H_2)	1	1	1 (--- or VFA), 1.95 (H_2)
samples[5]		A, S	A, CH_4	A, CH_4	A, S, CH_4, P	A	A, S	A

[1] sludge type: S = seed sludge, i.e. dye- and AQDS unadapted sludge from a distillery wastewater treatment plant (Nedalco, Bergen op Zoom, The Netherlands); R = reactor sludge, i.e. sludge from the dye- and AQDS exposed lab reactor.

[2] substrate type: VFA = neutralised mixture of volatile fatty acids (acetate/propionate/butyrate at a 1:1:1 COD based ratio); --- = no primary electron donor; C2 = acetate (neutralised); C3 = propionate (neutralised); C4 = butyrate (neutralised); H2 = hydrogen.

[3] concentration of the commercial dye preparation (dye purity ~50%)

[4] basal medium contained (mg l^{-1}): NH_4Cl (280), $CaCl_2$ (5.7), KH_2PO_4 (250), $MgSO_4 \cdot 7H_2O$ (100), H_3BO_3 (0.05), $FeCl_2 \cdot 4H_2O$ (2), $ZnCl_2$ (0.05), $MnCl_2 \cdot 4H_2O$ (0.5), $CuCl_2 \cdot 2H_2O$ (0.04), $(NH_4)_6Mo_7O_{24} \cdot 5H_2O$ (0.05), $CoCl_2 \cdot 6H_2O$ (1), $NiCl_2 \cdot 6H_2O$ (1), $Na_2SeO_3 \cdot 5H_2O$ (0.16). In all experiments except the AQDS reduction test, $NaHCO_3$ (5 g l^{-1}) was added to obtain a bicarbonate buffered system at pH 7.3 ± 0.3. In the AQDS reduction test, a phosphate buffer at pH 7.1 was used (2.72 g l^{-1} $NaH_2PO_4.2H_2O$ and 1.35 g l^{-1} $Na_2HPO_4.2H_2O$).

[5] samples: A = Absorbance at 539 nm; S = total-sulphide; CH_4 = headspace methane content; P = headspace pressure

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test). In control experiments, it was tested if RR2 could be decolourised abiotically, in a reaction with hydrogen, VFA or inactivated biomass.

In the AQDS gradient test, the rate of RR2 decolourisation was determined at ten different AQDS concentrations, ranging from 0 to 1180 μM.

The substrate dependency test was conducted to investigate the substrate specificity of direct and AQDS catalysed RR2 decolourisation and to investigate the effect of biomass adaptation. The experiments were performed in the presence of acetate, propionate, butyrate and hydrogen as the electron donating substrates and in the absence of an external substrate, with biomass-VSS as the only possible electron donor. In case of VFA substrates, injecting supplemental quantities of neutralised stock solutions if the concentration dropped below 1 g COD l^{-1} prevented substrate depletion during the course of the decolourisation process. Hydrogen was replenished every 24 hours, by bringing back the headspace to 2 atm. 80%:20% H_2/CO_2. The methane production in all serum vials was roughly determined, by following the headspace methane content in the first week of the experiment or, in case of the vials with hydrogen, by following the headspace pressure. The methane production rates were normalised for the VSS concentration to obtain a value for the SMA, the specific methanogenic activity (expressed in g CH_4-COD g^{-1} VSS d^{-1})

Control experiments were conducted to test if hydrogen or VFA can decolourise RR2 in a direct chemical reaction, as well as to quantify the abiotic decolourisation of RR2 by inactivated (autoclaved) biomass. In the abiotic controls without sludge, the absorbance was followed during 30 days. For studying the decolourisation of RR2 by reduced compounds in inactivated biomass, the dye was added to vials that were, after 3 days pre-incubation and flushing, cooked for 90 minutes in a pressure cooker.

RR2 toxicity experiments: In batch toxicity experiments, the inhibition of the methanogenic activity of seed sludge by RR2 was determined by following the build-up of methane in the headspace of 117-ml serum vials. Both acute and long-term toxicity was tested.

The acute toxicity of RR2 was tested in serum vials that were monitored during 7 hours immediately after dye injection. In the long-term toxicity test, incubation lasted much longer (21 days) and also a much lower sludge/dye ratio was applied. To investigate the effect of decolourisation on RR2 toxicity, the long-term test was performed at different AQDS levels. Furthermore, to investigate the effect of hydrolysis (i.e. the replacement of chloro groups by hydroxyl groups) on RR2 toxicity, the test was as well performed with a preparation of RR2 that was previously heated in a Na_2CO_3 solution [25]. When the methane content exceeded 35%, the headspace was flushed again and VFA was replenished. The experiment was accompanied by a parallel experiment in separate vials to measure the decolourisation.

AQDS reduction experiments: To determine whether adaptation of biomass to AQDS had led to a changed capability of reducing AQDS, a batch experiment was conducted to compare the reduction of AQDS by reactor sludge and by seed sludge, with acetate as the primary electron donor. Pre-incubated batch vials were supplemented with 0.41 mM AQDS. At intervals, liquid phase samples for absorbance measurements were taken to follow the formation of AH_2QDS. As AH_2QDS was found to oxidise very fast when being exposed to air, it was necessary to sample and analyse in an anaerobic

hood. As the absorbance factor of AH_2QDS was found sensitive to small changes in pH, a phosphate buffer instead of a bicarbonate buffer was used.

5.2.3 Analysis

RR2 colour was measured spectrophotometrically with a Spectronics 60 spectrophotometer (Milton Ray Analytical Products Division, Belgium) at the dye's wavelength of maximum absorbance (539 nm). At this wavelength, a 1 g l^{-1} solution of the commercial dye preparation (dye content ~50%) has an extinction of 21.6 absorbance units per cm. The estimated molar extinction coefficient at 539 nm is therefore approximately $26.6 \cdot 10^3$ cm^{-1} M^{-1}. Liquid phase samples (0.75 ml) were centrifuged (2 minutes at 10,000 rpm) and diluted up to an absorbance of less than 1 in a phosphate buffer (10.86 g l^{-1} $NaH_2PO_4 \cdot 2H_2O$; 5.38 g l^{-1} $Na_2HPO_4 \cdot H_2O$). The buffer contained freshly added ascorbic acid (~200 mg l^{-1}) to prevent autoxidation. Without dye, light absorbance of medium and buffer was less than 1% of the absorbance right after dye addition and could therefore be neglected.

Reduced AQDS (anthrahydroquinone-2,6-disulphonic acid, AH_2QDS) was measured spectrophotometrically at 450 nm. Liquid phase samples were centrifuged (2 minutes at 10,000 rpm) and diluted to an absorbance less than 1 in a phosphate buffer (10.86 g l^{-1} $NaH_2PO_4 \cdot 2H_2O$; 5.38 g l^{-1} $Na_2HPO_4 \cdot H_2O$). A 5.0 mM AH_2QDS standard solution (molar extinction coefficient at 450 nm $\sim 2.6 \cdot 10^3$ cm^{-1} M^{-1}) was prepared from a 5.0 mM AQDS solution by a palladium catalysed reaction with hydrogen during 4 hours at 50 °C. Sampling and analysis took place in an anaerobic chamber under a 96%:4% N_2/H_2 atmosphere.

Sulphide was determined according the method described by Trüper and Schlegel [328].

COD (Chemical Oxygen Demand) was measured using the micro-method described by Jirka and Carter [145].

Volatile Fatty Acids (VFA) and methane were determined by gas chromatography, as described in Chapter 4.

Methane was determined by gas chromatography. The chromatograph (Packard-Becker, Delft, The Netherlands) chromatograph equipped with a 2m x 2mm steel column packed with Poropak Q (80/100 mesh). The temperatures of the column, injection port and flame ionisation unit were respectively 60, 200 and 220 °C. The carrier gas was nitrogen (20 ml per minute). Gas samples were taken with a 100 µl pressure-lock syringe (Dynatech, Baton Rouge, USA).

The pH was determined with a Kinck 511 pH meter (Berlin, Germany) and a Schott Geräte N32A double electrode (Hofheim, Germany).

VSS (Volatile Suspended Solids) were determined according to standard methods [12].

5.2.4 Chemicals

Reactive Red 2 (Procion Red MX-5B, C.I. 18200) was purchased from either Aldrich (Gillingham, England) or as a commercially available dye powder. The dye purity was ~50%. The dye was used without further purification. The structure formula is plotted in Figure 5.1.

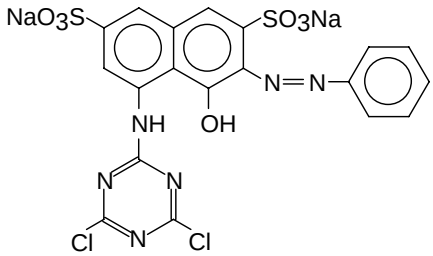

Figure 5.1 Structure formula of Reactive Red 2 (RR2), C.I. 18200.

5.3 Results

5.3.1 The effect of AQDS on the rate of Reactive Red 2 decolourisation

The decolourisation of RR2 by anaerobic granular sludge was followed in batch experiments with increasing concentrations of the redox mediator AQDS (see Figure 5.2, squares). First-order rate constants were determined to approximate the decolourisation rates. Without redox mediator the first-order rate constant was 0.21 d^{-1}. The addition of AQDS dramatically increased the rate constant: as shown in Figure 5.2, a 7-fold increase was obtained at 240 µM AQDS; and (not shown in graph) a 16-fold increase was obtained at the 1180 µM AQDS. The impact of AQDS was even evident at

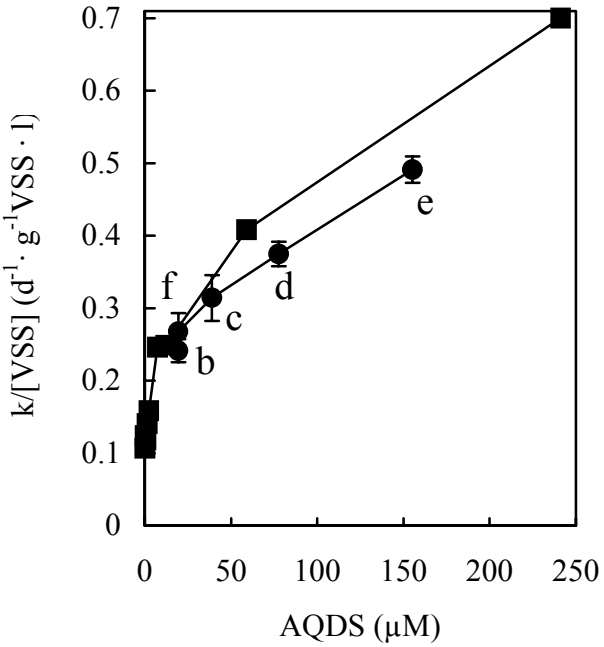

Figure 5.2 First-order rate constants (k) of the decolourisation of RR2 at different AQDS concentrations. The k-values are normalised for the biomass-VSS (volatile suspended solids) concentration, in a batch series with 2.0 ± 0.1 g VSS l^{-1} seed sludge (squares) and in the reactor with ~30 g VSS l^{-1} (circles). The letters refer to the different AQDS dosage periods in the reactor, as specified in Fig. 3 but with point b only referring to period b after day 121. The error bars represent the standard deviations.

micromolar concentrations. AQDS at 7 μM increased the decolourisation rate constant by 2-fold even though the molar ratio of AQDS:RR2 was only 1:16.

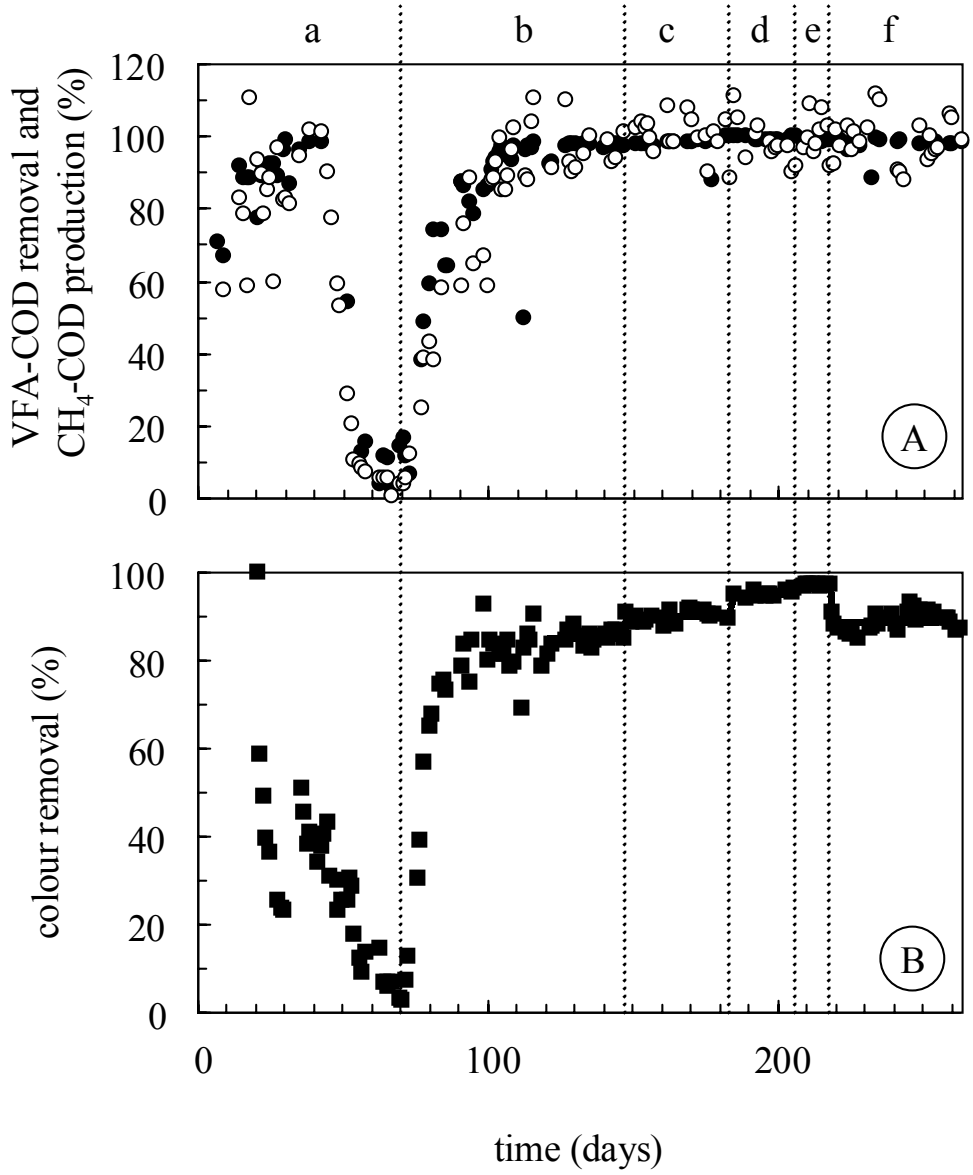

Figure 5.3 Reactor performance: A. VFA-COD removal (full circles) and CH4-COD-recovery (open circles) as percentages of the influent VFA-COD; B. Colour removal efficiency (squares). The dashed lines mark the periods with different AQDS concentrations: a (days 0-72) 0 μM; b (days 72-147) 19 μM; c (days 147-183) 39 μM; d (days 183-206) 78 μM; e (days 206-218) 155 μM; f (days 218-263) 19 μM.

5.3.2 Reactor performance

Dye decolourisation was also studied in a laboratory scale UASB reactor to determine if dye decolourisation with AQDS could be achieved and maintained under continuous operation. The reactor was fed with a VFA mixture as the primary electron donating substrate and tested in an initial period with RR2 without any redox mediator. On day 72 the redox mediator was introduced in the reactor influent and its concentration was incremented stepwise in time.

VFA removal: The performance of the bioreactor in terms of VFA removal is shown in Figure 5.3A. In the first three weeks (until day 21), the reactor was started up in the absence of dye. The influent flow was increased to about 5.5 l d^{-1}. The VFA removal efficiency increased to 85-90%. The dye (200 mg l^{-1}) was introduced into the reactor influent on day 21. The removal of VFA initially increased further to a maximum of 98%. However on day 43, three weeks after the start of dye dosing, the VFA removal efficiency declined drastically. Only ten days later, the bioreactor performance collapsed, resulting in VFA removal efficiencies as low as 5-10%, in the period between day 53 and 72. On day 72, micromolar concentrations of AQDS (19 µM) were introduced into the reactor influent. Consequently, a rapid change of the reactor performance was observed. The methanogenic conversion of VFA recovered steadily: the effluent butyrate concentration dropped to trace level (<25 mg COD l^{-1}) within four days and the effluent acetate and propionate concentrations showed a gradual decrease. In about three weeks, the bioreactor performance was completely recovered, with VFA removal efficiencies higher than 95% and a methane production that accounted for 101 ± 8 % of the influent VFA-COD. After day 105, the VFA removal efficiency remained stable at high levels (98 ± 1%), apart from during some minor short-term operational troubles.

Colour Removal: The performance of the bioreactor in terms of dye decolourisation is shown in Figure 5.3B. Immediately after Reactive Red 2 was added to the influent on day 21, considerable dye colour removal occurred up to day 30 and could be attributed to adsorption onto reactor sludge. As the adsorption capacity of the reactor sludge became exhausted, the reactor effluent became increasingly red-coloured and the colour removal efficiency decreased to about 25%. Granules sampled from the reactor sludge were dark red, indicating the presence of adsorbed dye. The sludge colour remained visually unchanged after washing the granules with water or after long-term storage in basal medium, suggesting that the adsorption was irreversible.

The reactor was temporarily operated without dye between day 30 and 35 due to limited dye supply. When a new preparation of Reactive Red 2 was again administered, the colour removal efficiency temporarily increased to levels of 30 to 50%. However, starting on day 43, simultaneous with the decline of the VFA removal, the colour removal dropped to lower levels (20-30%). Eventually, it dropped to only a few percent during the maximum inhibition of VFA removal. On day 72, when AQDS (19 µM) was introduced to the reactor influent, a rapid gradual increase of the colour removal efficiency was observed: 75% decolourisation was achieved within 10 days of AQDS dosing, which was followed by a slow further increase. Finally, a stable level of 85 ± 2% decolourisation or 0.64 ± 0.04 g RR2 l^{-1} reactor volume d^{-1} was reached (days 121-147). After day 148, the influent AQDS concentration was altered step-wise (period c to period f in Figure 5.3). Each increment in AQDS concentration was accompanied by a corresponding increase in the decolourisation efficiency, up to 98% decolourisation (or 0.81 ± 0.03 g RR2 l^{-1} reactor volume d^{-1}) in period f with 155 µM AQDS. Based on the assumption that the reaction follows first-order kinetics, equation [5.1] can be used to calculate, at each AQDS level, the first-order rate constant of RR2 decolourisation in the reactor (see equation [5.2]: the average HRT is taken as t, the absorbance of the reactor influent as A_0 and the absorbance of the reactor effluent as A_t.)

$$A_t = A_0 \cdot e^{-kt} \Rightarrow k = \frac{\ln\left(\frac{A_0}{A_t}\right)}{t} \Rightarrow k = \frac{\ln\left(\frac{A_{influent}}{A_{effluent}}\right)}{HRT} \quad [5.2]$$

The full circles in Figure 5.2 depict the relationship between AQDS concentration and k-value.

5.3.3 Dye toxicity

Both short-term and long-term toxicity effects were evaluated. The acute toxicity of RR2 was tested by following the build-up of methane in the headspace of acetate fed granular sludge in serum vials during 7 hours immediately after dye injection. The specific methanogenic activity after short-term exposure to dye (260 mg l^{-1}) did not significantly differ from that of the dye-free control (data not shown). In the continuous experiment, inhibition was observed after long term exposure to RR2. This corresponded to inhibition observed in long term toxicity batch tests, in which prolonged exposure of anaerobic granular sludge to RR2 led to a decline of the methane production in batches without AQDS (Figure 5.4). This delayed toxicity effect was less apparent when a small quantity of AQDS was present and the dye decolourised faster.

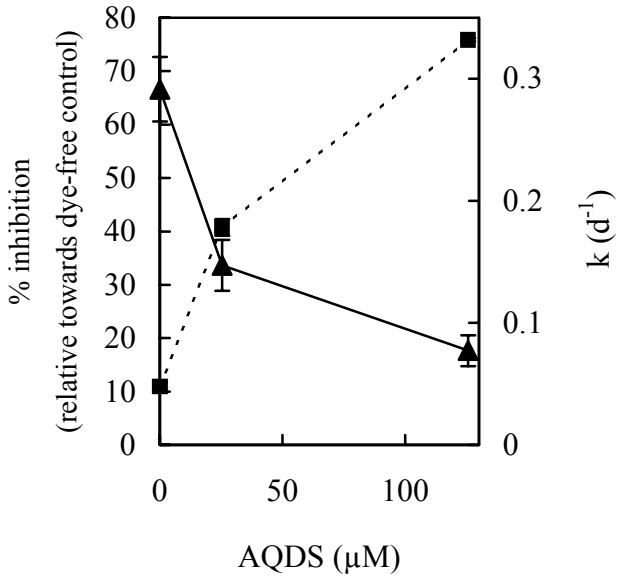

Figure 5.4 Inhibition of acetoclastic methanogenesis (triangles) and RR2 decolourisation rates (squares) at different AQDS concentrations. The average specific methane production rate of dye-exposed granular sludge during the last two weeks of a three-week incubation period is expressed as a percentage of the specific methane production rate by the same sludge in the absence of dye (1.2 ± 0.1 g COD $\cdot$ g^{-1} VSS $\cdot$ d^{-1}). All batch vials were incubated with seed sludge.

In another experiment (data not shown) it was found that pre-hydrolysis of the RR2 solution almost annihilated the RR2 toxicity, indicating that the presence of chloro groups on the triazyl residue were responsible for toxicity.

5.3.4 Substrate dependency of RR2 decolourisation

In the VFA fed reactor, RR2 is decolourised and acetate, propionate and butyrate are oxidised. Both acetate and interspecies hydrogen produced from propionate and butyrate as well as endogenous substrate (e.g. sludge organics) may serve as electron donors for dye reduction. As the thermodynamics of the different electron donating half-reactions are different, the reaction rate is likely to be influenced by the type of electron donor. Furthermore, as the reactor had been supplied with dye and mediator for more than one year, reactor sludge might have become different from the original seed sludge (Nedalco sludge) by enrichment of specific AQDS or RR2 reducing bacteria. The reduction of RR2 was therefore investigated in batch vials with either reactor sludge or seed sludge,

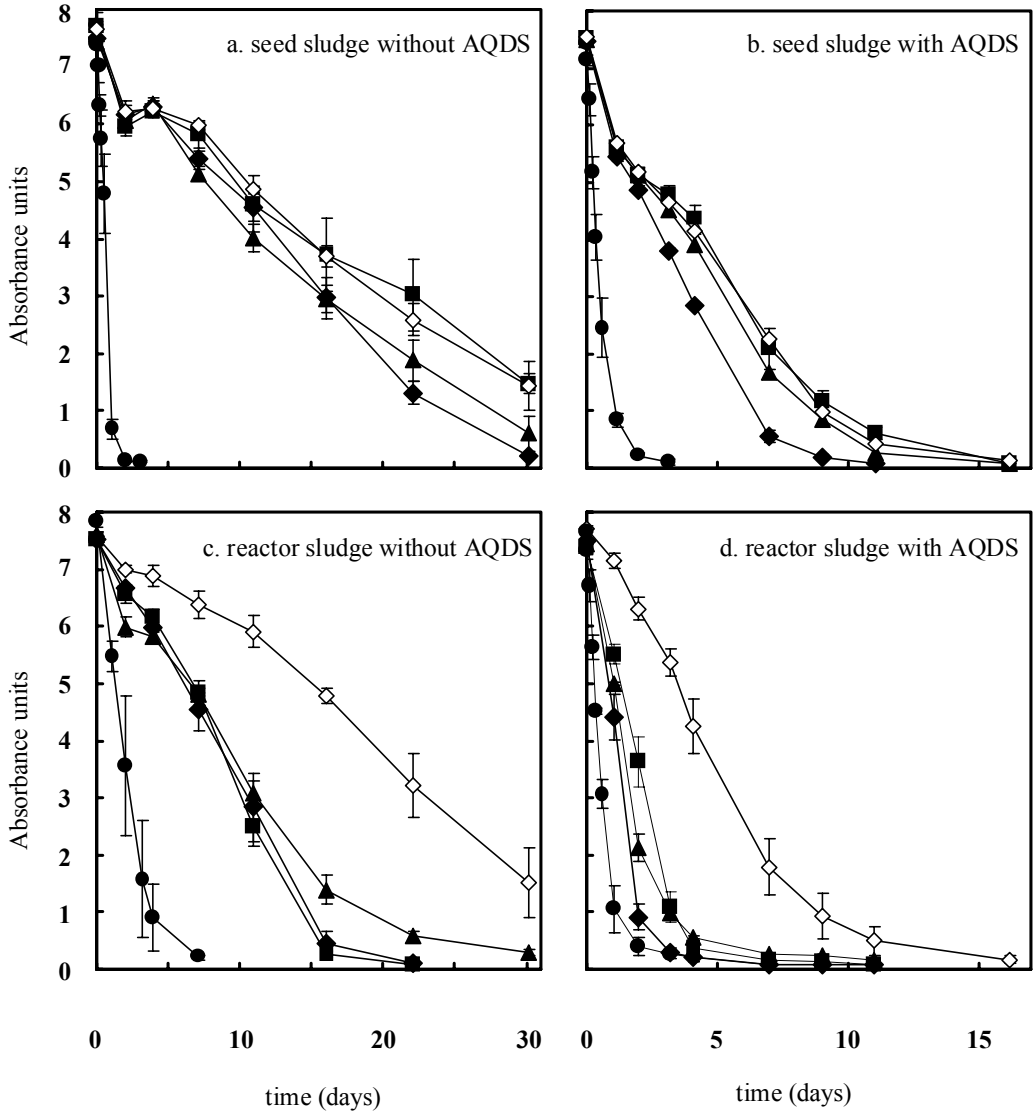

Figure 5.5 Decolourisation of RR2 by either seed sludge or reactor sludge, both in the absence and in the presence of 21 μM AQDS, with either no substrate (open diamonds) or acetate (full diamonds), propionate (squares), butyrate (triangles) or hydrogen (circles) as the electron donor. The error bars represent the standard deviations.

different electron donating substrates, both in the absence and in the presence of 20 μM AQDS. Figure 5.5 shows the decolourisation curves. The abiotic control experiments (data not shown) showed that direct chemical reduction of RR2 by VFA or hydrogen did not occur. Furthermore, due to the low

sulphide and VSS concentrations used in these experiments, the decolourisation by reduction and adsorption of RR2 by autoclaved reactor sludge and autoclaved seed sludge was limited to respectively less than 10% and less than 15% of the initial dye concentration (data not shown). The decolourisation processes reported in this section can therefore be considered as direct or indirect results of biological activity.

The effect of biomass adaptation on direct dye decolourisation (without AQDS): By comparing Figures 5.5a and 5.5c it is seen that reactor sludge and seed sludge showed different RR2 decolourisation behaviour. RR2 decolourisation by VFA fed seed sludge proceeded at more or less equal rate as in the absence of an external electron donor, whereas RR2 decolourisation by VFA fed reactor sludge was considerably faster than in the absence of an external electron donor. These results clearly show that reactor sludge contains an enriched bacterial population that utilises VFA substrates as electron donors to facilitate dye reduction.

The effect of substrate type on direct dye decolourisation (without AQDS): From Figures 5.5a and 5.5c it is clear that both sludge types reduced RR2, even if no external electron donor was present and the reaction depended fully on the production of endogenous reducing power. As the k-values for RR2 decolourisation with hydrogen were at least five times higher than with the other electron donors, hydrogen was clearly the preferred electron donor for both sludge types. Therefore, bacteria that couple dye reduction to hydrogen oxidation were naturally present in seed sludge.

In view of the high decolourisation rates with hydrogen as a bulk electron donor, it could be expected that, because of interspecies hydrogen produced by acetogens, the dye would decolourise faster with propionate and butyrate than with acetate. The similar decolourisation rates that were achieved with all three VFA substrates prove, however, that interspecies hydrogen is not available to dye reduction. When considering the very high methanogenic activity that both sludge types showed on hydrogen, it is highly probable that interspecies hydrogen is preferably consumed by hydrogenotrophic methanogens.

The effect of AQDS on dye decolourisation: AQDS accelerated the decolourisation of RR2 by reactor sludge and seed sludge, both with endogenous as well as with added electron donating substrates (see Figures 5.5b and 5.5d in comparison to Figures 5a and 5c, respectively). With seed sludge, the increase in the rate of decolourisation due to 20 μM AQDS for any given substrate was more or less similar to the increase observed without substrate addition. Thus, AQDS mainly accelerated the decolourisation associated with the oxidation of endogenous substrate. With reactor sludge, AQDS additionally stimulated dye decolourisation associated with hydrogen and VFA (especially acetate) oxidation: the stimulatory effect of AQDS in the presence of hydrogen or acetate was almost five times higher than the stimulation of the basal decolourisation rate when only endogenous substrate was present. These results suggest that, due to enrichment, the reactor sludge contained one or more cultures of specialised bacteria that utilise AQDS as the electron acceptor for the oxidation of hydrogen and acetate.

Methanogenic activity and decolourisation rate: The specific methanogenic activity (SMA) on hydrogen was 2 - 2.5 g CH_4-COD g^{-1} VSS d^{-1} and 3.5 g CH_4-COD g^{-1} VSS d^{-1} for reactor sludge and

seed sludge, respectively. On VFA, the SMA was at least 18 times lower. In the presence of hydrogen, the SMA of seed sludge was higher than the SMA of reactor sludge and the decolourisation also proceeded faster with the seed sludge ($k = 0.80 \pm 0.21$ d^{-1}) than with reactor sludge ($k = 0.46 \pm 0.12$ d^{-1}). When looking at the other substrates, there does not seem to be any correlation between methanogenic activity and decolourisation rate. For example, the SMA of seed sludge was almost zero on propionate and relatively high on acetate but the decolourisation rates did not differ. Likewise, though in the absence of an external electron donor, seed sludge had a 4 to 8 times higher SMA than reactor sludge, the decolourisation rates were almost equal.

AQDS had no noteworthy effect on the SMA. Its impact was therefore very exclusive on dye decolourisation.

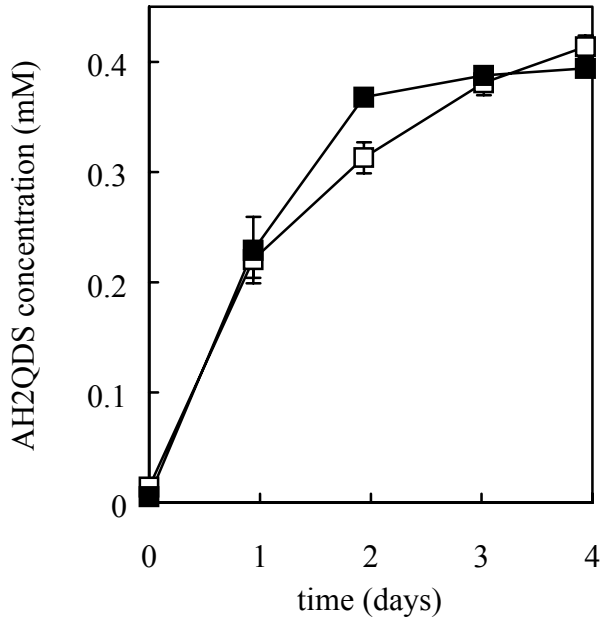

Figure 5.6 Reduction of AQDS (0.42 mM) by 1.0 g VSS l^{-1} reactor sludge (full squares) and by 1.0 g VSS l^{-1} seed sludge (open squares). The error bars represent the standard deviations.

5.3.5 Effect of biomass adaptation on the reduction of AQDS

To determine if an enriched acetate-oxidising, AQDS-reducing bacterial population had developed in the reactor sludge, the formation of AH_2QDS was followed in acetate-fed batch vials with either reactor sludge or seed sludge. The results are depicted in Figure 5.6. It is shown that both sludge types reduced AQDS at equal rate. Consequently, the experiment did not demonstrate the expected enrichment of acetate-oxidising quinone-respiring bacteria anticipated from the interaction of AQDS and dye in the reactor sludge. However, this AQDS reduction experiment probably did not adequately represent the selection pressure in the dye decolourisation experiments, requiring bacteria to utilise AQDS efficiently at only 20 μM.

5.4 Discussion

Reactive azo dyes, especially those with triazyl reactive groups, are often slowly decolourised in anaerobic sludge environments. The complete decolourisation of the model compound used in this study (the reactive azo dye RR2) and of several other azo dyes require long dye-sludge contact times (Chapter 2 and reference[25]). The extent of azo dye decolourisation in continuous anaerobic systems was often low unless long hydraulic retention times were applied (Chapter 1 and references [98, 247, 250, 298]).

In this study, a synthetic wastewater containing RR2 was treated in a UASB-reactor at a moderate hydraulic retention time of 5.9 ± 0.7 hours, realistic for wastewater treatment in practice. The decolourisation efficiency that was reached after adsorption breakthrough was very poor, which indicated that the rate of RR2-decolourisation was not sufficient and the bioreactor and would not work under practical conditions. This problem was aggravated by toxicity of the unreduced dye, which severely inhibited the biological activity of the methanogens in the reactor sludge.

The objective was to overcome the problems due to slow decolourisation rates by introducing a redox mediator, AQDS, to the anaerobic system. From previous experiments (Chapter 3) it had become clear that in batch systems, AQDS was a powerful catalyst in the reduction of azo dyes. Therefore, application of micromolar concentrations of AQDS during treatment of dye containing wastewater in anaerobic bioreactors may be a useful tool. Here, the effect of this redox mediator during long-term operation of a continuous bioreactor was evaluated.

5.4.1 Application of a redox mediator

The results of this study clearly demonstrated that with micromolar concentrations (19 μM) of AQDS, it was feasible to obtain highly efficient (87 ± 3%) continuous decolourisation of a recalcitrant azo dye in a UASB reactor at a practical HRT of 5.9 ± 0.7 hours. Increasing the AQDS concentration resulted in higher decolourisation efficiencies (up to >98%), reflecting the correlation between AQDS concentration and decolourisation rate previously found in batch experiments. In previous research (Chapter 2), a linear relationship was found between the VSS-concentration and the first-order decolourisation rate ($k \approx 0.12 \cdot [VSS]$). By normalising the k-values from the batch-series and the k-values from the reactor study to the biomass VSS concentration, two plots of k/[VSS] as a function of AQDS concentration were obtained (see Figure 5.2). These plots match very well, which indicates that batch-wise determined k-values are suitable to predict the decolourisation efficiency of a continuous reactor.

AQDS increases the reaction rate by acting as a redox mediator that shifts electrons between its oxidised, quinone, form (AQDS) and its reduced, hydroquinone, form (AH_2QDS). Quinones have been reported to act as redox mediators for azo dye reduction (Chapter 3 and references [23, 152, 161, 343]) as well as for other non-specific reductive transformations, i.e. reductive dehalogenation of organohalogens and reduction of nitroaromatics [96]. The mechanism of redox mediation by quinones comprises two reactions: the oxidation of the hydroquinone by a terminal electron acceptor (e.g. RR2) and the reduction of the quinone by an electron donor. As evidenced by the non-specificity of

anaerobic azo dye reduction processes, the hydroquinone will generally be oxidised by the azo dye in a direct chemical reaction. Likewise, quinone reduction has mostly been reported as a direct chemical reaction with reduced compounds like sulphide or ferrous iron [42, 74, 262-264]. However, also biological quinone reduction by specific bacteria that couple the oxidation of organic substrates to enzymatic quinone reduction has been reported, not only for situations in which a quinone acts as a terminal electron acceptor [29, 66, 195] but also for quinone mediated azo dye reduction [152, 161] and humic acid mediated dechlorination [68].

5.4.2 Role of biological activity on dye decolourisation

Conflicting views exist on the terminal reaction that is responsible for azo dye decolourisation in anaerobic sludge environments. It may be a direct chemical reaction with reduced compounds like sulphide, a reaction with reduced enzyme cofactors or a direct enzymatic reaction. Possibly, it is a mix of all three, with microorganisms regenerating chemical reducing equivalents and cofactors.

Evidence indicates that biological activity played an important role in this study: in batch vials with autoclaved sludge samples, hardly any decolourisation occurred and the decolourisation efficiency of the bioreactor was almost negligible during the period that the biological activity was severely inhibited. It should be noted, however, that sulphide, which is ubiquitous in anaerobic environments and known to be effective as a chemical dye reducer, was present in this study at concentrations that were too low (<10 μM) to account for substantial abiotic dye decolourisation.

Another evidence of the importance of biological activity for anaerobic azo dye reduction was the observation that dye decolourisation by reactor sludge was stimulated by addition of substrates. This indicates that the reactor sludge contained specialised bacteria transferring reducing equivalents to the dye while metabolising the substrates.

5.4.3 Role of electron donors

The basal dye decolourisation activity was supported by endogenous substrate in the sludge, possibly associated with hydrolysis of sludge biomass. Only a small portion of the sludge would have to be consumed to supply the required amount of reducing equivalents. In the batch vials, the amount of dye was low (5.6 μmole) in comparison to the amount of sludge (50 mg VSS). Theoretically, 22 μeq reducing equivalents or 0.36 mg COD are needed to decolourise this 5.6 μmole RR2. Assuming that 50 mg VSS equals 70 mg COD, this is about 0.5% of the sludge-COD, more or less equal to the basal amount of COD that reactor sludge converted to methane in one week (measured in sludge vials without external substrate).

Addition of electron donating primary substrates resulted generally in much higher dye decolourisation rates but VFA substrates were only effective with the adapted reactor sludge. This suggests that in the reactor, a bacterial population was enriched that could utilise reducing equivalents from these substrates to specifically reduce RR2. Hydrogen was by far the most favourable electron donor for dye decolourisation by both reactor and seed sludge. The effectiveness of hydrogen in the non-adapted seed sludge suggests the ubiquitous presence of microorganisms that utilise hydrogen to

reduce the dye.

As hydrogen will be formed as intermediate in the degradation of propionate and butyrate, it is reasonable to expect that these compounds will be preferred to acetate as electron donors for azo dye decolourisation. In accordance with this hypothesis was the observation that the azo dye Mordant Orange 1 was reduced much faster with ethanol than with acetate as the electron donating compound (Tan, et al., 1999). However, the decolourisation of RR2 in the absence of AQDS proceeded equally fast for all of the VFA, which demonstrates that interspecies hydrogen from propionate and butyrate was not available to dye reduction. Probably, the interspecies hydrogen was preferably utilised by methanogens because the sludge had a very high SMA on hydrogen, and maybe also because methanogens have a higher affinity for the low steady state concentrations than hydrogen utilising dye reducing bacteria.

AQDS accelerated the reduction of RR2 under all conditions tested. With seed sludge, the main effect of AQDS was to assist the transfer of electrons from endogenous sludge to the dye while the other substrate hardly increased the AQDS stimulated basal decolourisation rate. With reactor sludge, however, AQDS additionally stimulated the transfer of electrons from hydrogen and VFA substrates (especially acetate) to the dye. These results suggest that in the reactor sludge one or more cultures of specialised bacteria were enriched that could utilise AQDS as the electron acceptor for the oxidation of hydrogen and acetate.

5.4.4 Toxicity

After a prolonged exposure period, RR2 was very toxic to the methanogens in granular sludge. Most of the bacteria are located in biofilms containing polysaccharide slime that will delay the penetration of high molecular weight or charged inhibitors in much the same way as a molecular sieve. The delay of the full expression of this inhibition is therefore possibly due to the high molecular weight of the dye and to the negative charge of its sulphonate groups. Biologically reduced RR2 was clearly less toxic than RR2 itself. This was evident from the reduced toxicity when the time of exposure to RR2 was lowered as the reduction rates increased with increasing AQDS concentrations. Toxicity of azo dyes have been reported earlier [25, 46, 86]. Donlon *et al.*[86] reported detoxification by azo dye reduction: the IC50 concentration of Mordant Yellow 12 for acetoclastic methanogens was more than 10 times lower than that of its corresponding aromatic amines. AQDS can therefore play an important role in the detoxification of azo dyes by accelerating dye reduction. This study demonstrated that by adding AQDS, complete restoration of the biological activity of a dye inhibited bioreactor could be achieved.

Heating the dye in a soda solution also resulted in detoxification. This is probably due to hydrolytic dechlorination of the dye's triazyl group. In textile-processing industries, significant hydrolysis can be expected because of the alkaline dye batch conditions. A large fraction of the unreacted RR2 in textile effluents will therefore be less toxic than the RR2 preparations used in this study. The detoxification of triazyl-reactive azo dyes by hydrolysis is comparable to the loss of functionality of the pesticide atrazine as a result of hydrolytic dechlorination, a reaction that appears to be catalysed by acidic groups of humic substances [153, 175, 200, 241].

6

Activated carbon as redox mediator and electron acceptor during the anaerobic biotransformation of azo dyes

Abstract Redox mediators accelerate the non-specific reductive biotransformation of azo dyes by facilitating the transfer of electrons from biogenic or chemical electron donors to the dye. Dosage of soluble quinones has been successful in enhancing the reduction of azo dyes in anaerobic bioreactors. However, as soluble redox mediators are not retained in the reactors, continuous dosing will be necessary. Therefore, it would be desirable if the redox mediator could be immobilised in the bioreactor. Activated carbon (AC), which is known to contain quinone groups at its surface, may fit this role. To explore the feasibility of AC to act as a redox mediator, the reduction of a recalcitrant azo dye (hydrolysed Reactive Red 2) was studied in laboratory-scale anaerobic bioreactors, using volatile fatty acids as electron-donor. It was shown that incorporation of AC in the sludge bed greatly improved dye removal and formation of aniline, a dye reduction product. After six months of operation, the enhanced dye removal was up to 121-fold greater than the dye adsorption capacity of the AC supplied. These results indicated that AC acts as a redox mediator. Supporting evidence for this hypothesis was obtained in batch experiments. It was demonstrated that bacteria from crushed granular sludge, as well as bacteria from an acetate-oxidising quinone-reducing enrichment culture composed mainly of *Geobacter* sp., could oxidise acetate and concomitantly reduce AC. Furthermore, it was demonstrated that AC greatly accelerated the rate of chemical azo dye reduction by sulphide. The results taken as a whole clearly suggest that AC accepts electrons from the microbial oxidation of organic acids and transfers the electrons to azo dyes, accelerating the rate of their biological reduction. This constitutes the first example of biocatalysis mediated by AC.

Van der Zee, F.P., Bisschops, I.A.E. Lettinga, G. and Field, J.A. (2002) *Submitted*

6.1 Introduction

Removal of dyes is a major concern when treating textile-processing wastewater. The vast majority (60-70%) of the dyes applied in textile-processing industries are azo compounds, i.e. molecules with one or several azo (N=N) bridges linking substituted aromatic structures [46]. Discharge of dyes into the environment should be avoided, not only for aesthetic reasons, but also because many azo dyes and their breakdown products are toxic to aquatic life [61] and mutagenic to humans [59]. Azo dyes are generally persistent under aerobic conditions [251, 299]. However, under anaerobic conditions, they undergo reductive fission, yielding colourless aromatic amines, compounds that in turn generally require aerobic conditions for their biodegradation [35, 90]. Anaerobic azo dye reduction as the first stage in the complete anaerobic-aerobic degradation of azo dyes has been studied intensively and most researchers agree that it is a non-specific and presumably an extracellular process, in which reducing equivalents from either biological or chemical source are transferred to the dye.

Though most azo dyes are fortuitously reduced under anaerobic conditions, the rate of the reaction may be low, especially for reactive azo dyes (Chapter 2). This presents a problem for the application of high-rate anaerobic bioreactors for the treatment of dye-containing wastewater, as long hydraulic retention times may then be necessary to reach a satisfactory extent of dye reduction. However, this problem can be solved by making use of the property of redox mediating compounds, especially quinones, to speed up the rate of azo dye reduction by shuttling electrons from either biologically generated or chemical electron donors to the electron-accepting azo dye. In different experimental systems, redox mediators have been demonstrated to accelerate azo dye reduction. Enzyme cofactors like FAD are known as effective redox mediators for azo dye reduction [101, 111, 286] and also artificial quinones can act as redox mediators: in abiotic systems, quinones accelerated chemical azo dye reduction by sulphide (Chapter 3) as well as electrochemical azo dye reduction [23]. In biological systems, quinones were also shown to accelerate azo dye reduction by anaerobically incubated aerobic biomass [152, 161] as well as by anaerobic granular sludge (Chapters 4 and 5). Previous research in our laboratory demonstrated that continuous dosing of anthraquinone disulphonate (AQDS) at catalytic concentrations strongly increases the azo dye reduction efficiencies of anaerobic bioreactors operated at hydraulic retention times realistic for wastewater treatment practice (Chapter 5 and reference [49]). Though the effective AQDS dosage levels were low, continuous dosing implies continuous expenses related to procurement of the chemical, as well as continuous discharge of this biologically recalcitrant compound. Therefore, it is desirable to immobilise the redox mediator in the bioreactor. For this purpose, activated carbon (AC), which is known to contain many different active groups at its surface, including quinone structures [27, 97, 107], may fit.

In this study, we investigated the feasibility of activated carbon as a redox mediator for the anaerobic reduction of azo dyes. Its potential to improve the decolourisation activity of anaerobic granular sludge was tested in a number of lab-scale upflow anaerobic sludge bed (UASB) reactors and in supporting batch experiments.

6.2 Materials and methods

6.2.1 Sorption isotherm RR2

A sorption experiment was conducted to estimate the extent of adsorption of the prehydrolysed reactive dye Reactive Red 2 (RR2) to AC (Norit SA-4) in the AC-amended bioreactors. Like in the reactor experiments, RR2 was previously hydrolysed (i.e. the chloro groups were replaced by hydroxyl groups) by heating at alkaline pH [25]. This was done to prevent dye toxicity (Chapter 5).
Different quantities of RR2 were added from a 4 mM stock solution to serum vials containing 50 mg AC (Norit SA-4) in 50 ml 0.1 M phosphate buffer at pH = 7.0. The batch vials were sealed and incubated at 22 °C in a rotary shaker at 100 rpm. After 24 hours, samples were centrifuged and absorbance was measured spectrophotometrically at the λ_{max} (539 nm).

6.2.2 Reactor study

Lab-scale Upflow Anaerobic Sludge Blanket (UASB) reactors (liquid volume 0.25 l) were initiated with 35 g VSS l^{-1} anaerobic granular sludge from a distillery wastewater treatment plant (Nedalco, Bergen op Zoom, The Netherlands) and fed with a neutralised volatile fatty acid mixture (1.5 g COD l^{-1} at a 1:1:1 COD based ratio of acetate, propionate and butyrate) in basal nutrient medium containing (mg l^{-1}) NH_4Cl (280), $CaCl_2$ (5.7), KH_2PO_4 (250), $MgSO_4 \cdot 7H_2O$ (100), H_3BO_3 (0.05), $FeCl_2 \cdot 4H_2O$ (2), $ZnCl_2$ (0.05), $MnCl_2 \cdot 4H_2O$ (0.5), $CuCl_2 \cdot 2H_2O$ (0.04), $(NH_4)_6Mo_7O_{24} \cdot 5H_2O$ (0.05), $CoCl_2 \cdot 6H_2O$ (1), $NiCl_2 \cdot 6H_2O$ (1) and $Na_2SeO_3 \cdot 5H_2O$ (0.16). The hydraulic retention times of the reactors were kept constant at 5-5.5 h. Effluent was recycled at a 1:1 influent:effluent flow ratio. After a 15-days start-up phase in the absence of dye, the dye, pre-hydrolysed RR2 (45 mg/l ≈ 0.073 mM), was added to the influent of the reactors (day 0). Three reactors were used. One reactor was started up with 2.5 g Norit SA-4 mixed with the seed sludge, whereas another reactor was the control reactor to which no AC was added. The third reactor, originally operated identical to the control reactor, was amended with 0.1 g Norit SA-4 at day 46. The reactors' dye and VFA removal efficiencies were monitored regularly.

6.2.3 The effect of AC to the chemical reduction of AO7 by sulphide (batch)

To determine whether AC could accelerate chemical azo dye reduction, a series of batch experiments was conducted in which the course of the reduction of Acid Orange 7 (AO7) by sulphide was followed in the presence and in the absence of 100 mg l^{-1} AC. Controls without sulphide were incorporated to correct for dye adsorption, as well as to verify the stability of the dye. The experiment was performed with triplicate vials. AC (5.0 mg of either Norit SA-4 or Norit SX-4) was added to glass serum vials (V = 117 ml). A 60 mM $NaHCO_3$ solution was added to a liquid volume of 50 ml. Next, the vials were sealed with butyl rubber stoppers and the gas headspace was flushed for 5 minutes with oxygen-free flush gas (N_2:CO_2 80%:20%). Sulphide was added with a syringe from a 0.1 M Na_2S stock solution to obtain an initial total-sulphide concentration of either 0.5 or 1.7 mM. All vials were incubated at 25 °C in a rotary shaker at 50 rpm. After 1 day pre-incubation, the total-sulphide concentration was measured and AO7 was added from a concentrated stock solution to obtain an initial AO7 concentration of 0.14

mM. At selected intervals, absorbance was measured spectrophotometrically at λ_{max} (484 nm) and sulphanilic acid (SA) was measured by HPLC.

Furthermore, it was investigated whether AO7 adsorbed to AC could be reduced by sulphide. Fully suspended AC from sulphide-free control vials was sampled (7.5 ml, five aliquots of 1.5 ml) and each aliquot was centrifuged for 10 min. at 10,000 rpm. The liquid phase was decanted and the carbon pellet was washed (three times) with demineralised water. The washed pellet was mixed with 0.75 ml 5 g l^{-1} $NaHCO_3$ and the centrifuge cup was placed in a glass flask. Next, the flask was sealed with a rubber septum and the gas phase was flushed for 5 minutes with oxygen-free flush gas (N_2:CO_2 80%:20%). Sulphide (0.25 ml of a 0.1 M Na2S stock solution) was added with a syringe and the glass flask was incubated at 25°C. SA in the liquid phase was sampled after 1 hour and 1 day and measured by HPLC.

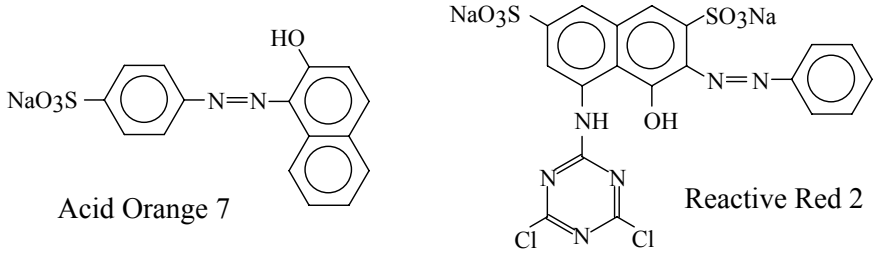

Figure 6.1 Structure formulas of the dyes used in this study

6.2.4 Biological AC reduction (batch)

To determine whether AC could act as the terminal electron acceptor for the biological oxidation of acetate, the course of the concentrations of acetate and reduced AC was followed in bacteria-inoculated batch vials with AC and acetate. A set of controls excluding either bacteria or AC or acetate was incorporated. The experiments were performed with triplicate vials. AC (1.00 or 2.00 g of either Norit SA-4 or Norit SX-4) was added to glass serum vials (V = 117 ml). A 60 mM $NaHCO_3$ solution in basal nutrient medium was added to a liquid volume of 50 ml and acetate was added by pipette from a neutralised stock solution. The vials were sealed with butyl rubber stoppers and the gas headspace was flushed for 5 minutes with oxygen-free flush gas (N_2:CO_2 70%:30%). Next, the vials were autoclaved in a pressure cooker (20 minutes at 120 °C) and allowed cooling down. Under sterile conditions, the methanogenesis inhibitor 2-Bromoethane-sulphonic acid (BES) was now added with a syringe from a filter-sterile concentrated stock solution, to a concentration of 30 mM. Finally, bacteria were added, either crushed granules from an anaerobic reactor in which acetate oxidation was coupled to the reduction of AQDS, or an AQDS/acetate enrichment culture derived from anaerobic sludge, which contained primarily *Geobacter* sp. [51].

At selected time intervals, medium or activated carbon was sampled under sterile or aseptic conditions, respectively. Acetate was measured by gas chromatography and the reduction equivalents of activated carbon were measured by reaction with Fe(III) and subsequent determination of the Fe(II) formed. At the end of the experiment, headspace samples for methane and medium samples for sulphide were taken.

6.2.5 Analysis

AO7 and RR2 colour was measured spectrophotometrically with a Spectronics 60 spectrophotometer (Milton Ray Analytical Products Division, Belgium) at the dyes' wavelengths of maximum absorbance (484 nm and 539 nm, respectively). The estimated molar extinction coefficients at these wavelengths are $22.9 \cdot 10^3$ and $38 \cdot 10^3$ cm^{-1} M^{-1} for AO7 and RR2 respectively. Liquid phase samples (0.75 ml) were centrifuged (2 minutes at 10,000 rpm) and diluted up to an absorbance of less than 0.8 in a 0.1 M phosphate buffer at pH = 7.0. The buffer contained freshly added ascorbic acid (200 mg l^{-1}) to prevent autoxidation (reactor experiments only). Without dye, light absorbance of medium and buffer was less than 1% of the absorbance right after dye addition and could therefore be neglected.

Sulphide was determined colorimetrically after reaction with *N*,*N*-dimethyl-*p*-phenylenediamine oxalate according the method described by Trüper and Schlegel [328]. Volatile Fatty Acids (VFA) and methane were determined by gas chromatography, as described in Chapter 4. Sulphanilic acid (SA) was measured by High Performance Liquid Chromatography following the method described in Chapter 3. To stop the reaction between AO7 and sulphide in the HPLC vials, sulphide was precipitated as Zinc sulphide by dilution with Zinc acetate followed by centrifugation (5', 10,000 rpm).

Analysis of reduced AC using the ferrozine technique [194] was carried out in an anaerobic chamber under N_2/H_2 (95%:5%) atmosphere. Samples reacted with Fe(III)citrate at low pH, yielding Fe(II). Next, the absorbance of the purple Fe(II)-ferrozine complex (molar extinction coefficient $28 \cdot 10^3$ cm^{-1} M^{-1}) was measured spectrophotometrically. When no Fe^{3+} was added, no Fe^{2+} was formed: the AC itself did not contain any ferrous iron.

Methane was determined by gas chromatography. The chromatograph (Packard-Becker, Delft, The Netherlands) chromatograph equipped with a 2m x 2mm steel column packed with Poropak Q (80/100 mesh). The temperatures of the column, injection port and flame ionisation unit were respectively 60, 200 and 220 °C. The carrier gas was nitrogen (20 ml per minute). Gas samples were taken with a 100 µl pressure-lock syringe (Dynatech, Baton Rouge, USA).

Volatile Suspended Solids (VSS) were determined according to standard methods [12].

6.3 Results

6.3.1 Sorption isotherm RR2

Data of the sorption of pre-hydrolysed RR2 to AC (Norit SA-4) showed almost complete dye sorption up to a dye:carbon w:w ratio of 5% and a maximum sorption capacity of 63 ± 3 mg per g. Figure 6.2 shows the sorption data, together with their fit to the sorption isotherm equations of Freundlich and Langmuir (Q = mg sorbed dye in per g AC; C = equilibrium concentration [mg/l]; K_f and n are constants of the Freundlich equation, Q_0 and K_l are constants of the Langmuir equation, with Q_0 = maximum sorption capacity [mg sorbed dye per g AC] and K_l = affinity constant [mg/l]. The

Freundlich equation provided a better fit compared to the Langmuir equation. The affinity of the carbon for the dye was very high.

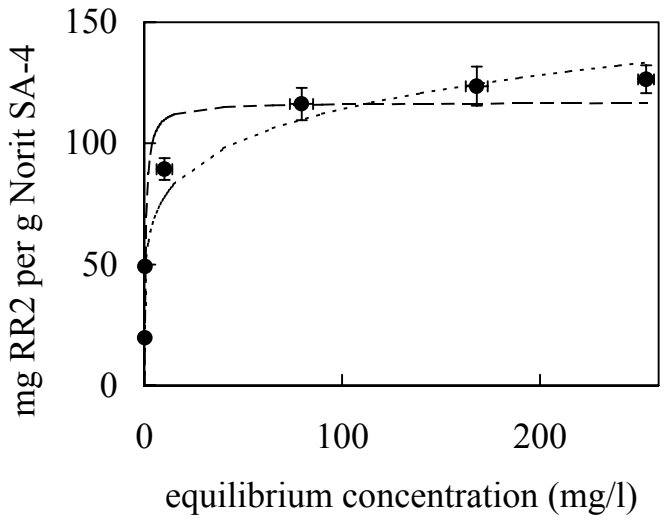

Figure 6.2 Adsorption isotherm for the adsorption of pre-hydrolysed RR2 on activated carbon (Norit SA-4). Experimental data (full circles) with standard deviations (error bars);
Freundlich fit: $Q = K \cdot C^n = 52.7 \cdot C^{0.17}$ (dotted line)
Langmuir fit: $Q = (Q_0 \cdot C)/(K+C) = (116.8 \cdot C)/(0.67+C)$ (dashed line)

6.3.2 Reactor study

The effect of AC on reductive biotransformation of azo dyes was studied in laboratory scale UASB reactors. The reactors were initiated with anaerobic granular sludge (8.75 g VSS) and fed with the VFA substrate (1.5 g COD l^{-1}) in basal nutrient medium. The dye, pre-hydrolysed RR2 (45 mg l^{-1}; 0.073 mM), was added to the influent after a 15-days start-up phase in the absence of dye. This was defined as the start of the experiment (day 0 in Figures 6.3 and 6.4). At this point of time, the removal of acetate and

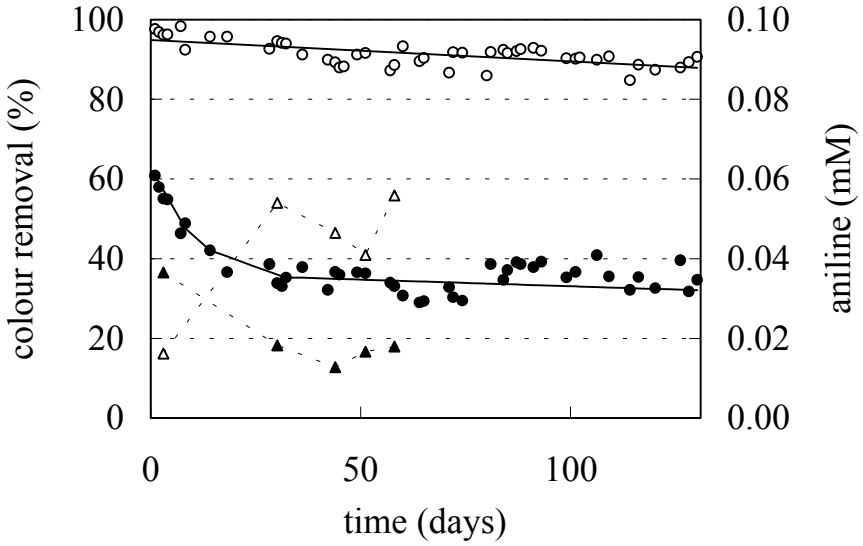

Figure 6.3 Dye removal efficiency (circles) and effluent aniline concentration (triangles) of the reactor with 2.5 g Norit SA-4 (open markers) and the control reactor without activated carbon (closed markers).

butyrate was complete and remained complete (>95%) during the entire period of reaction operation, whereas complete and stable propionate removal (>95%) was achieved from day 25.

In the first phase of the experiment, two reactors were used. One reactor was started up with 2.5 g Norit SA-4 mixed with the seed sludge, whereas the other reactor was the control reactor to which no AC was added. The dye removal efficiencies (based on λ_{max} absorbance measurements) of these reactors are depicted in Figure 6.3. The dye removal efficiency of the control reactor was much lower than that of the carbon-amended reactor.

In the control reactor, the dye removal during the first two to four weeks immediately after RR2 addition had started, could be partly attributed to adsorption of the dye onto the reactor sludge, and possibly also to wash-out of sludge material with mediating capacity. As the adsorption capacity of the sludge became exhausted, the dye removal efficiency decreased to about 35%. This level, presumably corresponding to biological RR2 reduction, remained more or less stable for the rest of the experiment (up to day 130). Granules sampled from the reactor sludge were dark red, indicating the presence of adsorbed dye. In the AC-amended reactor, the dye removal efficiency was almost complete (97%) immediately after RR2 addition, decreased to approximately 90% after 40 days and remained at that level for the rest of the experiment.

During the entire experiment (130 d), the AC-amended reactor had removed 5.895 g RR2, whereas the control reactor had removed 2.385 g RR2, i.e. 3.51 g RR2 had been additionally removed in the carbon-amended reactor. With the observed RR2 sorption capacity of the AC that was used in these experiments (maximum 63 ± 3 mg RR2 per g Norit SA-4), it can be estimated that at the maximum 0.1575 g RR2 would be removed by carbon sorption. Consequently, an additional dye removal of 3.51 g RR2 exceeds the sorption capacity by more than 22-times.

These data suggest that the major role of AC was to enhance the chemical conversion of dye rather than dye adsorption. To verify the enhanced chemical conversion of the dye, the reaction product aniline was monitored at selected time points between days 3 and 58. From the aniline data presented in Figure 6.3 it can be seen that the higher dye removal efficiency of the AC-amended reactor corresponds to higher aniline concentrations in the reactor effluent during steady state operation, confirming an enhanced reduction of the dye due to AC catalysis. This indicates that the carbon-amended reactor reduced a higher fraction of the dye than the control reactor without AC. In both reactors, the aniline recovery corresponded to the expected stoichiometry based on dye removal (68 ± 15% and 79 ± 12% for the control reactor and the carbon-amended reactor, respectively), indicating that the reduction was the major mechanism of dye removal.

An additional reactor was used in this experiment, in which a much smaller amount of AC was supplied. The reactor sludge was mixed with 0.1 g Norit SA-4. The performance of this reactor was compared with the performance of the control reactor described previously. Addition of AC was postponed until day 46 to verify that both the AC-amended and the control reactors performed the same before AC addition. Figure 6.4 confirms that both reactors had the same pattern of dye removal preceding the addition of AC. The three following the AC addition showed a relatively rapid decline of the colour removal efficiency (from ~78% to ~50%), presumably corresponding to wash-out of AC, followed by a

slight gradual further decline, to ~43% after 112 days of reactor operation with AC. The dye removal efficiency of the AC-amended reactor throughout the entire experiment remained significantly higher than that of the control reactor. During the 158 days of operation, the carbon-amended reactor had removed 3.6 g RR2, whereas the control reactor had removed 2.835 g RR2, i.e. 0.765 g RR2 had been additionally removed in the carbon-amended reactor. In this reactor, the estimated dye removal due to sorption is very limited: using the observed maximum RR2 sorption capacity of 63 mg RR2 per g Norit SA-4, carbon sorption would only account for the removal of 0.0063 g RR2. Consequently, the observed additional dye removal of 0.765 g RR2 exceeds the dye sorption capacity of AC by more than 121-times. The results from these continuous experiments clearly reveal a role of AC in the catalysis of azo dye reduction. In order to get more evidence for the catalysis, we conducted two supporting batch experiments. The first experiment was meant to assess catalysis of AC in the direct chemical reduction of an azo dye by sulphide. The second experiment was designed to demonstrate that AC could accept electrons from the microbial oxidation of the VFA substrate.

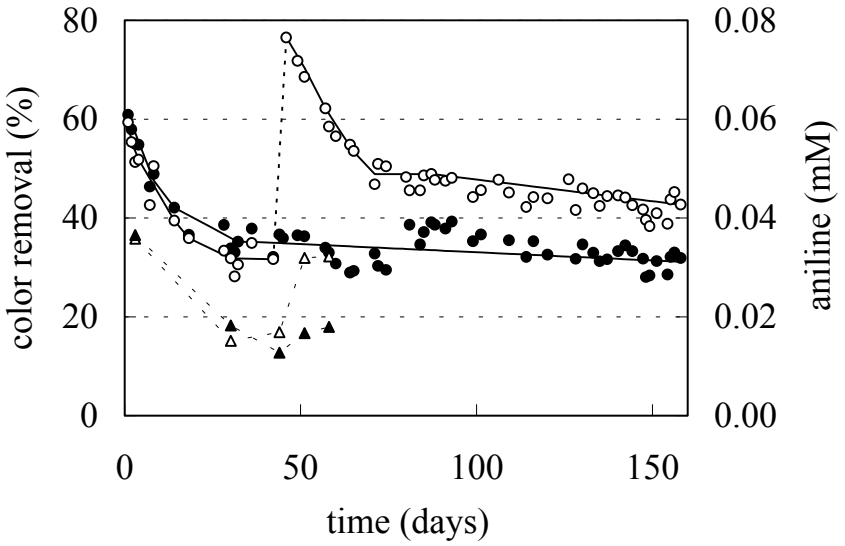

Figure 6.4 Dye removal efficiency (circles) and effluent aniline concentration (triangles) of the reactor with 0.1 g Norit SA-4 (open markers) and the control reactor without activated carbon (closed markers).

6.3.3 AC catalysed chemical azo dye reduction

A first series of batch experiments was performed to determine whether low amounts of activated carbon could accelerate the simple chemical reduction of azo dyes by sulphide. For that purpose, the decolourisation of AO7 and the production of sulphanilic acid (SA), one of the dye's reductive cleavage products was followed in time by monitoring the concentrations of the dye and SA in the presence and in the absence of a low amount of AC. Controls without sulphide were incorporated to determine the extent of dye adsorption and to verify the stability of the dye. The results of these experiments, presented in Figure 6.5, show that AC strongly accelerates the reduction of AO7 and the concomitant production of SA. With 100 mg l^{-1} Norit SA-4 and an initial sulphide concentration of 0.5 mM, the AO7 absorbance rapidly decreased by 80% within five days to its final level; whereas, without AC, only 40% was

removed within two weeks (Figure 6.5A). The reduced product, SA, was formed only in treatments with sulphide. In the sulphide-free controls with AC, 22% of the dye was removed but no SA was produced. These results indicate that the removal of AO7 in the presence of sulphide and AC was a chemical reaction, whereas the removal of AO7 in the presence of AC alone was only due to adsorption.

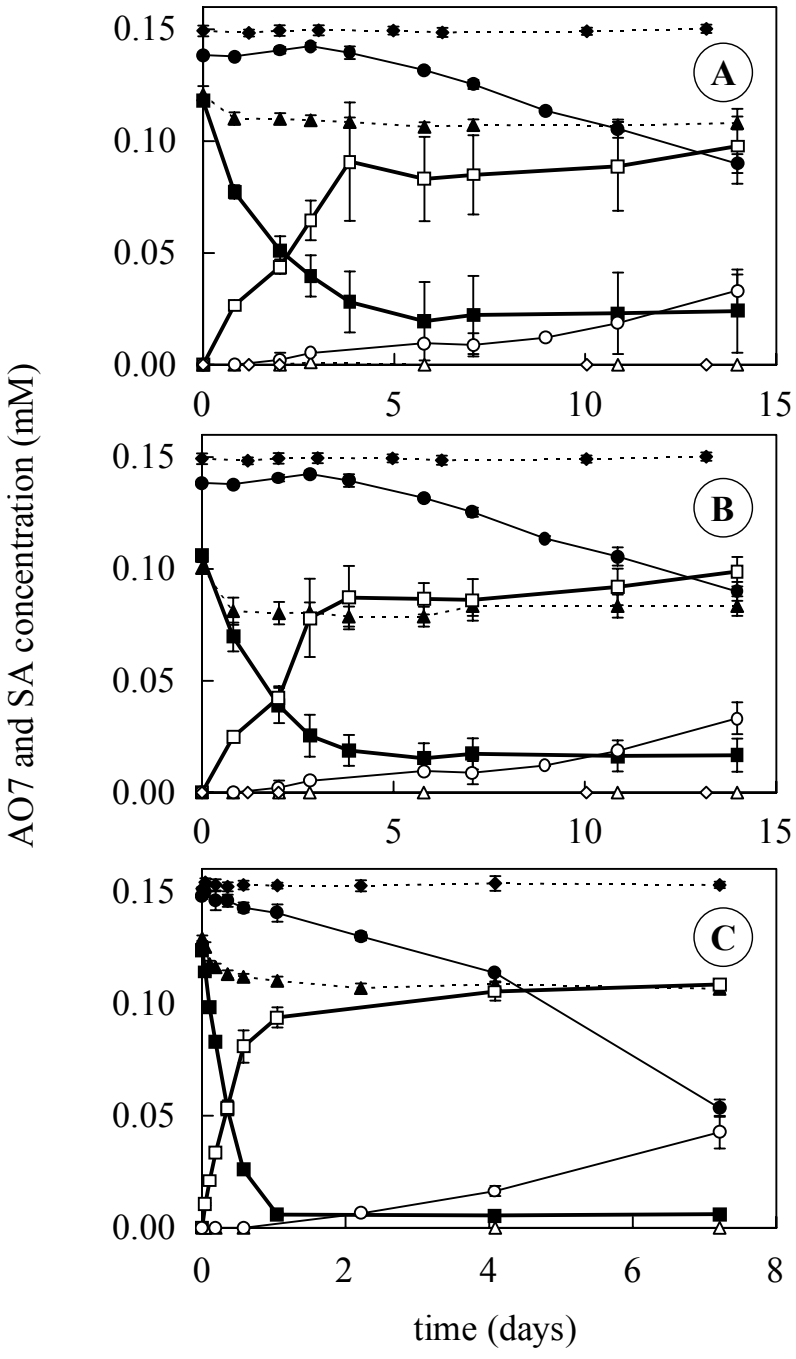

Figure 6.5 Activated carbon catalysis of AO7 reduction by sulphide. Dye concentration (full markers) and SA concentration (open markers) – squares: AO7 + sulphide + activated carbon; circles: control without activated carbon; triangles: control without sulphide; diamonds: dye only. The error bars represent standard deviations between triplicate measurements.

A. 100 mg l^{-1} Norit SA-4; initial total-sulphide concentration 0.51 ± 0.02 mM;
B. 100 mg l^{-1} Norit SX-4; initial total-sulphide concentration 0.51 ± 0.02 mM;
C. 100 mg l^{-1} Norit SA-4; initial total-sulphide concentration 1.73 ± 0.05 mM.

A second experiment was conducted using the same quantity (100 mg l^{-1}) of a different type of AC (Norit SX-4). The results of this experiment (Figure 6.5B) were similar to those of the experiment with Norit SA-4, aside from a relatively higher degree of dye adsorption (42%) by the AC in sulphide-free controls. At the end of this experiment, dye-sorbed carbon from vials with AC in the absence of sulphide was sampled, washed and centrifuged. Next, a concentrated sulphide solution (25 mM) was added and samples were taken to determine whether the adsorbed dye would be reduced. After one hour anaerobic incubation, 0.02 mmol SA was released per g Norit SX-4 and one day incubation resulted in the release of 0.59 mmol SA per g Norit SX-4, an amount almost equal to the amount of AO7 that was estimated to be sorbed. Therefore, the adsorbed dye is reversibly bound to the carbon and can be reduced.
In a third experiment, the AC Norit SA-4 was used again but with a higher initial sulphide concentration (1.73 ± 0.05 mM). Figure 6.5C shows that in this experiment, the decolourisation of AO7 and the concomitant formation of SA in the assays with AC and sulphide proceeded more rapidly, mainly within the first day of incubation. In contrast, reduction of the dye by sulphide in the absence of AC took more than 7 days. Also in this experiment, no sulphanilic acid was formed in the assays without sulphide.
The molar fraction of removed AO7 that was recovered as SA in the assays with AC and sulphide was 85 ± 1, 85 ± 3 and 80 ± 5 % for the experiments presented in Figures 6.5A, 6.5B and 6.5C, respectively. Possibly, the 15-20% gap between the recovered and the expected amount can be explained by delayed reduction of the AC sorbed dye, i.e. part of the sorbed dye may not have been reduced during the course of the experiment. However, the fraction of removed AO7 recovered as SA in the assays with sulphide in the absence of AC was lower, 69 ± 20% for the experiments present in Figures 6.5AB and only 43 ± 7 % for the experiment presented in Figure 6.5C. The reason for this low recovery is not known. Incomplete reduction, i.e. formation of colourless intermediates like the hydrazo complex, may be an explanation. Azo dye reduction has been suggested to involve two separate transfers of two electrons, with the hydrazo complex as the product of the first step [87].

6.3.4 Biological AC reduction

If AC accelerates the reduction of azo dyes via redox mediation, the bacteria in the sludge must be able to transfer electrons to AC. Therefore it should be feasible to demonstrate that AC is a terminal electron acceptor for the oxidation of organic substrates. To test the hypothesis for biological oxidation of acetate, two quinone respiring bacterial consortia were evaluated based on the assumption that quinone surface groups on AC were the redox mediating moieties. One of these consortia was crushed granular sludge from a laboratory-scale anaerobic bioreactor in which acetate oxidation was coupled to the reduction of AQDS. The other consortium was an acetate-oxidising-AQDS-reducing enrichment culture derived from the aforementioned sludge and predominated by *Geobacter* sp. The microbial consortia were incubated with acetate and a large amount of AC (20 or 40 g l^{-1}). To prevent the flow of electrons to methanogens, the cultures were supplemented with 30 mM 2-Bromoethanesulphonate (BES). The acetate concentration, as well as the concentration reduced carbon (measured after reaction with Fe^{3+} as Fe^{2+}-equivalents) was monitored in time. Controls without acetate, without AC and without bacteria, were incorporated. The time-course of acetate consumption

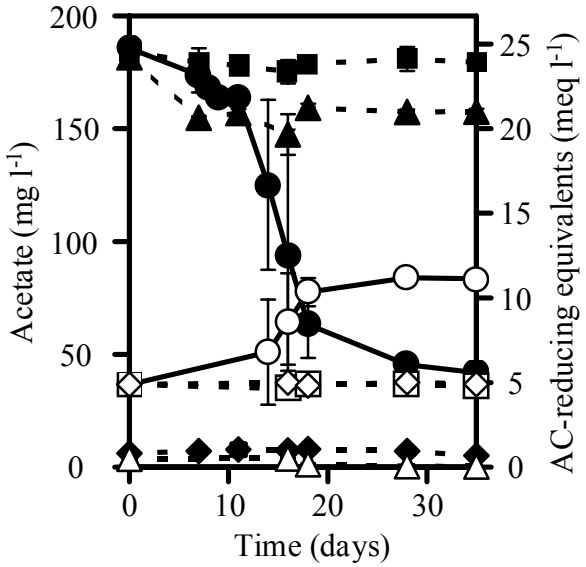

Figure 6.6 Oxidation of acetate and concomitant reduction of AC (20 g l^{-1} Norit SA-4) by a crushed granular sludge inoculum in basal nutrient medium with 60 mM $NaHCO_3$ buffer under a 80%:20% N_2:CO_2 atmosphere at pH 7.2 and T = 30 °C. Acetate concentration (full symbols) and AC-reducing equivalents concentration (open symbols): treatments with acetate, AC and inoculum (circles); treatments with acetate and inoculum (triangles); treatments with acetate and AC (squares); treatments with AC and inoculum (diamonds). The error bars represent standard deviations between triplicate measurements.

Table 6.1 Microbial acetate oxidation with AC as the terminal electron acceptor

nr.	AC type (concentration)[a]	culture	$\Delta C2$[b] meq-e^-/l[d]	ΔAC_{red}[c] meq e^-/1[d]	$\Delta AC_{red}/\Delta C2$ %
1	Norit SA-4 (20 g l^{-1})	crushed sludge	16.9 ± 0.7	6.3 ± 0.6	37
2	O_2 pre-treated Norit SX-4 (20 g l^{-1})	crushed sludge	19.2 ± 0.3	6.3 ± 0.3	39
3	Norit SX-4 (20 g l^{-1})	crushed sludge	13.6 ± 0.5	5.4 ± 0.3	51
4	Norit SX-4 (40 g l^{-1})	crushed sludge	28.4 ± 0.8	13.1 ± 1.5	52
5	Norit SA-4 (20 g l^{-1})	enrichment culture	15.9 ± 0.3	7.3	50
6	Norit SA-4 (20 g l^{-1})	enrichment culture	NA[e]	5.2 ± 0.6	NA[e]

[a] per litre liquid

[b] overall decrease in acetate concentration $\Delta C2 = \Delta C2_{C2,\ AC,\ bacteria} - \Delta C2_{C2,\ no\ AC,\ bacteria} - \Delta C2_{C2,\ AC,\ no\ bacteria} - \Delta C2_{AC,\ bacteria,\ no\ C2}$; the latter two terms are negligible

[c] overall increase in AC reducing equivalents per litre $\Delta AC_{red} = \Delta AC_{C2,\ AC,\ bacteria} - \Delta AC_{C2,\ no\ AC,\ bacteria} - \Delta AC_{C2,\ AC,\ no\ bacteria} - \Delta AC_{AC,\ bacteria,\ no\ C2}$; the latter three terms are negligible

[d] electron milli-equivalents per litre: acetate (8 meq-e^-/mmol), AC (1 meq-e^-/meq Fe^{3+} reduced to Fe^{2+}); standard deviations of triplicate measurements are given behind the ± -sign

[e] not available (failed acetate measurements).

and the concomitant formation of additional reducing equivalents in AC is shown in Figure 6.6 for a typical experiment with crushed sludge. The results clearly show the microbial consortium is only able to significantly consume acetate when AC is provided as an electron acceptor, and that AC is only reduced when acetate is provided as an electron donor. The results from additional experiments utilising either a different type of AC or the enrichment culture provided similar results as is summarised in Table 1. Headspace samples analysed for methane showed that methanogenesis did not occur in any of the assays (data not shown). The removal of acetate in the control assays without bacteria ($\Delta C2_{C2,\ AC,\ no\ bacteria}$) was negligible, even though sampling for reduced carbon measurements

could not be completely sterile. The removal of acetate in the controls without carbon ($\Delta C2_{C2,\ no\ AC,\ living}$) was less than 30 mg COD/l or 3.75 milli-electron equivalents (meq-e^-)/l. in all experiments. Reduction of AC did not occur in any of the control assays. The basal level of reducing equivalents in AC (about 6 meq-e^-/l) is due to the intrinsic reactivity of acidified AC with Fe^{3+}. A three-day pre-treatment of moist AC in a 100% O_2 atmosphere only reduced the basal level of reducing equivalents to 5 meq-e^-/l.

The significant differences between the test assays and the controls clearly show that the both the *Geobacter* predominated enrichment culture and the granular sludge biomass are capable of oxidising acetate with AC as the terminal electron acceptor. In Table I, the increase in the reducing equivalents of AC (ΔAC_{red}) is shown to be from 37 - 52% of the electron equivalents contained in the decreased acetate concentration ($\Delta C2 = \Delta C2_{C2,\ AC,\ bacteria} - \Delta C2_{C2,\ no\ AC,\ bacteria}$). The hole in the electron equivalent balance can be explained in part by cell yield and possibly also by incomplete recovery of soluble Fe^{2+} after reacting AC with Fe^{3+}.

6.4 Discussion

6.4.1 Evidence of role AC as electron acceptor and redox mediator

The results of this study taken as a whole suggest that microorganisms in anaerobic sludge can transfer electrons from substrate oxidation to electron accepting groups on AC. The reduced AC can then channel the electrons to the reduction of azo dyes, thereby enabling AC to act as a redox mediator. The main evidence for these observations are outlined below:

(i) The enhanced removal of RR2 in AC-amended as compared to non-amended continuous bioreactors was in large excess (up to 121-fold) of the adsorption capacity of the AC. The main effect of AC was clearly to enhance azo dye reduction rather than adsorption as is also evidenced by the improved production of aniline, a reduction product of RR2, in the AC-amended bioreactors.

(ii) Microorganisms incubated with acetate and AC, oxidised acetate and reduced AC, whereas no reaction took place in controls where one of the ingredients (acetate, AC or bacteria) was omitted. These results clearly demonstrate that bacteria are capable of utilising AC as a terminal electron to support the oxidation of acetate.

(iii) AC was shown to catalyse the reduction of the defined azo dye, AO7 by sulphide. There was very limited dye reduction in the presence of only sulphide and in the absence of sulphide, the dye was only partially adsorbed by AC. Significant formation of sulphanilic acid, the reduction product of AO7, only occurred if sulphide and AC were both present. These observations suggest that reduced AC is able to transfer electrons to the azo dye, AO7, and therefore function as a redox mediator.

6.4.2 Role of AC in Bioreactors

The application of AC in bioreactors is well established. However, application in such systems has been based on the adsorbent properties of AC. Several mechanisms have been proposed to explain how AC enhances the performance of bioreactors. The ability of AC to adsorb shock loads of inhibitory pollutants is one of the key mechanisms involved and the AC can be regenerated in situ by biodegradation of the slowly desorbing pollutants [24, 79, 311, 312].

6.4.3 Role of AC in Catalysis

Apart from its sorption properties, AC is generally considered as an inert material. To our knowledge, chemical catalysis caused by AC in combination with microorganisms has not been established before. In one previous study, a possible catalytic role of AC during the biological reduction of 2,4,6-trinitrotoluene (TNT) in an AC-amended anaerobic bioreactor, was suggested [220]. At high temperatures (>250°C), catalysis by AC is well established. AC has been reported to catalyse several chemical reactions, e.g. reductive dechlorination of chloroalkanes and hydrogenation of aldehydes [84], gas phase hydrogenolysis of substituted benzene compounds [14, 289] and oxidative dehydrogenation of ethylbenzene [259, 260]. The only well established example of cold catalysis by AC is the oxidation of sulphide to elemental sulphur by elemental oxygen [55, 75, 172]. In many respects the latter example is comparable to the observed catalysis in this study, in which sulphide oxidation was linked to azo dye reduction, only the dye replaces O_2 as the electron accepting species.

The mechanism of AC catalysis for redox reactions is generally not fully established but reactions involving quinone-groups on AC have been proposed [69, 259]. Quinone- moieties are known to be present on the AC surface [27, 97, 107]. Therefore, it is plausible that quinone reduction to hydroquinone is the first step in the catalysis. Hydroquinones are than expected to react with azo dyes. Many types of bacteria are known which are capable of reducing soluble quinone compounds, as well as quinone moieties in humus [50, 96, 195]. In this study, an acetate-oxidising quinone-reducing enrichment culture readily utilised AC as an electron acceptor. Therefore, it is justified to propose a mechanism of acetate oxidation that involves reduction of quinone-moieties in AC. While the electron accepting capacity of AC is low (only approximately 6-mg acetate could be oxidised per gram of AC), the electrons accepting moieties in AC were continuously regenerated by transferring electrons to azo dyes. By comparison, soluble quinones are known to greatly accelerate azo dye reduction at extremely substoichiometric concentrations [161] (Chapters 3-5).

The catalytic properties of AC enable its use as a biologically regenerable redox mediator in anaerobic bioreactors to accelerate the reduction of azo dyes. As AC can be retained in the reactor for prolonged time, it is an attractive alternative to soluble redox mediators (e.g. AQDS) that do not retain and thus need to be dosed continuously. The applicability of AC-amended bioreactors is probably not limited to enhancing the reduction of azo dyes, as also other reactions are known to be accelerated by quinone-type redox mediators, e.g. reductive dehalogenation and reduction of nitroaromatics [95]. With this respect, it is even possible that earlier reports of enhanced removal of several compounds in AC-amended bioreactors [24, 311] was at least partly due to redox mediation by AC

7

Summary and discussion

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7.1 Introduction

Dyeing of fabrics in textile-processing industries results in dye-containing wastewaters. Removal of dyes from these wastewaters is desired, not only because of their colour but also because of their toxicity, mutagenicity and carcinogenicity. Different physical, chemical and biological techniques can be used to remove dyes from wastewater. Each technique has technical and economic limitations. Most physicochemical dye removal methods have drawbacks because they are too expensive, have limited versatility, are greatly interfered by other wastewater compounds and/or generate waste products that must be handled. Alternatively, biological treatment holds promise as a relatively inexpensive way to remove dyes from wastewater. At least dyes belonging to the largest class of dyes, the azo dyes, are prone to bacterial biodegradation.

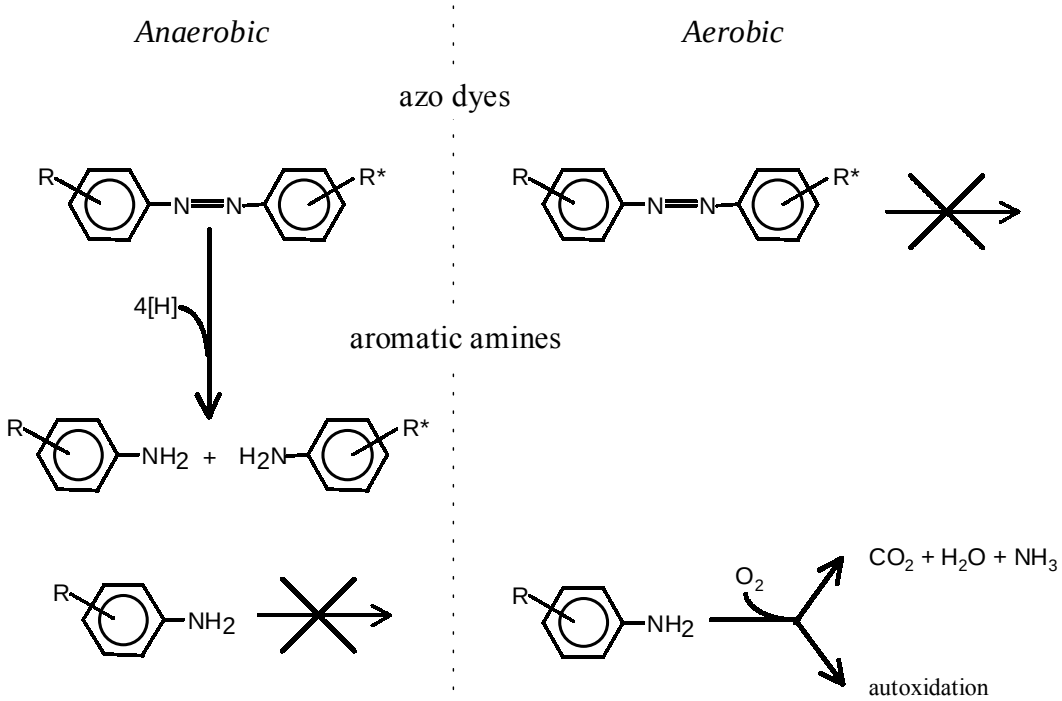

Figure 7.1 General overview of the fate of azo dyes and aromatic amines during anaerobic-aerobic treatment

Azo dyes are aromatic structures linked together by an azo (-N=N-) chromophore. As the electron-withdrawing nature of the azo linkage(s) obstructs the susceptibility of the dye molecules to oxidative reactions, azo dyes generally resist aerobic biodegradation. In contrast, reductive cleavage of azo linkages may fortuitously occur under anaerobic conditions. This anaerobic reduction implies decolourisation as the azo dyes are converted to –usually colourless but potentially harmful- aromatic amines. These aromatic amines are generally not further degraded under anaerobic conditions. However, under aerobic conditions, aromatic amines are likely to be removed from the water phase by a combination of biodegradation, autoxidation and adsorption processes. Sequential or integrated anaerobic-aerobic treatment is therefore the most logical strategy for the complete removal of azo dyes in biological systems (Figure 7.1)

Evaluation of the literature on anaerobic-aerobic treatment of azo dyes (Chapter 1) revealed two possible bottlenecks: (i) anaerobic azo dye reduction is a time-consuming process, reflected by the requirement of long reaction times; and (ii) the fate of aromatic amines during aerobic treatment is not conclusively elucidated.
This thesis discusses research that was done to solve the first possible bottleneck. The objective of this research was to optimise the first stage of the complete biodegradation of azo dyes, anaerobic azo dye reduction. This research was done by studying the reaction mechanism and by consequently applying the obtained insights.

7.2 General features of anaerobic azo dye reduction

Anaerobic azo dye reduction has generally being looked upon as a non-specific process in which the azo dye fortuitously accepts electrons released from the biological oxidation of primary electron donors. Chapter 2 of this thesis surveyed the reduction of twenty chemically distinct azo dyes by anaerobic granular sludge. The results were in agreement with this general view. All of the dyes tested were reduced by the non-adapted sludge, generally yielding colourless products. The reaction proceeded without lag-phase and followed first-order kinetics.
The reaction rates were found to vary greatly between the dyes: the half-life times of the dyes in the applied standard batch assay ranged from 1 to about 100 hours. Especially reactive dyes with a triazine reactive group reacted slowly. There was no correlation between a dye's half-life time and its molecular weight, which indicated that membrane penetration of the dyes was probably not important. This observation, combined with the non-specificity and lack of any lag-phase, pointed to an extracellular mechanism involving reduced compounds or non-specific enzymes. However, biological activity is not a prerequisite for anaerobic azo dye reduction, as it was furthermore demonstrated that the azo dyes could be reduced purely chemically by sulphide.

7.3 Biotic versus abiotic azo dye reduction

As dye containing wastewaters usually contain sulphate and other sulphur species that will be biologically reduced to sulphide during treatment in anaerobic bioreactors, azo dye reduction in anaerobic bioreactors will be a combination of biotic and abiotic processes. To evaluate the relative contribution of biological and chemical azo dye reduction in anaerobic sludge, the reduction rates of two azo dyes were compared in batch assays over a range of sulphide concentrations in the absence or presence of living or inactivated anaerobic granular sludge (Chapter 4).
The importance of biological azo dye reduction was clearly demonstrated by the observation that in assays lacking sulphur compounds, azo dye reduction only readily occurred in the presence of living granular sludge. Introduction of sulphide (or sulphate in accordance to biogenic sulphide formation from sulphate reduction) resulted in additional azo dye reduction due to chemical reactivity. This

effect was additive, as the rate of azo dye reduction in those cases corresponded to the sum of the biological rate and the chemical rate. The mechanisms are mutually independent, i.e.

- living or γ-irradiated sludge does not affect chemical azo dye reduction by sulphide;
- sulphide does not affect biological azo dye reduction, which demonstrates that the terminal reduction of azo dyes is not based on a reaction with biologically recycled sulphide;
- sulphate does not affect biological azo dye reduction, which demonstrates that sulphate does not compete with the dye as an electron acceptor.

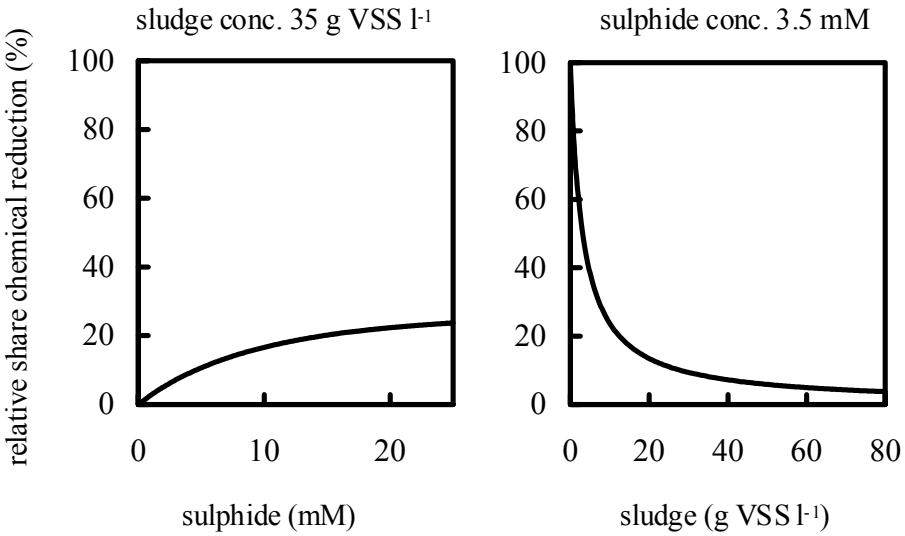

Figure 7.2 Estimation of the relative share of chemical azo dye reduction in the reduction of AO7 by anaerobic granular sludge in the presence of sulphide: (A) at different sulphide concentrations in the presence of 35 g VSS l^{-1} anaerobic granular sludge; (B) at different sludge concentrations in the presence of 3.5 mM sulphide. Based on kinetic data presented in Chapter 4.

Analysis of the kinetics indicated that the relative importance of chemical azo dye reduction in high-rate anaerobic bioreactors would be small due to the high biomass levels in the reactors. Chemical azo dye reduction by sulphide will generally be negligible, even when the bulk sulphide concentration is largely in excess over the dye concentration. In anaerobic reactors, chemical azo dye reduction by sulphide will therefore only substantially contribute to the overall azo dye reduction when the biomass concentrations are very low or –to a lesser extent- when the sulphide concentrations are extremely high (Figure 7.2). In continuous experiments in lab-scale upward-flow anaerobic sludge blanket (UASB) reactors, the low contribution of chemical azo dye reduction was confirmed by the observation that sulphide, formed upon the introduction of sulphate, did not affect the dye removal efficiency

The mechanism of biological azo dye reduction is further discussed in section 7.5.

7.4 Role of redox mediators

The observation that chemical azo dye reduction by sulphide proceeds relatively slow, even when the sulphide concentration is in large excess of the dye concentration (Chapters 2, 3 and 4), points at a bottleneck in the transfer of electrons from sulphide to the azo linkage. However, there are ways to solve this problem. In chapter 3, when chemical azo dye reduction was further explored it was noticed that the reaction between Acid Orange 7 (AO7) and sulphide appeared to be accelerated in time

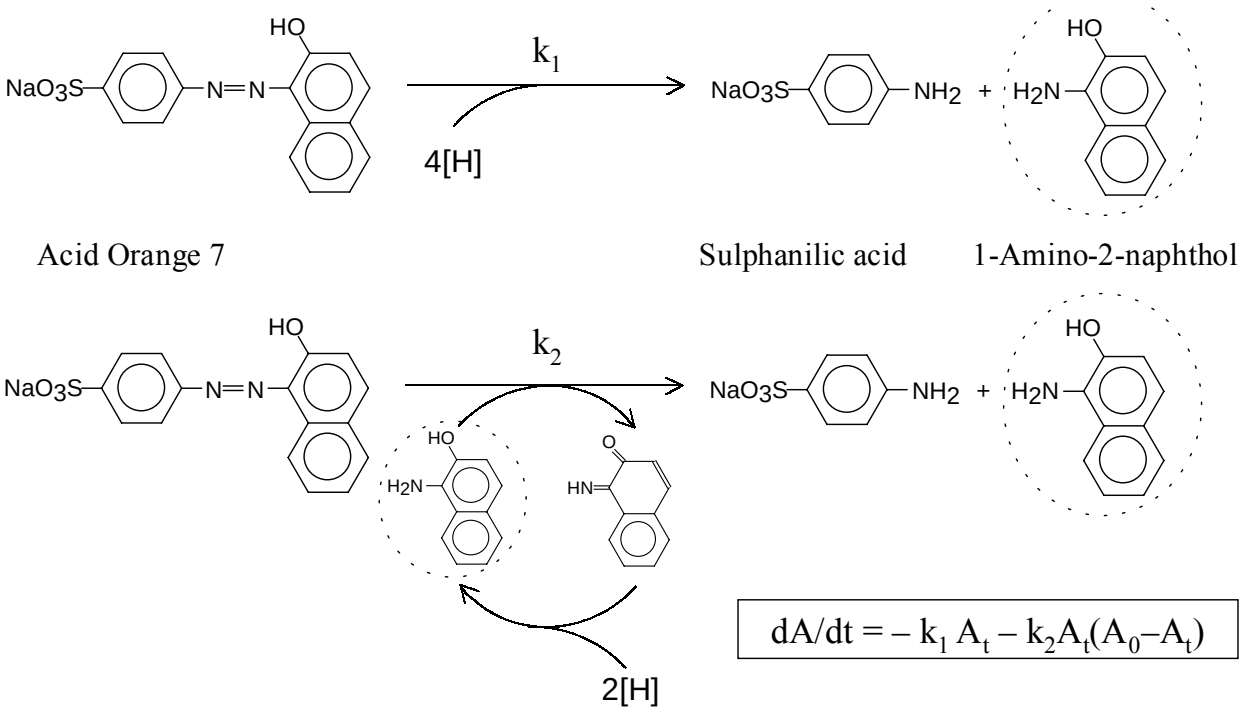

Figure 7.3 Schematic and mathematical representation of autocatalysed AO7 reduction. [H] = reducing equivalent; k_1 and k_2 refer to the reaction constants of the mathematical description given in the frame: k_1 = first-order constant for the direct chemical reaction; k_2 = second-order rate constant for the autocatalytic reaction; A_t = dye concentration at time t; A_0 = dye concentration at t=0.

according to the extent to which the dye was reduced. Mathematical evaluation of the experimental results pointed out that autocatalysis played an important role in the chemical reduction of AO7. Further tests made clear that 1-amino-2-naphthol was the dye's constituent aromatic amine that accelerated the reduction process. This observation was analogous with the reported catalysis of azo dye reduction by quinone compounds [23, 161]. The effect of 1-amino-2-naphthol is therefore possibly based on redox mediation. According to the proposed mechanism (Figure 7.3), 1-amino-2-naphthol (interchanging with its oxidised aminoquinone form) shuttles electrons from sulphide to the dye, thereby accelerating the reaction.

The impact of redox mediators was further evaluated by testing the effect of anthraquinone-2,6-disulphonate (AQDS), a humic analogue that has often been used as a redox mediator for reductive transformations. AQDS appeared to be a much more powerful redox mediator than 1-amino-2-naphthol. For comparison, while 1-amino-2-naphthol increased the first-order chemical reduction rate

constants by a factor 2 to 10, AQDS was 10 times more powerful, increasing the rate constants by a factor 10 to 100.

In the course of this thesis research, several other compounds and substances have been found to stimulate the reduction of azo dyes by sulphide. Among those were the artificial quinone compounds anthraquinone-2-sulphonate (AQS) and resazurin as well as the flavin enzyme cofactor precursor, riboflavin (Figure 7.4). Furthermore, redox mediation by activated carbon (see section 7.6.2) and autoclaved sludge was demonstrated.

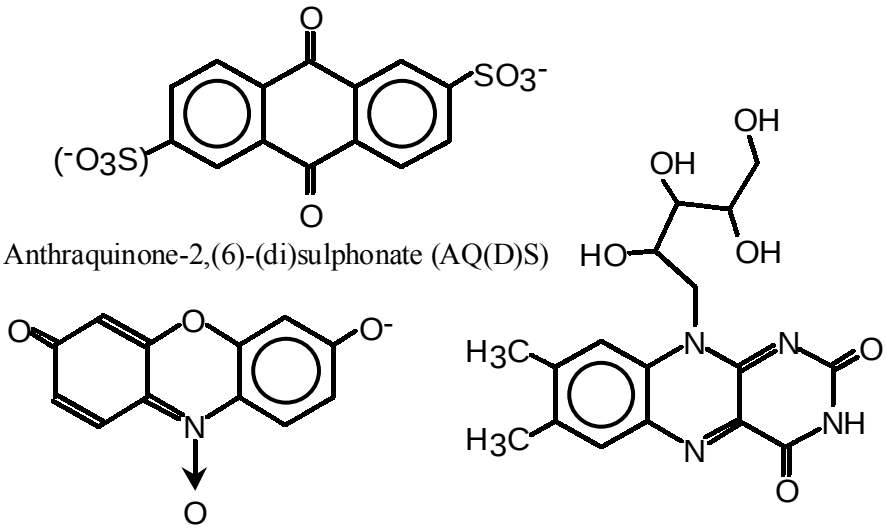

Figure 7.4 Structure formulas of some of the redox mediators used in this research

Stimulation of azo dye reduction by autoclaved sludge, first reported in Chapter 3, was further investigated in Chapter 4. Here, the effect of autoclaved sludge was compared with the effect of γ-irradiated sludge. It was observed that autoclaved sludge greatly stimulated the rate of azo dye reduction by sulphide, in sharp contrast to γ-irradiated sludge, which had no stimulatory effect at all. As autoclaving disrupts the cells, whereas irradiation inactivates biological activity while leaving the cell structure intact, these observations suggested that redox mediating enzyme cofactors released by cell lysis contribute to the stimulatory effect. This hypothesis was supported by the great acceleration of chemical azo dye reduction by riboflavin. Riboflavin is representative of the heat stable redox-mediating moieties of common occurring flavin enzyme cofactors

Redox mediator catalysis of azo dye reduction is not restricted to the chemical reaction with sulphide but it also applies to the biological azo dye reduction mechanism. This was demonstrated in Chapters 4 and 5, by the observation that catalytic concentrations of AQDS greatly stimulated the reduction of azo dyes by living granular sludge in the absence of sulphur compounds.

7.5 Role of bacteria

7.5.1 Biological azo dye reduction

As discussed above, the non-specific and presumably extracellular reduction of azo dyes in anaerobic sludge is mainly a biological process. Azo dyes are biologically reduced in a direct enzymatic reaction (Figure 7.5 –scheme I) or indirectly, in a reaction with reduced enzyme cofactors (Figure 7.5 –scheme II).

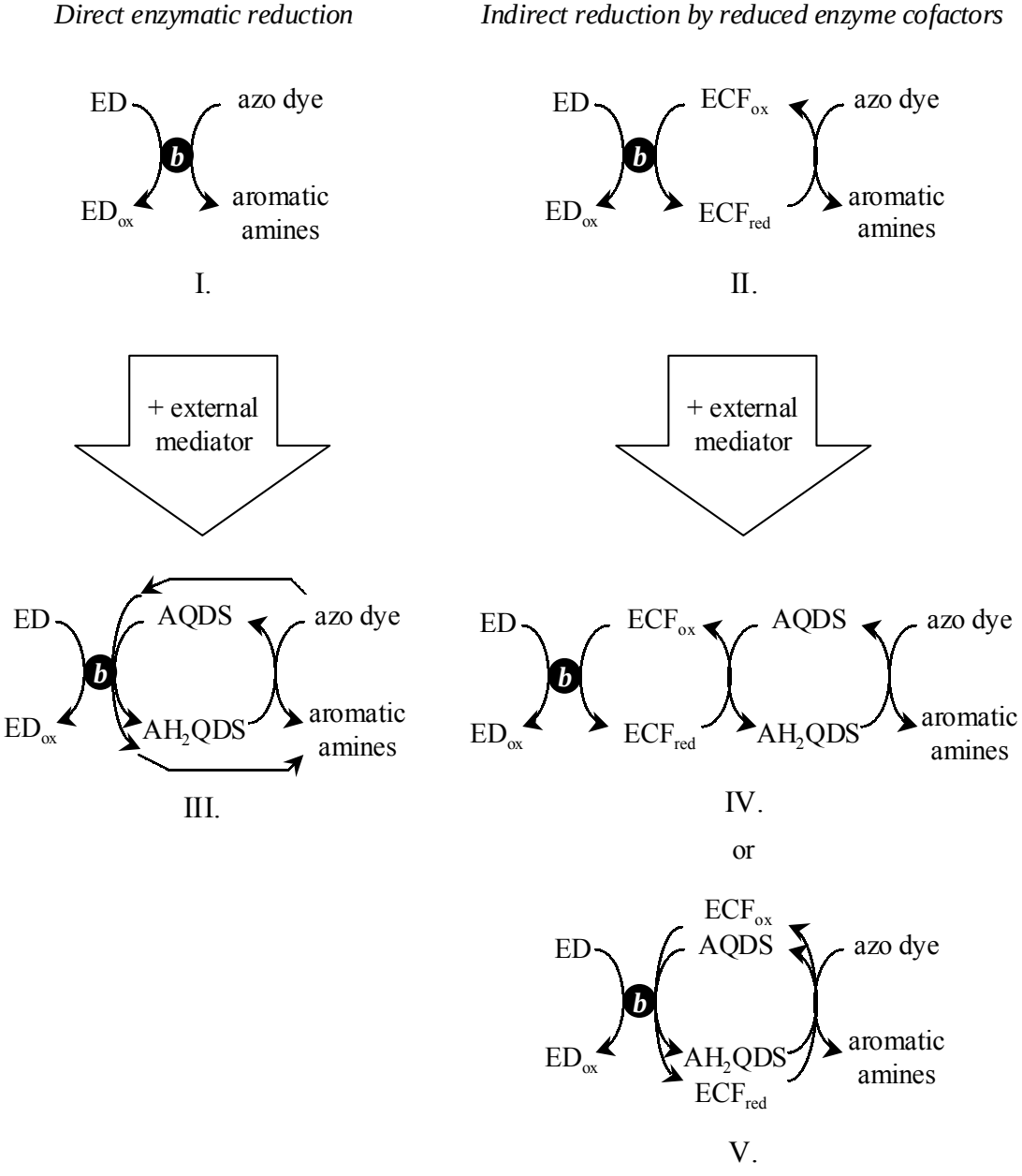

Figure 7.5 Schematic representation of direct enzymatic azo dye reduction (scheme I), azo dye reduction by reduced enzyme cofactors (scheme II) and AQDS stimulation of both mechanisms (scheme III and schemes IV and V, respectively).
ED = primary electron donor; *b* = bacteria; ECF = Enzyme cofactor, ox. = oxidised; red. = reduced

According to the direct enzymatic mechanism, azo dye reducing bacteria possess enzymes, for instance flavoproteins, that catalyse the transfer of reducing equivalents originating from the oxidation of organic substrates to the azo dyes. So far, all azo dye reducing enzymes isolated from (facultative) anaerobes were found capable of reducing many distinct azo compounds. This indicates that the redox active moieties of these enzymes (e.g. flavins in the case of flavoproteins) are highly unspecific with respect to the electron acceptor. According to the indirect mechanism, azo dyes are reduced by reduced enzyme cofactors, e.g. by $FADH_2$.

External redox mediators, e.g. artificial redox mediators like AQDS, can stimulate both direct enzymatic and indirect biological azo dye reduction (Figure 7.5 –schemes III, IV and V). In the case of direct enzymatic azo dye reduction, the stimulating effect of AQDS will be due to enzymatic AQDS reduction (section 7.5.2) in addition to enzymatic reduction of the azo dye (Figure 7.5 –scheme III). Possibly, both reactions will be catalysed by the same non-specific periplasmic enzymes. In the case of azo dye reduction by reduced enzyme cofactors, the stimulating effect of AQDS will either be due to an electron shuttle between the reduced enzyme cofactor and AQDS (Figure 7.5 –scheme IV) or it will be due to enzymatic AQDS reduction in addition to enzymatic reduction of the enzyme cofactor (Figure 7.5 –scheme III). In the latter case, introduction of AQDS simply increases the pool of electron carriers.

The research discussed in this thesis does not allow a conclusive judgement on which mechanism is responsible for azo dye reduction in anaerobic sludge. The similar rates of azo dye reduction by sulphide in the absence of sludge and in the presence of γ-irradiated sludge (Chapter 4) may seem to indicate that natural electron carriers are not available to (extracellular) dye reduction, supporting the direct enzymatic mechanism of biological azo dye reduction.

Though the capability to non-specifically reduce azo dyes seems to be an almost ubiquitous property of anaerobically incubated bacteria, experiments evaluating the effect of biomass adaptation for azo dye reduction (Chapter 5) showed interesting differences with respect to the utilisation of different electron donors. In these experiments, the substrate dependency for the reduction of the azo dye Reactive Red 2 (RR2) by dye-unadapted granular sludge was compared to that of granular sludge from a UASB reactor that treated RR2 containing synthetic wastewater with volatile fatty acids as the primary electron donating substrates. Hydrogen, added in bulk, was clearly the preferred electron donor for RR2 reduction. Bacteria that couple dye decolourisation to hydrogen oxidation were naturally present in the unadapted seed sludge. However, only the RR2-adapted sludge could utilise electrons from volatile fatty acids for dye reduction, indicating enrichment of RR2 reducing bacteria and/or enzyme systems.

7.5.2 Biological AQDS reduction

The mechanism of redox mediator stimulated biological azo dye reduction according schemes III and V in Figure 7.5 involves biological reduction (regeneration) of the mediating compound, e.g. biological reduction of AQDS to its hydroquinone, anthrahydroquinone-2,6-disulphonate (AH_2QDS).

The capability of microorganisms to reduce AQDS and other quinones is known as 'humus or quinone respiration', i.e. the utilisation of quinones (in humic substances) as an electron acceptor in the oxidation of electron donating substrates. This property, which appears to be ubiquitous under several distinct groups of microorganisms, has only relatively recently been recognised [195]. The mechanism of quinone respiration probably involves the activity of periplasmic enzymes with a low specificity with respect to the electron acceptor, e.g. like the periplasmic hydrogenase from *Desulfovibrio vulgaris* that was found to quickly reduce several quinone compounds [324]. Biological azo dye reduction, being either a direct enzymatic reaction or a reaction with enzymatically generated reduced electron carriers, may very well depend on the same enzymes.

The presence and viability of quinone-respiring microorganisms in anaerobic sludge has been demonstrated in recent research [48, 52]. Reduction of quinones like AQDS is thermodynamically favoured over methanogenesis and competition studies confirmed that quinone-respiring bacteria out-competed methanogens for acetate oxidation when anaerobic granular sludge was supplemented with stoichiometric quantities of AQDS [52]. Therefore, it can be excluded that application of redox mediators to accelerate azo dye reduction in anaerobic bioreactors will be restricted by unavailability of quinone-reducing power.

Though the capability to reduce quinones seems to be an almost ubiquitous property of anaerobically incubated bacteria [50, 66], experiments evaluating the effect of biomass adaptation for AQDS catalysed azo dye reduction (Chapter 5) showed interesting differences with respect to the utilisation of different electron donors. In these experiments, the substrate dependency of AQDS catalysed azo dye reduction by AQDS-unadapted granular sludge was compared to that of granular sludge from a UASB reactor that treated an azo dye and AQDS containing synthetic wastewater with volatile fatty acids as the primary electron donating substrates. The stimulatory effect of AQDS on RR2 decolourisation by AQDS-unadapted sludge was mainly due to assisting the electron transfer from endogenous substrates in the sludge to the dye. The stimulatory effect of AQDS on RR2 decolourisation by sludge from the AQDS-exposed reactor was in addition strongly associated with the transfer of electrons from hydrogen and acetate to the dye, probably due to enrichment of specialised AQDS-reducing bacteria.

7.6 Application of redox mediators to accelerate azo dye reduction in anaerobic bioreactors

7.6.1 AQDS

Chapter 5 presents the results of a study that evaluated the application of AQDS as a redox mediator during the continuous treatment of azo dyes in anaerobic bioreactors. For that purpose, continuous treatment of a synthetic wastewater containing the azo dye Reactive Red 2 (RR2) was studied in a laboratory-scale UASB reactor. In the initial phase without AQDS, the dye removal was low, less than 30%. Consequently, severe toxicity problems occurred, eventually resulting in almost complete

inhibition of the methanogenic activity. Addition of catalytic concentrations of AQDS to the reactor influent caused an immediate increase in the dye removal efficiency and recovery of the methane production. Ultimately, RR2 removal efficiencies stabilised at a high level (88%), and higher AQDS loads resulted in even higher RR2 removal efficiencies (up to 98%). Stable operation was maintained for more than one year. Moreover, AQDS dosing to a UASB-reactor that treated AO7 in a shorter parallel experiment with AO7 was also found to increase the dye removal efficiency [49]. It is clear, therefore, that AQDS can be successfully applied to accelerate azo dye reduction in anaerobic bioreactors.

7.6.2 Activated carbon

Though the required effective AQDS dosage levels are low, continuous dosing implies continuous expenses related to procurement of the chemical, as well as continuous discharge of this biologically recalcitrant compound. Therefore, incorporation and retention of an alternative mediator in the sludge bed would be a great improvement of the redox mediator application concept. The research presented in Chapter 6 demonstrated that activated carbon (AC), which is known to contain quinone groups at its surface [27, 97, 107], can be used as such an alternative, non-soluble redox mediator. Laboratory-scale UASB reactors with AC-amended granular sludge showed better removal of hydrolysed RR2 as compared to the control reactor without AC. The effect of AC was in large excess of its dye adsorption capacity, indicating improved dye reduction, a phenomenon that was also featured by higher concentration of aniline, one of the dye's constituent aromatic amines, in the reactor effluent. Supporting evidence for the hypothesis that AC can act as a redox mediator was obtained in batch experiments. It was demonstrated that bacteria from crushed granular sludge, as well as bacteria from an acetate-oxidising quinone-reducing enrichment culture composed mainly of *Geobacter* sp., could oxidise acetate and concomitantly reduce AC. Furthermore, it was demonstrated that AC greatly accelerated the rate of chemical azo dye reduction by sulphide. The results taken as a whole clearly suggest that AC accepts electrons from the microbial oxidation of organic acids and transfers the electrons to azo dyes, thereby accelerating their biological reduction. This constitutes the first example of biocatalysis mediated by AC and puts the role of AC in a new light.

AC has a long history of application in environmental technology as an adsorbent of pollutants for the purification of drinking- and wastewaters [205]. In bioreactors, AC is utilised to enhance performance by a well-established mechanism of adsorbing shock loads of inhibitory pollutants. AC is furthermore known as a catalyst of several chemical reactions at high temperature (>250 °C), e.g. reductive dechlorination of chloroalkanes and hydrogenation of aldehydes [84], gas phase hydrogenolysis of substituted benzene compounds [14, 289] and oxidative dehydrogenation of ethylbenzene [259, 260]. The only well established example of cold catalysis by AC is the oxidation of sulphide to elemental sulphur by elemental oxygen [55, 75, 172]. To our knowledge, the use of AC to catalyse biological reactions has not been reported before.

The mechanism of AC catalysis for redox reactions is generally not fully established but reactions involving the quinone-groups on AC have been proposed [69, 259]. Therefore, it is justified to propose a mechanism that involves mediation by quinone-moieties in AC.

7.7 Concluding remarks and perspectives

From the above it is clear that the reduction of azo dyes can be accelerated by utilising redox mediators, i.e. by continuous dosing of soluble quinones or by incorporation of AC in the sludge blanket. Shortening the long reaction times needed for the reduction of several azo dyes, redox mediators enable bioreactor operation at much shorter hydraulic retention times than otherwise would have been necessary for the reduction of those dyes. Consequently, redox mediator application provides a useful tool for the optimisation of the anaerobic first stage of the complete biodegradation of azo dyes. Thus optimised, the anaerobic reactor can be expected to reduce the lion's share of the azo compounds in the wastewater. The aerobic reactor, i.e. the second stage of the complete biodegradation of azo dyes, will therefore hardly receive any non-reduced, aerobically non-biodegradable azo dyes. Instead, the aerobic reactor will mainly receive aromatic amines, which are presumably aerobically removable, either by biodegradation or by autoxidation.

The potential of using redox mediators is probably not limited to enhancing the reduction of azo dyes. Also other reactions are known to be accelerated by quinone-type redox mediators, e.g. reductive dehalogenation [20, 42, 68, 74, 96, 246, 262, 263, 301] and reduction of nitroaromatics [38, 88, 96, 118, 129, 294, 327]. Therefore, anaerobic treatment of wastewaters containing such compounds will benefit from application of redox mediators because the reaction time requirements will be decreased, especially if the non-mediated reduction rates are low, as well because reduction of many of these compounds implies lowering of their toxicity towards methanogens [85, 92]. The potential of using redox mediators is furthermore probably not limited to wastewater treatment but may also apply to bioremediation of soils polluted with e.g. polychlorinated solvents or nitroaromatic pesticides. Also in these environments, addition redox mediators may help to transfer electrons from either naturally occurring or externally added primary electron donors.

7’

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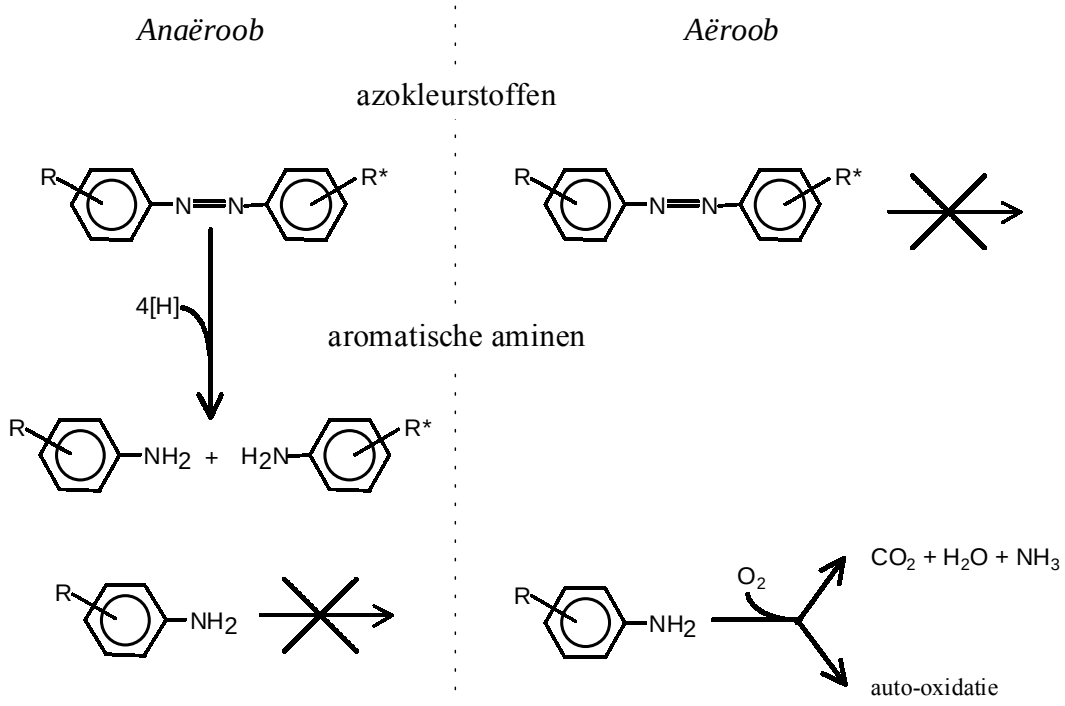
7.1' Inleiding

Het verven van doeken in de textielverwerkende industrie leidt tot kleurstofhoudende afvalwaters. De verwijdering van kleurstoffen uit degelijke afvalwaters is gewenst, niet alleen vanwege de kleur, maar ook vanwege de giftigheid en kankerverwekkendheid van deze stoffen. Er bestaan verschillende fysische, chemische en biologische technieken om kleurstoffen uit het afvalwater te halen. Iedere techniek kent technische en economische beperkingen. Veel fysisch-chemische kleurverwijdering-methoden zijn te duur of niet veelzijdig genoeg, ondervinden in te sterke mate invloed van andere stoffen in het afvalwater en/of leiden tot de vorming van afval dat dan vervolgens weer moet worden behandeld. Biologische behandeling is mogelijk een relatief goedkoop alternatief, want ten minste de kleurstoffen uit de meest gebruikte groep van kleurstoffen, de azokleurstoffen, zijn vatbaar voor biologische afbraak.



Figuur 7.1' Algemeen overzicht van het lot van azokleurstoffen en aromatische aminen in gecombineerd anaëroob-aërobe biologische zuiveringsystemen.

Azokleurstoffen zijn aromatische structuren die onderling verbonden zijn door een azo(-N=N-)-chromofoor. De elektronenzuigende aard van dergelijke azobruggen vormt echter een obstakel voor oxidatieve reacties. Doorgaans weerstaan azokleurstoffen daarom aërobe biodegradatie. Daar staat tegenover dat reductieve splitsing van de azobruggen spontaan mogelijk is onder anaërobe omstandigheden. Anaërobe reductie houdt in, dat azokleurstoffen worden omgezet in –meestal kleurloze, maar mogelijk schadelijke- aromatische aminen. Deze aromatische aminen worden in het algemeen onder anaërobe omstandigheden niet verder afgebroken. Onder aërobe omstandigheden

daarentegen, is het aannemelijk dat aromatische aminen uit de waterfase worden verwijderd als gevolg van een combinatie van biodegradatie, auto-oxidatie en adsorptie. Sequentiële of geïntegreerde anaëroob-aërobe behandeling is daarom de meest logische biologische behandelingsstrategie ter verwijdering van azokleurstoffen uit afvalwater (Figuur 7.1').

Evaluatie van de literatuur over anaëroob-aërobe behandeling van azokleurstoffen (Hoofdstuk 1) bracht twee mogelijk zwakke punten van het proces aan het licht: (i) anaërobe reductie van azokleurstoffen vergt nogal wat tijd, hetgeen blijkt uit de vereiste lange reactietijden; en (ii) over het lot van aromatische aminen is niet duidelijk uitsluitsel te geven.

Dit proefschrift bespreekt onderzoek dat is uitgevoerd om het eerste zwakke punt op te heffen. Het doel van dit onderzoek was om de eerste fase van de volledige biologische afbraak, de anaërobe reductie van azokleurstoffen, te optimaliseren. Het reactiemechanisme werd bestudeerd en vervolgens werden de verkregen inzichten toegepast.

7.2' Algemene eigenschappen van de anaërobe reductie van azokleurstoffen

Anaërobe azokleurstofreductie wordt in het algemeen beschouwd als een aspecifiek proces, waarin de kleurstof min of meer spontaan de elektronen opneemt die vrijkomen bij de biologische oxidatie van organische elektronendonors. Hoofdstuk 2 van dit proefschrift behelsde een verkenning van de reductie van een twintigtal chemisch verschillende azokleurstoffen door anaëroob korrelslib. De resultaten van deze verkenning waren in lijn met dit algemene beeld. Alle geteste kleurstoffen werden gereduceerd door anaëroob korrelslib en in het algemeen leidde dit tot de vorming van kleurloze producten. De reactie verliep zonder lagfase en volgde eersteordekinetiek.

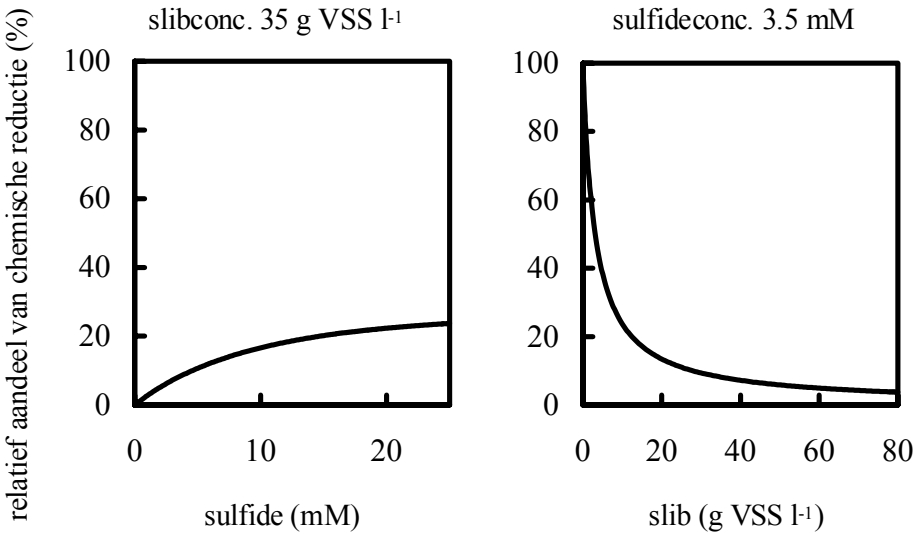
De reactiesnelheden bleken sterk te variëren tussen de verschillende kleurstoffen: de halfwaardetijden van de kleurstoffen in de uitgevoerde standaard-batchtests reikten van 1 tot ongeveer 100 uur. Vooral reactieve kleurstoffen met een triazinestructuur als reactieve groep reageerden langzaam. Er bestond geen verband tussen de halfwaardetijd en het molecuulgewicht van de kleurstof. Celwandpenetratie van kleurstoffen maakt daarom waarschijnlijk geen deel uit van het mechanisme. Deze waarneming, in combinatie met de aspecificiteit en het uitblijven van een lagfase, wees op een extracellulair mechanisme waarbij gereduceerde verbindingen of aspecifieke enzymen betrokken zijn. Biologische activiteit is overigens geen absolute vereiste voor anaërobe azokleurstofreductie, want het bleek dat azokleurstoffen ook in een puur chemisch gereduceerd kunnen worden, in een reactie met sulfide.

7.3' Biotische versus abiotische azokleurstofreductie

Aangezien kleurstofhoudende afvalwaters doorgaans sulfaat en andere zwavelcomponenten bevatten die tijdens behandeling in anaërobe bioreactoren biologisch tot sulfide gereduceerd worden, zal azokleurstofreductie een combinatie zijn van biotische en abiotische processen. Om de relatieve bijdrage van biologische en chemische azokleurstofreductie in anaëroob slib in te kunnen schatten

werd van twee kleurstoffen de reductiesnelheid gemeten in afwezigheid en in aanwezigheid van levend of gedood slib, in batchtests over een reeks van sulfideconcentraties (Hoofdstuk 4).
Het belang van biologische azokleurstofreductie werd duidelijk aangetoond door de waarneming dat azokleurstofreductie in afwezigheid van zwavelcomponenten alleen plaatsvond in aanwezigheid van levend slib. Het toevoegen van sulfide (of sulfaat, dat vervolgens werd gereduceerd tot sulfide) leidde tot extra azokleurstofreductie, ten gevolge van de chemische reactie. Dit betrof een additioneel effect, want de snelheid van azokleurstofreductie kwam overeen met de som van de snelheden van biologische en chemische azokleurstofreductie. Onderling waren beide mechanismen onafhankelijk, i.e.

- levend of γ-bestraald slib heeft geen invloed op chemische azokleurstofreductie door sulfide;
- sulfide heeft geen invloed op biologische azokleurstofreductie, wat aantoont dat de uiteindelijke reductie van azokleurstoffen niet gebaseerd is op een reactie met biologisch gerecycleerd sulfide;
- sulfaat heeft geen invloed op biologische azokleurstofreductie, wat aantoont dat sulfaat niet de kleurstof concurreert als elektronenacceptor.



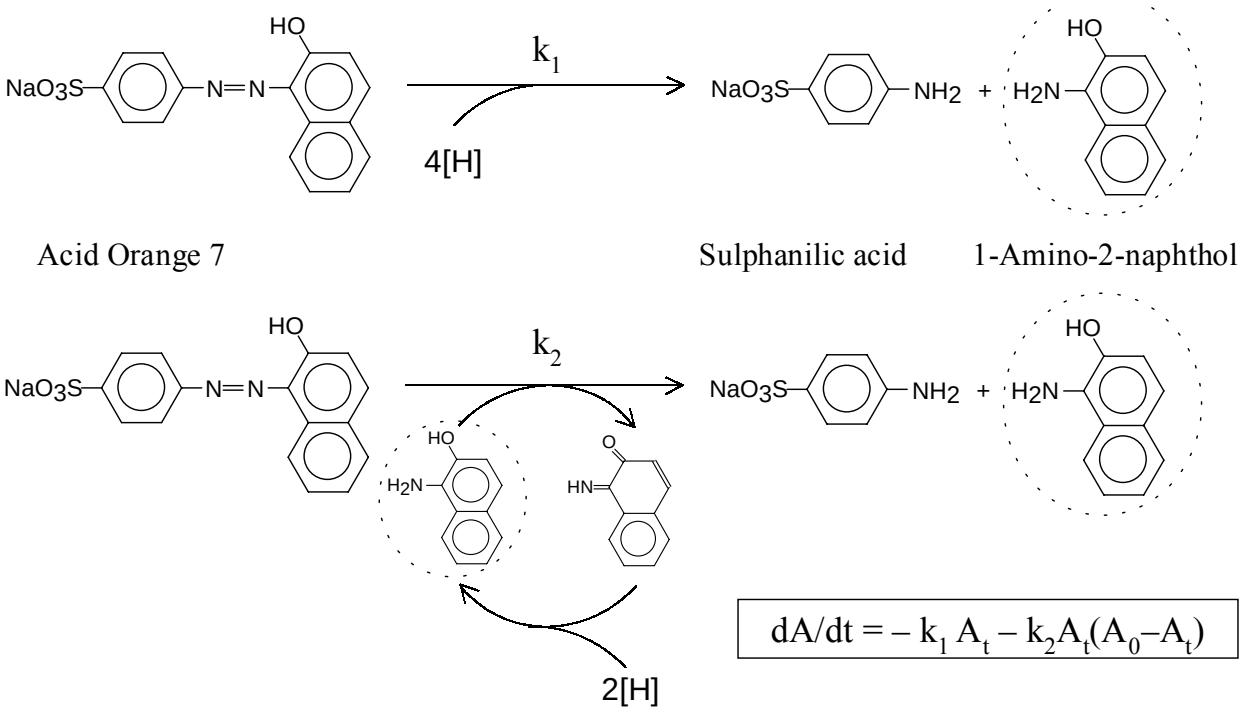
Figuur 7.2' Schatting van het relatieve aandeel van chemische reductie in de reductie van AO7 door anaëroob korrelslib in aanwezigheid van sulfide: (A) bij oplopende sulfideconcentraties in aanwezigheid van 35 g VSS l^{-1} anaëroob korrelslib; (B) bij verschillende slibconcentraties in aanwezigheid van 3.5 mM sulfide. Gebaseerd op de kinetische data die in Hoofstuk 4 gepresenteerd werden.

Analyse van de reactiekinetiek liet zien dat chemische azokleurstofreductie een relatief geringe rol speelt in 'high-rate' anaërobe bioreactoren wegens de hoge biomassaconcentratie in deze reactoren. Het aandeel van chemische azokleurstofreductie door sulfide zal in het algemeen verwaarloosbaar zijn, zelfs als de concentratie sulfide in de bulk de kleurstofconcentratie in grote mate overtreft. Chemische reductie door sulfide zal alleen bij zeer lage biomassaconcentraties of –in mindere mate- bij extreem hoge sulfideconcentraties een substantiële bijdrage leveren aan de totale reductie van azokleurstoffen in anaërobe bioreactoren (Figuur 7.2'). Continue experimenten in laboratoriumschaal opwaarts doorstroomde slibbedreactoren (UASB-reactoren) bevestigden dat de rol van chemische

azokleurstofreductie klein is, want het sulfide dat gevormd werd uit de reductie van het geïntroduceerde sulfaat had geen invloed op de kleurstofverwijderingsefficiëntie.
Op het mechanisme van biologische azokleurstofreductie wordt nader ingegaan in paragraaf 7.5'.

7.4' De rol van redoxmediatoren

De waarneming dat chemische reductie van azokleurstoffen relatief langzaam verloopt, zelfs wanneer sulfide in grote overmaat voorhanden is (Hoofdstukken 2, 3 en 4), duidt op een bottleneck in de elektronenoverdracht van sulfide op de azobrug. Er bestaan echter manieren om dit probleem op te lossen. In hoofstuk 3, waar de chemische reductie van azokleurstoffen verder werd verkend, werd opgemerkt dat de reactie tussen Acid Orange 7 (AO7) en sulfide in de tijd, d.w.z. naarmate er meer kleurstof gereduceerd was, versneld werd. Mathematische beoordeling van de experimentele resultaten wezen erop, dat autokatalyse een belangrijke rol speelde in de chemische reductie van AO7. Verdere experimenten brachten aan het licht dat 1-amino-2-naftol, een van de twee aromatische aminen die gevormd worden uit de reductie van AO7, het reactieproces versnelde. Deze waarneming kwam overeen met de in de literatuur vermelde katalyse van azokleurstofreductie door chinonverbindingen [23, 161]. Het effect van 1-amino-2-naftol is daarom mogelijkerwijze gebaseerd op redoxmediatie. Volgens

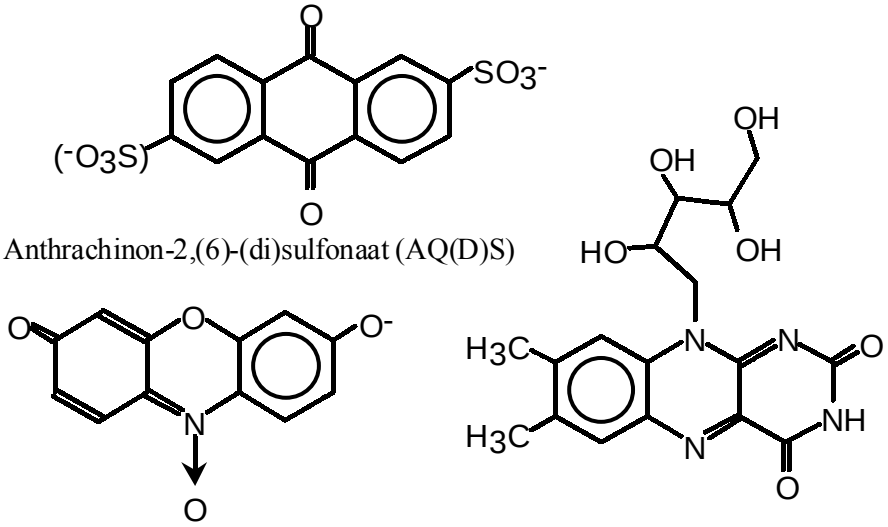


Figuur 7.3' Schematische en mathematische voorstelling van autokatalytische AO7-reductie. [H] = reductie-equivalent; k_1 en k_2 verwijzen naar de reactiesnelheidsconstanten volgens de formule in het kader: k_1 = eersteordeconstante van de directe reactie tussen AO7 en sulfide; k_2 = tweedeordeconstante van de autokatalytische reactie; A_t = kleurstofconcentratie op tijdstip t; A_0 = kleurstofconcentratie op t = 0.

het voorgestelde mechanisme (Figuur 7.3'), brengt 1-amino-2-naftol, stuivertje wisselend met het corresponderende -geoxideerde- aminochinon, elektronen van sulfide over op de kleurstof en versnelt het daarmee de reactie.

De invloed van redoxmediatoren werd nader bekeken door het effect te testen van antrachinon-2,6-disulfonaat (AQDS), een humuszuuranaloge verbinding die vaker is gebruikt als redoxmediator in reductieve transformaties. AQDS bleek een tien keer zo krachtige redoxmediator te zijn als 1-amino-2-naftol. Ter vergelijking: bij concentraties waarop 1-amino-2-naftol de eersteordereactiesnelheid-constante met een factor 2 tot 10 verhoogde, verhoogde AQDS deze constante met een factor 10 tot 100.

In het verloop van dit promotieonderzoek is van verscheidene andere stoffen vastgesteld dat ze de reductie van azokleurstoffen door sulfide versnellen. Onder deze stoffen waren zowel de kunstmatige chinonverbindingen antrachinon-2-sulfonaat en resazurine, als de uitgangsstof van de flavine enzymcofactor, riboflavine (Figuur 7.4'). Bovendien werd redoxmediatie door actieve kool (paragraaf 7.6.2) en geautoclaveerd slib vastgesteld.



Figuur 7.4' Structuurformules van enkele in dit onderzoek gebruikte redoxmediatoren

Stimulering van azokleurstofreductie door geautoclaveerd slib, als eerste opgemerkt in Hoofstuk 3, werd nader onderzocht in Hoofdstuk 4. Hier werd het effect van geautoclaveerd slib vergeleken met het effect van met gammastralen bestraald slib. Het bleek dat geautoclaveerd slib de reductie van azokleurstoffen enorm sterk versnelde, terwijl γ-bestraald slib in het geheel geen stimulerend effect had. Aangezien autoclaveren de cellen kapot maakt, terwijl bestraling de biologische activiteit tenietdoet maar de celstructuur intact laat, doen deze waarnemingen vermoeden dat enzymcofactoren die vrijkomen tijdens cellysis als redoxmediatoren bijdragen aan het stimulerende effect. Deze hypothese werd ondersteund door de sterke versnelling van chemische azokleurstofreductie door riboflavine. Riboflavine is een vertegenwoordiger van de hittestabiele redoxmediatorstructuren van veelvoorkomende flavine enzymcofactoren.

Katalyse van de reductie van azokleurstoffen door redoxmediatoren beperkt zich niet tot de chemische reactie met sulfide, maar geldt ook voor het biologische mechanisme van azokleurstofreductie. Dit is aangetoond in de Hoofdstukken 4 en 5, met de waarneming dat katalytische hoeveelheden AQDS de

reductie van azokleurstoffen door levend korrelslib in afwezigheid van zwavelcomponenten sterk stimuleren.

7.5' De rol van bacteriën

7.5.1' Biologische azokleurstofreductie

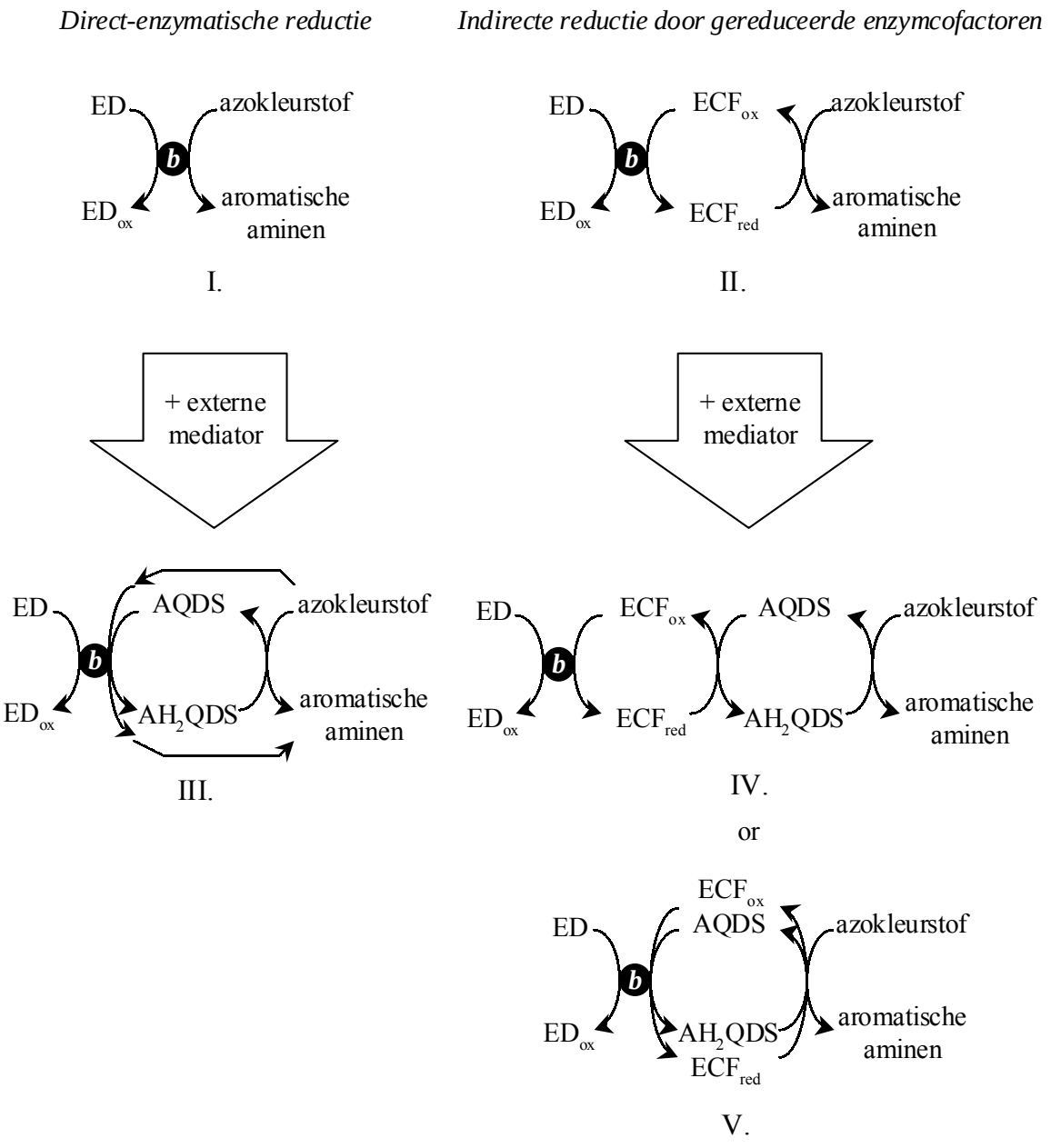
Zoals bleek uit het hierboven besprokene is de aspecifieke en vermoedelijk extracellulaire reductie van azokleurstoffen voornamelijk een biologisch proces. Azokleurstoffen worden biologisch gereduceerd in een direct-enzymatische reactie (Figuur 7.5' – schema I) of indirect, in een reactie met gereduceerde enzymcofactoren (Figuur 7.5' – schema II).

Volgens het direct-enzymatische mechanisme bezitten azokleurstofreducerende bacteriën enzymen, bijvoorbeeld flavoproteïnen, die de overdracht katalyseren van reductie-equivalenten die afkomstig zijn van de oxidatie van organische substraten, naar de azokleurstof. Totnogtoe bleken alle uit (facultatief) anaërobe bacteriën geïsoleerde azokleurstofreducerende enzymen in staat tot reductie van vele verschillende azoverbindingen. Dit duidt erop dat de actieve structuren in deze enzymen (bijvoorbeeld flavinen in geval van flavoproteïnen) nogal aspecifiek zijn voor wat betreft de elektronenacceptor. Volgens het indirecte mechanisme worden azokleurstoffen gereduceerd door gereduceerde enzymcofactoren, bijvoorbeeld door $FADH_2$.

Extern toegevoegde redoxmediatoren, bijvoorbeeld kunstmatige redoxmediatoren zoals AQDS, kunnen zowel de direct-enzymatische als de indirecte biologische azokleurstofreductie stimuleren (Figuur 7.5' – schema's III, IV en V). In het geval van direct-enzymatische azokleurstofreductie zal het stimulerende effect van AQDS veroorzaakt worden door enzymatische AQDS-reductie (paragraaf 7.5.2') naast enzymatische kleurstofreductie (Figuur 7.5' – schema III). Beide reacties worden mogelijkerwijze door dezelfde aspecifieke periplasmatische enzymen gekatalyseerd. In het geval van azokleurstofreductie door gereduceerde enzymcofactoren zal het stimulerende effect van AQDS veroorzaakt worden door de versnelde overdracht van elektronen van de gereduceerde enzymcofactor op AQDS (Figuur 7.5' – schema IV) of door enzymatische AQDS-reductie naast enzymatische reductie van de enzymcofactor (Figuur 7.5' – schema III). In het laatste geval zal de introductie van AQDS simpelweg de hoeveelheid elektronentransporteurs vergroten.

Het onderzoek dat in dit proefschrift werd besproken staat niet toe een afdoend oordeel te vellen over het mechanisme dat verantwoordelijk is voor de biologische reductie van azokleurstoffen door anaëroob slib. De vergelijkbare snelheden van azokleurstofreductie door sulfide in afwezigheid van slib en die in aanwezigheid van γ-bestraald slib (Hoofdstuk 4) lijken aan te tonen dat natuurlijke elektronentransporteurs niet beschikbaar zijn voor (extracellulaire) kleurstofreductie, hetgeen wijst op het direct-enzymatische mechanisme van biologische azokleurstofreductie. Hoewel het vermogen tot aspecifieke azokleurstofreductie zowat een alomtegenwoordige eigenschap van anaëroob geïncubeerde bacteriën lijkt te zijn, toonden experimenten die het effect van biomassa-adaptatie aan azokleurstofreductie evalueerden aan, dat er interessante verschillen zijn wat betreft het gebruik van

verschillende elektronendonoren. In deze experimenten werd de substraatafhankelijkheid van de reductie van de azokleurstof Reactive Red 2 (RR2) door ongeadapteerd korrelslib vergeleken met die van korrelslib uit een UASB-reactor die synthetisch afvalwater behandelde dat RR2 en vluchtige vetzuren bevatte. Waterstof, toegevoegd als bulkelektronendonor, was duidelijk de geprefereerde elektronendonor voor RR2-reductie. Bacteriën die de reductie van azokleurstoffen koppelden aan de oxidatie van waterstof waren reeds aanwezig in het ongeadapteerde entslib. Alleen het RR2-geadapteerde slib kon de elektronen die afkomstig waren van vluchtige vetzuren aanwenden voor kleurstofreductie, hetgeen wees op verrijking van vetzuuroxiderende RR2-reducerende bacteriën en/of enzymsystemen.



Figuur 7.5' Schematische voorstelling van direct-enzymatische azokleurstofreductie (schema I) en azokleurstofreductie door gerduceerde enzymcofactoren (schema II), alsmede van het stimulerende effect van AQDS in beide mechanismen (respectievelijk schema III en schema's IVen V).
ED = primaire elektronendonor; *b* = bacteriën; ECF = Enzymcofactor, ox. = geoxideerd; red. = gereduceerd

7.5.2' Biologische AQDS-reductie

Het mechanisme van redoxmediatorgestimuleerde biologische azokleurstofreductie (schema's III en V in Figuur 7.5') behelst biologische reductie (regeneratie) van de mediator, bijvoorbeeld biologische reductie van AQDS naar het corresponderende hydrochinon, antrahydrochinon-2,6-disulfonaat (AH_2QDS). Het vermogen van micro-organismen om AQDS en andere chinonen te reduceren staat bekend als 'humus- of chinonademhaling', i.e. het gebruik van chinonen (in humusstoffen) als elektronenacceptor voor de oxidatie van elektronendonerende substraten. Deze eigenschap, die alomtegenwoordig lijkt te zijn onder een brede verscheidenheid aam micro-organismen, is pas relatief recentelijk onderkend [195]. Het mechanisme van chinonademhaling behelst waarschijnlijk de activiteit van periplasmatische enzymen met een lage specificiteit voor wat betreft de elektronenacceptor, zoals het periplasmatische hydrogenase van *Desulfovibrio vulgaris* waarvan is geconstanteerd dat het verschillende chinonverbindingen snel kon reduceren [324]. Biologische azokleurstofreductie, om het even of het nu een direct-enzymatische reactie of een reactie met gereduceerde elektronentransporteurs betreft, zou heel goed van dezelfde enzymen afhankelijk kunnen zijn.

De aanwezigheid en levensvatbaarheid van chinonrespirerende micro-organismen in anaëroob slib is in recent onderzoek aangetoond [48, 52]. Reductie van AQDS en vele andere chinonverbindingen is thermodynamisch voordeliger dan methanogenese. Competitiestudies met anaëroob slib bevestigden dat chinonrespirerende bacteriën methanogene bacteriën verdrongen voor de oxidatie van acetaat [52]. Het is daarom uitgesloten dat het toepassen van redoxmediatoren ter versnelling van de reductie van azokleurstoffen in anaërobe bioreactoren belemmerd zal worden door beperkte beschikbaarheid van chinonreducerende kracht.

Hoewel het vermogen om chinonen te reduceren zowat een alomtegenwoordige eigenschap van anaëroob geïncubeerde bacteriën is [50, 66], lieten experimenten waarin het effect van biomassa-adaptatie voor AQDS-gekatalyseerde azokleurstofreductie werd onderzocht (Hoofdstuk 5) interessante verschillen zien voor wat betreft het aanwenden van verschillende elektronendonoren. In deze experimenten werd de substraatafhankelijkheid van AQDS-gekatalyseerde kleurstofreductie door AQDS-ongeadapteerd korrelslib vergeleken met die van korrelslib uit een UASB-reactor die een synthetisch afvalwater behandelde dat RR2, AQDS en vluchtige vetzuren bevatte. Het bleek dat het stimulerende effect van AQDS op de ontkleuring van RR2 door ongeadapteerd slib vooral verband hield met de overdracht van elektronen uit endogeen substraat naar de kleurstof. Het stimulerende effect van AQDS op de ontkleuring van RR2 door slib uit de reactor die blootgesteld was aan AQDS was daarenboven eveneens verbonden met de overdracht van elektronen van waterstof en vetzuren (in het bijzonder acetaat) naar de kleurstof, vermoedelijk door verrijking van het slib met gespecialiseerde AQDS-reducerende bacteriën.

7.6' Toepassing van redoxmediatoren ter versnelling van azokleurstofreductie in anaërobe bioreactoren

7.6.1' AQDS

In Hoofdstuk 5 werden de resultaten gepresenteerd van een studie naar de toepassing van AQDS als redoxmediator gedurende de continue behandeling van azokleurstoffen in bioreactoren. Voor dat doel werd de continue behandeling van een synthetisch afvalwater dat de azokleurstof Reactive Red 2 (RR2) bevatte in een laboratoriumschaal UASB-reactor bestudeerd. In de initiële fase zonder AQDS was de kleurstofverwijdering laag, minder dan 30%. Het gevolg hiervan was dat er toxiciteitproblemen optraden die uiteindelijk resulteerden in de volledige remming van de methanogene activiteit. Het toevoegen van katalytische concentraties AQDS aan het reactorinfluent leidde onmiddellijk tot toename van de kleurverwijderingsefficiëntie en herstel van de methaanproductie. Uiteindelijk stabiliseerde de RR2-kleurverwijderingsefficiëntie zich op een hoog niveau (88%) en hogere AQDS-doseringen leidden zelfs tot nog hogere RR2-kleurverwijderingsefficiënties (tot 98%). Stabiel reactorbedrijf werd langer dan een jaar volgehouden. Ook in een korter durend parallelexperiment met een UASB-reactor die een andere azokleurstof (AO7) behandelde leidde continue AQDS-dosering tot verhoogde kleurverwijderingsefficiënties [49]. Het is dan ook duidelijk dat AQDS met succes kan worden toegepast ter versnelling van azokleurstofreductie in anaërobe bioreactoren.

7.6.2' Actieve kool

Hoewel de vereiste effectieve AQDS-doses laag zijn brengt continue dosering met zich mee dat er continu kosten zijn in verband met het chemicaliënverbruik en ook dat er voorturend lozing plaatsvindt van deze recalcitrante verbinding. Het zou daarom een belangrijke verbetering zijn als er gebruik kon worden gemaakt van een alternatieve redoxmediator die zich in het slibbed laat inbouwen. Het onderzoek dat in Hoofdstuk 6 werd gepresenteerd liet zien dat actieve kool (AC), waarvan bekend is dat het oppervlak chinongroepen bevat [27, 97, 107], als zo een alternatieve, onopgeloste redoxmediator dienst kan doen. Laboratoriumschaal UASB-reactoren waarvan het korrelslib gemengd was met AC waren beter in staat gehydrolyseerd RR2 te verwijderen dan de controlereactor zonder AC. Het effect van AC overschreed de adsorptiecapaciteit van de kool vele malen, hetgeen duidde op verbeterde azokleurstofreductie. Dit verschijnsel uitte zich ook in hogere concentraties aniline, een van de aromatische aminen die ontstaan bij reductie van RR2, in het effluent van de reactoren. Batchexperimenten leverden aanvullende ondersteuning voor de hypothese dat AC zich als redoxmediator kan gedragen. Het werd aangetoond dat bacteriën uit vermalen korrelslib, net als bacteriën van een acetaatoxiderende chinonreducerende verrijkingscultuur die voornamelijk uit *Geobacter* sp. bestond, acetaat konden reduceren onder gelijktijdige reductie van AC. Voorts werd aangetoond dat AC de snelheid van chemische azokleurstofreductie door sulfide in belangrijke mate versnelde. Wanneer dit alles bij elkaar in ogenschouw wordt genomen lijkt het er sterk op dat AC elektronen accepteert die afkomstig zijn van de biologische oxidatie van organische zuren en deze

elektronen overdraagt op de azokleurstoffen, waarmee de biologische reductie van azokleurstoffen versneld wordt. Dit vormt het eerste voorbeeld van biokatalyse door AC als redoxmediator en plaatst de rol van AC in een nieuw licht.

AC kent een lange geschiedenis van milieutechnologische toepassing als adsorbens van verontreinigingen tijden de zuivering van drink- en afvalwaters [205]. Een gevestigde toepassing van AC in bioreactoren is het gebruik ervan als adsorbens van schokbelastingen van remmende verontreinigingen. Daarnaast staat AC bekend als katalysator van verschillende chemische reacties bij hoge temperaturen (> 250 °C), bijvoorbeeld de reductieve dechlorering van chlooralkanen en de hydrogenering van aldehyden [84], gasfase-hydrogenolyse van gesubstitueerde benzeenverbindingen [14, 289] en oxidatieve dehydrogenering van ethylbenzeen [259, 260]. Het enige bekende voorbeeld van koude katalyse door AC is de oxidatie van sulfide naar elementair zwavel door elementair zuurstof [55, 75, 172]. Voor zover ons bekend is het gebruik van AC om biologische reacties te katalyseren nooit eerder gerapporteerd.

Het mechanisme van AC-gekatalyseerde redoxreacties is niet volledig bekend, maar men heeft geopperd dat het gaat om reacties met chinongroepen aan het oppervlak van de kool [69, 259]. Het is daarom gerechtvaardigd om een mechanisme voor te stellen dat uitgaat van redoxmediatie door chinonstructuren in AC.

7.7’ Concluderende opmerkingen en perspectieven

Uit wat hierboven is besproken is het duidelijk dat de reductie van azokleurstoffen kan worden versneld door gebruik te maken van redoxmediatoren, i.e. door opgeloste chinonverbindingen continu te doseren of door de inbouw van AC in het slibbed. Door de lange reactietijd die nodig is voor de reductie van verscheidene azokleurstoffen te verkorten maken redoxmediatoren het mogelijk om bioreactoren te bedrijven bij veel kortere hydraulische verblijftijden dan anders nodig zouden zijn voor de reductie van deze kleurstoffen. Toepassing van redoxmediatoren is daarom een handige truc ter optimalisering van de anaërobe eerste stap in de volledige biologische afbraak van azokleurstoffen. Van een anaërobe reactor die op een dergelijke manier is geoptimaliseerd kan worden verwacht dat deze het leeuwendeel van de azoverbindingen in het afvalwater zal reduceren. De aërobe reactor, i.e. de tweede stap in de volledige biologische afbraak van azokleurstoffen, zal daarom nauwelijks enige niet-gereduceerde, aëroob niet-afbreekbare azokleurstoffen ontvangen. In plaats daarvan zal de aërobe reactor voornamelijk aromatische aminen ontvangen, die vermoedelijk aëroob te verwijderen zijn door een combinatie van biologische afbraak en auto-oxidatie.

De potentie van het gebruik van redoxmediatoren beperkt zich waarschijnlijk niet tot het verbeteren van de reductie van azokleurstoffen. Ook van andere reacties is bekend dat ze versneld worden door redoxmediatoren van het chinontype, bijvoorbeeld reductieve dehalogenering [20, 42, 68, 74, 96, 246, 262, 263, 301] en reductie van nitroaromaten [38, 88, 96, 118, 129, 294, 327]. Anaërobe behandeling van afvalwaters die dergelijke stoffen bevatten zal baat hebben bij het gebruik van redoxmediatoren omdat de vereiste tijd zal worden teruggebracht, vooral als de niet-gekatalyseerde reductiesnelheden laag zijn of als reductie

van dergelijke stoffen detoxificering impliceert [85, 92]. De potentie van het gebruik van redoxmediatoren is voorts waarschijnlijk evenmin beperkt tot afvalwaterbehandeling, maar zou ook kunnen gelden voor de bioremediatie van bodems die verontreinigd zijn met bijvoorbeeld meervoudig gechloreerde oplosmiddelen of nitroaromatische pesticiden. Ook in deze milieus kan het toevoegen van redoxmediatoren helpen om elektronen van natuurlijke of extern toegevoegde elektronendonoren over te dragen.

References

1. Abdo, M.S.E. and Al-Ameeri, R.S. (1987) Anodic oxidation of a direct dye in an electrochemical reactor. *J. Environ. Sci. Heal A*, **22**: 27-42.
2. Abo-Elala, S.I. and El-Dib, M.A. (1987) Color removal via adsorption on wood shavings. *Sci. Total Environ.*, **66**: 269-273.
3. Ahmed, M.N. and Ram, R.N. (1992) Removal of basic dye from waste-water using silica as adsorbent. *Environmental pollution*, **77**: 79-86.
4. Al-Duri, B., McKay, G., El Geundi, M.S. and Abdul Wahab, M.Z. (1990) Three resistance transport model for dye adsorption onto bagasse pith. *J. Environ. Eng.-ASCE*, **116**: 487-502.
5. Altinbas, U., Dokmeci, S. and Baristiran, A. (1995) Treatability study of wastewater from textile industry. *Environ. Technol.*, **16**: 389-394.
6. An, H., Qian, Y., Gu, X.S. and Tang, W.Z. (1996) Biological treatment of dye wastewaters using an anaerobic-oxic system. *Chemosphere*, **33**: 2533-2542.
7. Anliker, R. (1979) Ecotoxicology of Dyestuffs - A joint effort by industry. *Ecotoxicol. Environ. Saf.*, **3**: 59-74.
8. Anliker, R., Clarke, E.A. and Moser, P. (1981) Use of the partition coefficient as anindicator of bioaccumulation tendency of dyestuffs in fish. *Chemosphere*, **10**: 263-274.
9. Anliker, R. and Moser, P. (1987) The limits of bioaccumulation of organic pigments in fish: their relation to the partition coefficient and the solubility in water octanol. *Ecotoxicol. Environ. Saf.*, **13**: 43-52.
10. Anliker, R., Moser, P. and Poppinger, D. (1988) Bioaccumulation of dyestuffs and organic pigments in fish. Relationships to hydrophobicity and steric factors. *Chemosphere*, **17**: 1631-1644.
11. Antal, M., Ebringerova, A. and Simkovic, I. (1984) New aspects in cationization of lignocellulose materials containing quaternary ammonium groups. *J. Appl. Polym. Sci.*, **29**: 637-642.
12. APHA, *Standard methods for examination of water and wastewaters*. 16 ed. American Public Health Association. 1985, Washington DC, USA.
13. Aplin, R. and Wait, T.D. (2000) Comparison of three advanced oxidation processes for degradation of textile dyes. *Water Sci. Technol.*, **42**: 345-354.
14. Arends, I.W.C.E., Ophorst, W.R., Louw, R. and Mulder, P. (1996) Gas phase hydrolysis mediated by activated carbon: monsubstituted benzenes. *Carbon*, **34**: 581-588.
15. Azmi, W., Sani, R.K. and Banerjee, U.C. (1998) Biodegradation of triphenylmethane dyes. *Enzyme and Microbiol. Technol.*, **22**: 185-191.
16. Baird, R., Camona, L. and Jenkins, R.L. (1977) Behavior of benzidine and other aromatic amines in aerobic wastewater treatment. *J. Water Pollut. Control Fed.*, **49**: 1609-1615.
17. Ball, A.S., Betts, W.B. and McCarthy, A.J. (1989) Degradation of lignin-related compounds by actinomycetes. *Appl. Environ. Microbiol.*, **55**: 1642-1644.
18. Banat, I.M., Nigam, P., Singh, D. and Marchant, R. (1996) Microbial decolorization of textile-dye-containing effluents: a review. *Biores. Technol.*, **58**: 217-227.
19. Bandara, J., Nadtochenko, V., Kiwi, J. and Pulgarin, C. (1997) Dynamics of oxidant addition as a parameter in the modelling of dye mineralization (Orange II) via advanced oxidation technologies. *Water Sci. Technol.*, **35**: 87-93.
20. Barkovskii, A.L. and Adriaens, P. (1998) Impact of humic constituents on microbial dechlorination of polychlorinated dioxins. *Environ. Toxicol. Chem.*, **17**: 1013-1020.
21. Basibuyuk, M. and Forster, C.F. (1997) The use of sequential anaerobic/aerobic processes for the biotreatment of a simulated dyeing wastewater. *Environ. Technol.*, **18**: 843-848.
22. Bauer, C., Jacques, P. and Kalt, A. (2001) Photooxidation of an azo dye induced by visible light incident on the surface of TiO2. *J. Photoch. Photobio. A*, **140**: 87-92.
23. Bechtold, T., Burtscher, E. and Turcanu, A. (1999) Anthraquinones as mediators for the indirect cathodic reduction of dispersed organic dyestuffs. *J. Electroanal. Chem.*, **465**: 80-87.

24. Benedek, A., *Simultaneous biodegradation and activated carbon adsorption - a mechanistic look*, in *Activated carbon adsorption of organics from the aqueous phase*, I.H. Suffet and McGuire, M.J., Editors. 1980, An Arbor Science Publishers, Inc.: Ann Arbor, Michigan, USA. p. 273-302.
25. Beydilli, M.I., Pavlostathis, S.G. and Tincher, W.C. (1998) Decolorization and toxicity screening of selected reactive azo dyes under methanogenic conditions. *Water Sci. Technol.*, **38**: 225-232.
26. Beydilli, M.I., Matthews, R.D. and Pavlostathis, S.G. (2000) Decolorization of a reactive copper-phthalocyanine dye under methanogenic conditions. In: *1st World Water Congress of the International Water Association*(Eds.), Paris, 3-7 July, 2000. p. 215-222
27. Boehm, H.P. (1994) Some aspects of the surface chemistry of carbon blacks and other carbons. *Carbon*, **32**: 759-769.
28. Bousher, A., Shen, X. and Edyvean, R.G.J. (1997) Removal of coloured organic matter by adsorption onto low-cost waste materials. *Water Res.*, **31**: 2084-2092.
29. Bradley, P.M., Chapelle, F.H. and Lovley, D.R. (1998) Humic acids as electron acceptors for anaerobic microbial oxidation of vinyl chloride and dichloroethene. *Appl. Environ. Microbiol.*, **64**: 3102-3105.
30. Bragger, J.L., Lloyd, A.W., Soozandehfar, S.H., Bloomfield, S.F., Marriott, C. and Martin, G.P. (1997) Investigations into the azo reducing activity of a common colonic microorganism. *Int. J. Pharm.*, **157**: 61-71.
31. Brahimi-Horn, M.C., Lim, K.K., Liang, S.L. and Mou, D.G. (1992) Binding of textile azo dyes by Myrothecium verrucaria. *J. Ind. Microbiol.*, **10**: 31-36.
32. Brock, B.J. and Gold, M.H. (1996) 1,4-Benzoquinone reductase from the basidiomycete *Phanerochaete chrysosporium*: spectral and kinetic analysis. *Arch. Biochem. Biophys.*, **331**: 31-40.
33. Brohm, K. and Frohwein, E. (1937) Nachweis von durch Säueringentfarbten künstlichen Eigelbfrabstoffen in Milchspeiseeis. *Zbl. Lebensmitt. Forsch.*, **73**: 30.
34. Bromly-Challenor, K.C.A., Knapp, J.S., Zhang, Z., Gray, N.C.C., Hetheridge, M.J. and Evans, M.R. (2000) Decolorization of an azo dye by unacclimated activated sludge under anaerobic conditions. *Water Res.*, **34**: 4410-4418.
35. Brown, D. and Laboureur, P. (1983) The aerobic biodegradability of primary aromatic amines. *Chemosphere*, **12**: 405-414.
36. Brown, D. and Laboureur, P. (1983) The degradation of dyestuffs: Part I - Primary biodegradation under anaerobic conditions. *Chemosphere*, **12**: 397-404.
37. Brown, D. (1987) Effects of colorants in the aquatic environment. *Ecotoxicol. Environ. Saf.*, **13**: 139-147.
38. Brown, J.P. (1981) Reduction of polymeric azo and nitro dyes by intestinal bacteria. *Appl. Environ. Microbiol.*, **41**: 1283-1286.
39. Brown, M.A. and DeVito, S.C. (1993) Predicting azo dye toxicity. *Crit. Rev. Env. Sci. Tec.*, **23**: 249-324.
40. Buckley, C.A. (1992) Membrane technology for the treatment of dyehouse effluents. *Water Sci. Technol.*, **22**: 265-274.
41. Bumpus, J.A. (1995) Microbial degradation of azo dyes. *Prog. Ind. Microbiol.*, **32**: 157-176.
42. Butler, E.C. and Hayes, K.F. (1998) Effects of solution composition and pH on the reductive dechlorination of hexachloroethane by iron sulfide. *Environ. Sci. Technol.*, **32**: 1276-1284.
43. Cao, J.S., Wei, L.P., Huang, Q.G., Wang, L.S. and Han, S.K. (1999) Reducing degradation of azo dye by zero-valent iron in aqueous solution. *Chemosphere*, **38**: 565-571.
44. Carliell, C.M., Barclay, S.J., Naidoo, N., Buckley, C.A., Mulholland, D.A. and Senior, E. (1994) Anaerobic decolorisation of reactive dyes in conventional sewage treatment processes. *Water SA*, **20**: 341-344.
45. Carliell, C.M., Godefroy, S.J., Naidoo, N., Buckley, C.A., Senior, E., Mulholland, D. and Martineigh, B.S. (1994) Anaerobic decolourisation of azo dyes. In: *Seventh International Symposium on Anaerobic Digestion*(Eds.), Cape Town, South Africa, 1994. RSA Litho (Pty) Ltd, Goodwood, South Africa, p. 303-306

46. Carliell, C.M., Barclay, S.J., Naidoo, N., Buckley, C.A., Mulholland, D.A. and Senior, E. (1995) Microbial decolourisation of a reactive azo dye under anaerobic conditions. *Water SA*, **21**: 61-69.
47. Cartwright, R.A. (1983) Historical and modern epidemiological studies on populations exposed to N-substituted aryl compounds. *Environ. Health Persp.*,: 4913-9.
48. Cervantes, F.J., van der Velde, S., Lettinga, G. and Field, J.A. (2000) Competition between methanogenesis and quinone respiration for ecologically important substrates in anaerobic consortia. *FEMS Microbiol. Ecol.*, **34**: 161-171.
49. Cervantes, F.J., Van der Zee, F.P., Lettinga, G. and Field, J.A. (2001) Enhanced decolourisation of Acid Orange 7 in a continuous UASB reactor with quinones as redox mediators. *Water Sci. Technol.*, **44**: 123-128.
50. Cervantes, F.J., De Bok, F.A.M., Duong-Dac, T., Stams, A.J.M., Lettinga, G. and Field, J.A. (in press) Reduction of humic substances by halorespiring, sulfate-reducing and methanogenic microorganisms. *Environ. Microbiol.*.
51. Cervantes, F.J., Duong-Dac, T., Ivanova, A.E., Roest, K., Akkermans, A.D.L., Lettinga, G. and Field, J.A. (submitted) Quinones as terminal electron acceptors in anaerobic granular sludge.
52. Cervantes-Carillo, F.J., *Quinones as electron acceptors and redox mediators for the anaerobic biotransformation of prority pollutants*, in *Agrotechnology and Food Sciences, Sub-department of Environmental Technology*. 2002, Wageningen University, The Netherlands: Wageningen, The Netherlands. p. 166.
53. 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 Res.*, **34**: 2223-2232.
54. Chippindale, C. and Taçon, P.S.C., *The Archaeology of Rock-Art*. 1998: Cambridge University Press.
55. Choi, J.J., Hirai, M. and Shoda, M. (1991) Catalytic oxidation of hydrogen sulfide by air over an activated carbon filter. *Appl. Cat. A-Gen.*, **79**: 241-248.
56. Choi, Y.S. and Cho, J.H. (1996) Color removal from dye wastewater using vermiculite. *Environ. Technol.*, **17**: 1169-1180.
57. Chu, W. and Ma Chi, W. (2000) Quantitative prediction of direct and indirect dye ozonation kinetics. *Water Res.*, **34**: 3153-3160.
58. Chung, K.T., Fulk, G.E. and Egan, M. (1978) Reduction of azo dyes by intestinal anaerobes. *Appl. Environ. Microbiol.*, **35**: 558-562.
59. Chung, K.T. and Cerniglia, C.E. (1992) Mutagenicity of azo dyes: Structure-activity relationships. *Mutat. Res.*, **277**: 201-220.
60. Chung, K.T., Stevens, S.E.J. and Cerniglia, C.E. (1992) The reduction of azo dyes by the intestinal microflora. *Crit. Rev. Microbiol.*, **18**: 175-197.
61. Chung, K.T. and Stevens, S.E.J. (1993) Degradation of azo dyes by environmental microorganisms and helminths. *Environ. Toxicol. Chem.*, **12**: 2121-2132.
62. Churchley, J. and Upton, J. (1997) Latest developments in textile colour removal studies. In: *Upgrading of water and waste water systems*(Eds.), Kalmar, Sweden, 1997. p. 1-9
63. Churchley, J.H. (1994) Removal of dyewaste colour from sewage effluent: The use of a full scale ozone plant. *Water Sci. Technol.*, **30**: 275-284.
64. Churchley, J.H., *The water company's view*, in *Colour in dyehouse effluent*, P. Cooper, Editor. 1995, Society of Dyers and Colourists: Bradford, England. p. 31-43.
65. Clarke, A.E. and Anliker, R., *Organic dyes and pigments*, in *The handbook of enviromental chemistry*, Hutzinger, Editor. 1980, Springer-Verlag: Berlin.
66. Coates, J.D., Ellis, D.J., Blunt-Harris, E.L., Gaw, C.V., Roden, E.E. and Lovley, D.R. (1998) Recovery of humic-reducing bacteria from a diversity of environments. *Appl. Environ. Microbiol.*, **64**: 1504-1509.
67. Cockett, K.R.F. and Webb, M., *Macrosorb colour treatment systems*, in *Colour in dyehouse effluent*, P. Cooper, Editor. 1995, Society of Dyers and Colourists: Bradford, England. p. 113-127.
68. Collins, R. and Picardal, F. (1999) Enhanced anaerobic transformations of carbon tetrachloride by soil organic matter. *Environ. Toxicol. Chem.*, **18**: 2703-2710.

69. Cookson, J.T., *Physicochemical changes of substances by or within carbon adsorption beds*, in *Activated carbon adsorption of organics from the aqueous phase*, I.H. Suffet and McGuire, M.J., Editors. 1980, An Arbor Science Publishers, Inc.: Ann Arbor, Michigan, USA. p. 379-396.
70. Cooper, P. (1993) Removing colour from dyehouse waste waters - a critical review of technology available. *J. Soc. Dyers Col.*, **109**: 97-100.
71. Costa, C. and Marquez, M.C. (1998) Kinetics of the PACT process. *Water Res.*, **32**: 107-114.
72. Crossley, C., *Membrane technology for the separation of dyehouse effluent*, in *Colour in dyehouse effluent*, P. Cooper, Editor. 1995, Society of Dyers and Colourists: Bradford, England. p. 155-170.
73. Cruz, A. and Buitrón, G. (2001) Biodegradation of Disperse Blue 79 using sequenced anaerobic/aerobic biofilters. *Water Sci. Technol.*, **44**: 159-166.
74. Curtis, G.P. and Reinhard, M. (1994) Reductive dehalogenation of hexachloroethane, carbon tetrachloride, and bromoform by anthraquinone disulfonate and humic acid. *Environ. Sci. Technol.*, **28**: 2393-2401.
75. Dalai, A.K., Majumdar, A. and Tollefson, E.L. (1999) Low temperature catalytic oxidation of hydrogen sulfide in sour produced wastewater using activated carbon catalysts. *Environ. Sci. Technol.*, **33**: 2241-2246.
76. Davis, R.J., Gainer, J.L., O'Neal, G. and Wu, I.W. (1994) Photocatalytic decolorization of wastewater dyes. *Water Environnent Research*, **66**: 50-53.
77. Davis, V.M. and Bailey, J.E.J. (1993) Chemical reduction of FD and C yellow No. 5 to determine combined benzidine. *J. Chromatogr.*, **635**: 160-164.
78. De Angelis, D.F. and Rodrigues, G.S. (1987) Azo dyes removal from industrial effluents using yeast biomass. *Arquivos de Biologia e Tecnologia*, **30**: 301-309.
79. De Jonge, R.J., Breure, A.M. and Van Andel, J.G. (1996) Bioregeneration of powdered activated carbon (PAC) loaded with aromatic compounds. *Water Res.*, **30**: 875-882.
80. DeFazio, S.A.K. and Lemley, A.T. (1999) Electrochemical treatment of acid dye systems: Sodium meta-bisulfite addition to the Andco system. *J. Environ. Sci. Heal A*, **34**: 217-240.
81. Delee, W., O'Neill, C., Hawkes, F.R. and Pinheiro, H.M. (1998) Anaerobic treatment of textile effluents: a review. *J. Chem. Technol. Biotechnol.*, **73**: 323-335.
82. Dieckhues, B. (1960) Untersuchungen zur reduktiven Spaltung der Azofarbstoffe durch Bakterien. *Zbl. Bakt. Paras. Inf. Abt. I*, **180**: 244-249.
83. Do, J.S. and Chen, M.L. (1994) Decolorization of dye-containing solutions by electrocoagulation. *J. Appl. Electrochem.*, **24**: 785-790.
84. Dockner, T. (1988) Reduction and hydrogenation with the system hydrocarbon/carbon. *Angew. Chem. Int. Ed. Engl.*, **27**: 679-682.
85. Donlon, B.A., Razo-Flores, E., Lettinga, G. and Field, J.A. (1996) Continuous detoxification, transformation and degradation of nitrophenols in upflow anaerobic sludge blanket (UASB) reactors. *Biotechnol. Bioeng.*, **51**: 439-449.
86. Donlon, B.A., Razo-Flores, E., Luijten, M., Swarts, H., Lettinga, G. and Field, J.A. (1997) Detoxification and partial mineralization of the azo dye mordant orange 1 in a continuous upflow anaerobic sludge-blanket reactor. *Appl. Microbiol. Biotechnol.*, **47**: 83-90.
87. Dubin, P. and Wright, K.L. (1975) Reduction of azo food dyes in cultures of *Proteus vulgaris*. *Xenobiotica*, **5**: 563-571.
88. Dunnivant, F.M., Schwarzenbach, R.P. and Macalady, D.L. (1992) Reduction of substituted nitrobenzenes in aqueous solutions containing natural organic matter. *Environ. Sci. Technol.*, **26**: 2133-2141.
89. Easton, J.R., *The dye maker's view*, in *Colour in dyehouse effluent*, P. Cooper, Editor. 1995, Society of Dyers and Colourists: Bradford, England. p. 9-21.
90. Ekici, P., Leupold, G. and Parlar, H. (2001) Degradability of selected azo dye metabolits in activated sludge systems. *Chemosphere*, **44**: 721-728.
91. El Geundi, M.S. (1991) Colour removal from textile effluents by adsorption techniques. *Water Res.*, **25**: 271-274.
92. Ennik-Maarsen, K.A., Louwerse, A., Roelofsen, W. and Stams, A.J.M. (1998) Influence of monochlorophenols on methanogenic activity in granular sludge. *Water Res.*, **32**: 2977-2982.

93. Fernandez, J., Bandara, J., Lopez, A., Buffat, P. and Kiwi, J. (1999) Photassisted Fenton degradation of nonbiodegradalbe azo dye (Orange II) in Fe-free solutions mediated by cation transfer membranes. *Langmuir*, **15**: 185-192.
94. Fewson, C.A. (1988) Biodegradation of xenobiotics and other persistent compounds: the causes of recalcitrance. *Trends Biotechnol.*, **6**: 148-153.
95. Field, J.A., Stams, A.J.M., Kato, M. and Schraa, G. (1995) Enhanced biodegradation of aromatic pollutants in cocultures of anaerobic and aerobic bacterial consortia. *Anton Leeuw. Int. J. G.*, **67**: 47-77.
96. Field, J.A., Cervantes, F.J., Van der Zee, F.P. and Lettinga, G. (2000) Role of quinones in the biodegradation of priority pollutants: a review. *Water Sci. Technol.*, **42**: 215-222.
97. Figueiredo, J.L., Pereira, M.F.R., Freitas, M.M.A. and Órfão, J.M.M. (1999) Modification of the surface chemistry of activated carbons. *Carbon*, **37**: 1379-1389.
98. FitzGerald, S.W. and Bishop, P.L. (1995) Two stage anaerobic/aerobic treatment of sulfonated azo dyes. *J. Environ. Sci. Heal A*, **30**: 1251-1276.
99. Fontenot, E.J., Beydilli, M.I., Lee, Y.H. and Pavlostathis, S.G. (2001) Kinetics and inhibition during the decolorization of reactive anthraquinone dyes under methanogenic conditions. In: *9th World Congress Anaerobic Digestion 2001 - Anaerobic conversion for sustainability*.A.F.M. Van Velsen and Verstraete, W.H. (Eds.), Antwerpen, Belgium, 2-6 Sept., 2001. Technologisch Instituut vzw, p. 215-220
100. Fu, Y. and Viraraghavan, T. (2001) Fungal decolorization of dye wastewaters: a review. *Biores. Technol.*, **79**: 251-262.
101. Fujita, S. and Peisach, J. (1982) The stimulation of microsomal azoreduction by flavins. *Biochim. Biophys. Acta*, **719**: 178-189.
102. Gähr, F., Hermanutz, F. and Oppermann, W. (1994) Ozonation - an important technique to comply with new German laws for textile wastewater treatment. *Water Sci. Technol.*, **30**: 255-263.
103. Ganesh, R., Boardman, G.D. and Michelsen, D. (1994) Fate of azo dyes in sludges. *Water Res.*, **28**: 1367-1376.
104. Gangneux, A. and Wattiez, D. (1976) Synthese et étude de celluloses échangeuses d'ions. Leur emploi dans l'épuration de eaux residuaires de l'industrie textile - I. *Europ. Polym. J.*, **12**: 536-541.
105. Gangneux, A. and Wattiez, D. (1976) Synthese et étude de celluloses échangeuses d'ions. Leur emploi dans l'épuration de eaux residuaires de l'industrie textile - II. *Europ. Polym. J.*, **12**: 543-549.
106. Gangneux, A. and Wattiez, D. (1976) Synthese et étude de celluloses échangeuses d'ions. Leur emploi dans l'épuration de eaux residuaires de l'industrie textile - III. *Europ. Polym. J.*, **12**: 551-557.
107. Garten, V.A. and Weiss, D.E. (1955) The quinone-hydroquine character of activated carbon and carbon black. *Australian Journal of Chemistry*, **8**: 68-95.
108. Ghosh, D.K., Mandal, A. and Chaudhuri, J. (1992) Purification and partial characterization of two azoreductases from Shigella dysenteriae type 1. *FEMS Microbiol. Lett.*, **98**: 229-234.
109. Ghosh, D.K., Ghosh, S., Sadhukhan, P., Mandal, A. and Chaudhuri, J. (1993) Purification of two azoreductases from Escherichia coli K12. *Ind. J. Exp. Biol.*, **31**: 951-954.
110. Giles, C.H. and Hassan, A.S.A. (1958) Adsorption at organic surfaces. V. A study aof the adsorption of dyes and other solutes by cellulose and chitin. *J. Soc. Dyers Colour.*, **74**: 874-857.
111. Gingell, R. and Walker, R. (1971) Mechanism of azo reduction by *Streptococcus faecalis* II. The role of soluble flavins. *Xenobiotica*, **1**: 231-239.
112. Glenn, J.K. and Gold, M.H. (1983) Decolorization of several polymeric dyes by the lignin-degrading basidiomycete Phanerochaete chrysosporium. *Appl. Environ. Microbiol.*, **45**: 1741-1747.
113. Glover, B. (1993) Getting rid of colour. *J. Soc. Dyers Colour.*, **109**: 273.
114. Gomes de Moreas, S., Sanches Freire, R. and Durán, N. (2000) Degradation and toxicity reduction of textile effluent by combined photocatalytic and ozonation processes. *Chemosphere*, **40**: 369-373.

115. Gonçalves, M.S.T., Oliveira Campos, A.M.F., Pinto, E.M.M.S., Plasencia, P.M.S. and Queiroz Maria, J.R.P. (1999) Photochemical treatment of solutions of azo dyes containing TiO2. *Chemosphere*, **39**: 781-786.
116. Grau, P. (1991) Textile industry wastewaters treatment. *Water Sci. Technol.*, **24**: 97-103.
117. Greene, J.C. and Baughman, G.l. (1996) Effects of 46 dyes on population growth of freshwater green alga *Selenastrum capricornutum. Text. Chem. Color.*, **28**: 23-30.
118. Haderlein, S.B. and P., S.R., *Environmental processes influencing the rate of abiotic reduction of nitroaromatic compounds in the subsurface*, in *Biodegradation of nitroaromatic compounds*, J.C. Spain, Editor. 1995, Plenum Press: New York and London.
119. Ham, S.W., Song, J.H., Kim, H.I. and Jin, S. (2000) Mechanism of cell cycle arrest by menadione. *B. Kor. Chem. Soc.*, **21**: 1173-1174.
120. Hao, O.J., H., K. and Chang, P.C. (2000) Decolorization of wastewater. *Crit. Rev. Env. Sci. Tec.*, **30**: 449-505.
121. Harmer, C. and Bishop, P. (1992) Transformation of azo dye AO-7 by wastewater biofilms. *Water Sci. Technol.*, **26**: 627-636.
122. Haug, W., Schmidt, A., Nortemann, B., Hempel, D.C., Stolz, A. and Knackmuss, H.J. (1991) Mineralization of the sulfonated azo dye Mordant Yellow 3 by a 6-aminonaphthalene-2-sulfonate-degrading bacterial consortium. *Appl. Environ. Microbiol.*, **57**: 3144-3149.
123. Hazel, B.G., *Industry evaluation of colour reduction and removal - the DEMOS project*, in *Colour in dyehouse effluent*, P. Cooper, Editor. 1995, Society of Dyers and Colourists: Bradford, England. p. 59-70.
124. Henderson, A.L., Schmitt, T.C., Heinze, T.M. and Cerniglia, C.E. (1997) Reduction of malachite green to leucomalachite green by intestinal bacteria. *Appl. Environ. Microbiol.*, **63**: 4099-4101.
125. Hernandez, P.H., Gilette, R. and Mazel, P. (1967) Studies on the mechanism of action of mammalian hepatic azoreductase. I. Azoreductase activity of reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase. *Biochem. Pharmacol.*, **16**: 1859-1875.
126. Hernandez, P.H., Gilette, R. and Mazel, P. (1967) Studies on the mechanism of action of mammalian hepatic azoreductase. II. The effect of phenobarbital and 3-methylcholanthrene on carbon monoxide sensitive and insensitive azoreductase activity. *Biochem. Pharmacol.*, **16**: 1877-1888.
127. Ho, Y.S. and McKay, G. (1998) The kinetics of sorption of basic dyes from aqueous solution by sphagnum moss peat. *Can. J. Chem. Eng.*, **76**: 822-827.
128. Ho, Y.S. and McKay, G. (1998) A two-stage batch sorption optimized design for dye removal to minimize contact time. *Process Saf. Environ.*, **76**: 313-318.
129. Hofstetter, T.B., Heijman, C.G., Haderlein, S.B., Holliger, C. and Swarzenbach, R.P. (1999) Complete reduction of TNT and other (poly)nitroaromatic compounds under iron-reducing subsurface conditions. *Environ. Sci. Technol.*, **33**: 1479-1487.
130. Hu, T.L. (1992) Sorption of reactive dyes by Aeromonas biomass. *Water Sci. Technol.*, **26**: 357-366.
131. Hung, C.H., Chiang, P.C., Yuan, C. and Chou, C.Y. (2001) Photocatalytic degradation of azo dye in TiO2 suspended solution. *Water Sci. Technol.*, **43**: 313-320.
132. Hwang, M.C. and Chen, K.M. (1993) The removal of color from effluents using polyamide-epichlorohydrin-cellulose. III. Use in anionic dye removal in a batch process. *J. Appl. Polym. Sci.*, **50**: 753-744.
133. Hwang, M.C. and Chen, K.M. (1993) The removal of color from effluents using polyamide-epichlorohydrin-cellulose. II. Use in acid dye removal. *J. Appl. Polym. Sci.*, **49**: 975-989.
134. Hwang, M.C. and Chen, K.M. (1993) The removal of color from effluents using polyamide-epichlorohydrin-cellulose. I. Preparation and use in direct dye removal. *J. Appl. Polym. Sci.*, **48**: 299-311.
135. IARC, *Some aromatic azo compounds*. Monographs on the evaluation of the carcinogenic risk of chemicals to humans. Vol. 8. 1975, Lyon, France: IARC (World Health Organization International Agency for Research on Cancer).

136. IARC, *Some industrial chemicals and dyestuffs*. Monographs on the evaluation of the carcinogenic risk of chemicals to humans. Vol. 29. 1982, Lyon, France: IARC (World Health Organization International Agency for Research on Cancer).
137. Ince, N.H. and Gonenc, D.T. (1997) Treatability of a textile azo dye by UV/H-2O-2. *Environ. Technol.*, **18**: 179-185.
138. Ince, N.H. and Tezcanli, G. (1999) Treatability of textile dye-bath effluents by advanced oxidation: Preparation for reuse. *Water Sci. Technol.*, **40**: 183-190.
139. Jank, M., Koser, H., Lucking, F., Martienssen, M. and Wittchen, S. (1998) Decolorization and degradation of Erioglaucine (acid blue 9) dye in wastewater. *Environ. Technol.*, **19**: 741-747.
140. Jia, J., Yang, J., Liao, J., Wang, W. and Wang, Z. (1999) Treatment of dyeing wastewater with ACF electrodes. *Water Res.*, **33**: 881-884.
141. Jiang, H. and Bishop, P.L. (1994) Aerobic biodegradation of azo dyes in biofilms. *Water Sci. Technol.*, **29**: 525-530.
142. Jianrong, Z., Yanru, Y., Huren, A. and Yi, Q. (1994) A study of dyewaste treatment using anaerobic-aerobic process. In: *Seventh International Symposium on Anaerobic Digestion*(Eds.), Cape Town, South Africa, 1994. p. 360-363
143. Jimenez, B., Noyola, A. and Capdeville, B. (1988) Selected dyes for residence time distribution evaluation in bioreactors. *Biotechnol. Techniques*, **2**: 77-82.
144. Jinqi, L. and Houtian, L. (1992) Degradation of azo dyes by algae. *Environmental Pollution*, **75**: 273-278.
145. Jirka, A. and Carter, M.J. (1975) Micro semi-automated analysis of surface and waste waters for chemical oxygen demand. *Anal. Chem.*, **47**: 1397-1401.
146. Juang, R.S., Tseng, R.L., Wu, F.C. and Lee, S.H. (1997) Adsorption behavior of reactive dyes from aqueous solutions on chitosan. *J. Chem. Technol. Biotechnol.*, **70**: 391-399.
147. Judkins Jr, J.F. (1984) Textile waste-water. *J. Water Pollut. Control Fed.*, **56**: 642.
148. Jung, R., Steinle, D. and Anliker, R. (1992) A compilation of genotoxicity and carcinogenicity data on aromatic aminosulphonic acids. *Food Chem. Toxicol.*, **30**: 635-660.
149. Kadirvelu, K., Palanival, M., Kalpana, R. and Rajeswari, S. (2000) Activated carbon from an agricultural by-product, for the treatment of dyeing wastewater. *Biores. Technol.*, **74**: 263-265.
150. Kalyuzhnyi, S. and Sklyar, V. (2000) Biomineralisation of azo dyes and their breakdown products in anaerobic-aerobic hybrid and UASB reactors. *Water Sci. Technol.*, **41**: 23-30.
151. Kang, S.F. and Chang, H.M. (1997) Coagulation of textile secondary effluents with Fenton's reagent. *Water Sci. Technol.*, **36**: 215-222.
152. Keck, A., Klein, J., Kudlich, M., Stolz, A., Knackmuss, H.J. and Mattes, R. (1997) Reduction of azo dyes by redox mediators originating in the naphthalenesulfonic acid degradation pathway of *Sphingomonas* sp. strain BN6. *Appl. Environ. Microbiol.*, **63**: 3684-3690.
153. Khan, S.U. (1978) Kinetics of hydrolysis of atrazine in aqueous fulvic acid solution. *Pesticide Science*, **9**: 39-43.
154. Kirk, T.K. and Farrell, R.L. (1987) Enzymatic 'combustion'. The microbial degradation of lignin. *Annu. Rev. Microbiol.*, **41**: 465-505.
155. Klibanov, A.M. and Morris, E.D. (1981) Horsheradish peroxidase for the removal of carcinogenic aromatic amines from water. *Enzyme and Microbiol. Technol.*, **3**: 119-122.
156. Knackmuss, H.J. (1996) Basic knowledge and perspectives of bioelimination of xenobiotic compounds. *J. Biotechnol.*, **51**: 287-295.
157. Konopka, A. (1993) Isolation and characterization of a subsurface bacterium that degrades aniline and methylanilines. *FEMS Microbiol. Lett.*, **111**: 93-99.
158. Koprivanac, N., Bosanac, G., Grabaric, Z. and Papic, S. (1993) Treatment of wastewaters from dye industry. *Environ. Technol.*, **14**: 385-390.
159. Krupa, N.E. and Cannon, F.S. (1996) GAC: Pore structure versus dye adsorption. *Am. Water Works Ass. J.*, **88**: 94-108.
160. Kuai, L., De Vreese, I., Vandevivere, P. and Verstraete, W. (1998) GAC-amended UASB reactor for the stable treatment of toxic textile wastewater. *Environ. Technol.*, **19**: 1111-1117.
161. 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. *Appl. Environ. Microbiol.*, **63**: 3691-3694.
162. Kudlich, M., Hetheridge, M.J., Knackmuss, H.J. and Stolz, A. (1999) Autoxidation reactions of different aromatic o-aminohydroxynaphthalenes that are formed during the anaerobic reduction of sulfonated azo dyes. *Environ. Sci. Technol.*, **33**: 896-901.
163. Kulla, H.G., Klausener, F., Meyer, U., Luedeke, B. and Leisinger, T. (1983) Evolution of new bacterial enzyme activities during adaptation to azo dyes. *Arch. Microbiol.*, **135**: 1-7.
164. Kulla, H.G., Klausener, F.K., Meyer, U., Ludeke, B. and Leisinger, T. (1983) Interference of aromatic sulfo groups in the microbial degradation of the azo dyes Orange I and Orange II. *Arch. Microbiol.*, **135**: 1-7.
165. Kuo, W.G. (1992) Decolorizing dye wastewater with Fenton's reagent. *Water Res.*, **26**: 881-886.
166. Lambert, S.D., Graham, N.J.D., Sollars, C.J. and Fowler, G.D. (1997) Evaluation of inorganic adsorbents for the removal of problematic textile dyes and pesticides. *Water Sci. Technol.*, **36**: 173-180.
167. Laszlo, J.A. (1994) Removing acid dyes from textile wastewater using biomass for decolorization. *Am. Dyest. Rep.*, **83**: 17-21.
168. Laszlo, J.A. (1996) Preparing an ion exchange resin from sugarcane bagasse to remove reactive dye from wastewater. *Text. Chem. Color.*, **28**: 13-17.
169. Laszlo, J.A. (1997) Regeneration of dye-saturated quaternized cellulose by bisulfite-mediated borohydride reduction of azo groups: an improved process for decolorization of textile wastewaters. *Environ. Sci. Technol.*, **31**: 3647-3653.
170. Laszlo, J.A. (2000) Regeneration of azo-dye-saturated cellulosic anion exchange resin by *Burholderia cepacia* anaerobic dye reduction. *Environ. Sci. Technol.*, **34**: 164-172.
171. Lee, C.K., Low, K.S. and Chow, S.W. (1996) Chrome sludge as an adsorbent for colour removal. *Environ. Technol.*, **17**: 1023-1028.
172. Lefers, J.B., Koetsier, W.T. and Van Swaaij, W.P.M. (1978) The oxidation of sulphide in aqueous solutions. *Chem. Eng. J.*, **15**: 112-120.
173. Lens, P.N., De, P.M.P., Cronenberg, C.C. and Verstraete, W.H. (1995) Sulfate reducing and methane producing bacteria in aerobic wastewater treatment systems. *Water Res.*, **29**: 871-880.
174. Levine, W.G. (1991) Metabolism of azo dyes: implication for detoxification and activation. *Drug Metabol. Rev.*, **23**: 253-309.
175. Li, G.C. and Felbeck jr., G.T. (1972) Atrazine hydrolysis as catalysed by humic acids. *Soil Sci.*, **114**: 201-209.
176. Li, X.Z. and Zhao, Y.G. (1997) On-site treatment of dyeing wastewater by a bio-photoreactor system. *Water Sci. Technol.*, **36**: 165-172.
177. Li, X.Z. and Zhao, Y.G. (1999) Advanced treatment of dyeing wastewater for reuse. *Water Sci. Technol.*, **39**: 249-255.
178. Liakou, S., Kornaros, M. and Lyberatos, G. (1997) Pretreatment of azo dyes using ozone. *Water Sci. Technol.*, **36**: 155-163.
179. Liakou, S., Pavlou, S. and Lyberatos, G. (1997) Ozonation of azo dyes. *Water Sci. Technol.*, **35**: 279-286.
180. Libra, J., Yoo, E.S., Borchert, M. and Wiesmann, U. (1997) Mechanisms of biological decolorization: their engineering applications. In: *Treatment of wastewaters from textile processing*(Eds.), Berlin, 1997. p. 245-265
181. Lin, S.H. and Lin, C.M. (1991) Decolorization of textile waste effluents by ozonation. *J. Environ. Syst.*, **21**: 143-156.
182. Lin, S.H. (1993) Adsorption of disperse dye by various adsorbents. *J. Chem. Technol. Biotechnol.*, **58**: 159-163.
183. Lin, S.H. (1993) Adsorption of disperse dye by powdered activated carbon. *J. Chem. Technol. Biotechnol.*, **57**: 387-391.
184. Lin, S.H. and Peng, C.F. (1994) Treatment of textile wastewater by electrochemical method. *Water Res.*, **28**: 277-282.
185. Lin, S.H. and Peng, C.F. (1995) A continuous Fenton's process for treatment of textile wastewater. *Environ. Technol.*, **16**: 693-699.

186. Lin, S.H. and Peng, C.F. (1996) Continuous treatment of textile wastewater by combined coagulation, electrochemical oxidation and activated sludge. *Water Res.*, **30**: 587-592.
187. Lin, S.H. and Chen, M.l. (1997) Purification of textile wastewater effluents by a combined Fenton process and ion exchange. *Desalination*, **109**: 121-130.
188. Little, L.W. and Chillingworth, M.A., in *Reports on selected dyes and their effect*, A.D.M. Institute, Editor. 1974, American Dye Manufacturers Institute: New York.
189. Liversidge, R.M., Lloyd, G.J., Wase, D.A.J. and Forster, C.F. (1997) Removal of Basic Blue 41 dye from aqueous solution by linseed cake. *Process Biochem.*, **32**: 437-477.
190. Loidl, M., Hinteregger, C., Ditzelmuller, G., Ferschl, A. and Streichsbier, F. (1990) Degradation of aniline and monochlorinated anilines by soil-born Pseudomonas acidovorans strains. *Arch. Microbiol.*, **155**: 56-61.
191. Longhinotti, E., Pozza, F., Furlan, L., Sanchez, M., Klug, M., Laranjeira, M.C.M. and Favere, V.T. (1998) Adsorption of anionic dyes on the biopolymer chitin. *J. Brazil. Chem. Soc.*, **9**: 435-440.
192. Lourenço, N.D., Novais, J.M. and Pinheiro, H.M. (2000) Reactive textile dye colour removal in a sequencing batch reactor. *Water Sci. Technol.*, **42**: 321-328.
193. Lourenço, N.D., Novais, J.M. and Pinheiro, H.M. (2001) Effect of some operational parameters on textile dye biodegradation in a sequential batch reactor. *J. Biotechnol.*, **89**: 163-174.
194. Lovley, D.R. and Phillips, E.J.P. (1986) Availability of ferric iron for microbial reduction in bottom sediments of the fresh-water tidal Potomac River. *Appl. Environ. Microbiol.*, **52**: 751-757.
195. Lovley, D.R., Coates, J.D., Blunt Harris, E.L., Phillips, E.J.P. and Woodward, J.C. (1996) Humic substances as electron acceptors for microbial respiration. *Nature*, **382**: 445-448.
196. Luangdilok, W. and Paswad, T. (2000) Effect of chemical structures of reactive dyes on color removal by an anaerobic-aerobic process. *Water Sci. Technol.*, **42**: 377-382.
197. Luther, M. and Soeder, C.J. (1987) Some naphthalenesulfonic acids as sulfur sources for the green microalga, *Scenedesmus obliquus. Chemosphere*, **16**: 1565-1578.
198. Luther, M. and Soeder, C.J. (1991) 1-Naphthalenesulfonic acid and sulfate as sulfur sources for the green alga Scenedesmus obliquus. *Water Res.*, **25**: 299-308.
199. Madigan, M.T., Martinko, J.M. and Parker, J., *Brock biology of microorganisms*. 8th ed. 1996, Upper Saddle River, New Jersey, USA: Prentice-Hall, Inc., Simon & Schuster/ A Viacom Company.
200. Mandelbaum, R.T., Wackett, L.P. and Allan, D.L. (1993) Rapid hydrolysis of atrazine to hydroxyatrazine by soil bacteria. *Environ. Sci. Technol.*, **27**: 1943-1946.
201. Marmagne, O. and Coste, C. (1996) Color removal from textile plant effluents. *Am. Dyest. Rep.*, **84**: 15-21.
202. Marmier Dussoubs, D., Mazet, M. and Pronost, J. (1991) Removal of dyestuffs by wood charcoal. *Environ. Technol.*, **12**: 625-634.
203. Marquez, M.C. and Costa, C. (1996) Biomass concentration in pact process. *Water Res.*, **30**: 2079-2085.
204. McClung, S. and Lemley, A.T. (1994) Electrochemical treatment and HPLC analysis of wastewater containing acid dyes. *Textile Chemist Colorist*, **24**: 17-22.
205. McGuire, M.J. and Suffet, I.H., *Activated carob adsorption of organics from the aqueous phase*. 1980, Ann Arbor, Michigan, USA: Ann Arbor Science Publishers, Inc.
206. McKay, G., Blair, H.S. and Gardner, J. (1982) Adsorption of dyestuffs onto chitin. External mass transfer process. *J. Appl. Polym. Sci.*, **27**: 4251-4261.
207. McKay, G., Otterburn, M.S. and Aga, J.A. (1985) Fuller's earth and fired clay as adsorbents for dyestuffs. *Water Air Soil Poll.*, **24**: 307-322.
208. McKay, G., El Geundi, M. and Nassar, M.M. (1987) Equilibrium studies during the removal of dyestuffs from aqueous solutions using bagasse pith. *Water Res.*, **21**: 1513-1520.
209. McKay, G., Ramprasad, G. and Mowli, P. (1987) Desorption and regeneration of dye colours from low-cost materials. *Water Res.*, **21**: 375-377.
210. McKay, G., El Geundi, E. and Nassar, M.M. (1988) External mass transport processes during the adsorption of dyes onto bagasse pith. *Water Res.*, **22**: 1527-1533.

211. McKay, G., El Geundi, E. and Nassar, M.M. (1996) Pore diffusion during the adsorption of dyes onto bagasse pith. *Process Saf. Environ.*, **74**: 487-502.
212. McMullan, G., Meehan, C., Conneely, A., Nirby, N., Robinson, T., Nigam, P., Banat, I.M., Marchant, R. and Smyth, W.F. (2001) Mini Review: Microbial decolourisation and degradation of textile dyes. *Appl. Microbiol. Biotechnol.*.
213. Mechsner, K. and Wuhrmann, K. (1982) Cell permeability as a rate limiting factor in the microbial reduction of sulfonated azo dyes. *Eur. J. Appl. Microbiol. Biotechnol.*, **15**: 123-126.
214. Meulenberg, R., Rijnaarts, H.H.M., Doddema, H.J. and Field, J.A. (1997) Partially oxidized polycyclic aromatic hydrocarbons show an increased bioavailability and biodegradability. *FEMS Microbiology Letters*, **152**: 45-49.
215. Meyer, V., Carlsson, F.H.H. and Oellermann, R.A. (1992) Decolourization of textile effluent using a low cost natural adsorbent material. *Water Sci. Technol.*, **26**: 1205-1211.
216. Mielgo, I., Moreira, M.T., Feijoo, G. and Lema, J.M. (2001) A packed-bed fungal bioreactor for the continuous decolourisation of azo-dyes (Orange II). *J. Biotechnol.*, **89 (2-3)**: 99-106.
217. Mittal, A.K. and Gupta, S.K. (1996) Biosorption of cationic dyes by dead macro fungus Fomitopsis carnea: Batch studies. *Water Sci. Technol.*, **34**: 81-87.
218. Morais, L.C., Freitas, O.M., Goncalves, E.P., Vasconcelos, L.T. and Gonzalez Beca, C.G. (1998) Reactive dyes removal from wastewaters by adsorption on eucalyptus bark: Variables that define the process. *Water Res.*, **33**: 979-988.
219. Morais, L.C., Goncalves, E.P., Vasconcelos, L.T. and Beca, C.G.G. (2000) Reactive dyes removal from wastewaters by adsorption on eucalyptus bark - adsorption equilibria. *Environ. Technol.*, **21**: 577-583.
220. Moteleb, M.A., Suidan, M.T., Kim, J., Davel, J.L. and Adrian, N.R. (2001) Anaerobic degradation of 2,4,6-trinitrotoluene in granular activated carbon fluidized bed and batch reactors. *Water Sci. Technol.*, **43**: 67-75.
221. Mou, D.G., Lim, K.M. and Shen, H.P. (1991) Microbial agents for decolorization of dye wastewater. *Biotechnological Advance*, **9**: 613-622.
222. Murray, C.J. and Webb, T. (1991) General acid catalysis of the reduction of *p*-benzoquinone by an NADH analogue. *J. Am. Chem. Soc.*, **113**: 7426-7427.
223. Nam, S. and Renganathan, V. (2000) Non-enzymatic reduction of azo dyes by NADH. *Chemosphere*, **40**: 351-357.
224. Nam, S. and Tratnyek, P.G. (2000) Reduction of azo dyes with zero-valent iron. *Water Res.*, **34**: 1837-1845.
225. Namasivayam, C. and Yamuna, R.T. (1992) Removal of Rhodamine-B by biogas waste slurry from aqueous solution. *Water Air Soil Poll.*, **65**: 101-109.
226. Namasivayam, C. and Yamuna, R.T. (1992) Removal of Congo Red from aqueous solutions by biogas waste slurry. *J. Chem. Technol. Biotechnol.*, **53**: 153-157.
227. Namasivayam, C. and Kadirvelu, K. (1994) Coirpith, an agricultural waste by-product, for the treatment of dyeing wastewater. *Biores. Technol.*, **48**: 79-81.
228. Namasivayam, C., D., P. and M., K. (1998) Removal of Direct Red and Acid Brilliant Blue by adsorption on to banana pith. *Biores. Technol.*, **64**: 77-79.
229. Namboodri, C.G. and Walsh, W.K. (1995) Decolorizing spent dyebath with hot peroxide. *Am. Dyest. Rep.*, **84**: 86-95.
230. Nasr, C., Vinodgopal, K., Fisher, L., Hotchandani, S., Chattopadhyay, A.K. and Kamat, P.V. (1996) Environmental photochemistry on semiconductor surfaces. Visible light induced degradation of a textile diazo dye, naphthol blue black, on TiO2 nanoparticles. *J. Phys. Chem.-US*, **100**: 8436-8442.
231. Nassar, M.M. and El Geundi, M.S. (1991) Comparative cost of color removal from textile effluents using natural adsorbents. *J. Chem. Technol. Biotechnol.*, **50**: 257-264.
232. Nassar, M.M., Hamoda, M.F. and Radwan, G.H. (1995) Adsorption equilibria of basic dyestuff onto palm-fruit bunch particles. *Water Sci. Technol.*, **32**: 27-32.
233. Nassar, M.M. and Magdy, Y.H. (1997) Removal of different basic dyes from aqueous solutions by adsorption on palm-fruit bunch particles. *Chem. Eng. J.*, **66**: 223-226.
234. Nassar, M.M. (1999) Intraparticle diffusion of basic red and basic yellow dyes on palm fruit bunch. *Water Sci. Technol.*, **40**: 133-139.

235. Naumczyk, J., Szpyrkowicz, L. and Zilio Grandi, F. (1996) Electrochemical treatment of textile wastewater. *Water Sci. Technol.*, **34**: 17-24.
236. Nawar, S.S. and Doma, H.S. (1989) Removal of dyes from effluents using low-cost agricultural by-products. *Sci. Total Environ.*, **79**: 271-279.
237. Newman, D.K. and Kolter, R. (2000) A role for excreted quinones in extracellular electron transfer. *Nature*, **405**: 97-97.
238. Nicolaou, M. and Hadjivassilis, I. (1992) Treatment of wastewater from the textile industry. *Water Sci. Technol.*, **25**: 31-35.
239. Nigam, P., Banat, I.M., Singh, D. and Marchant, R. (1996) Microbial process for the decolorization of textile effluent containing azo, diazo and reactive dyes. *Process Biochem.*, **31**: 435-442.
240. Nigam, P., Armour, G., Banat, I.M., Singh, D. and Marchant, R. (2000) Physical removal of textile dyes from effluents and solid-state fermentation of dye-adsorbed agricultural residues. *Biores. Technol.*, **72**: 219-226.
241. Noblet, J.A., Smith, L.A. and Suffet, I.H. (1996) Influence of natural dissolved organic matter, temperature, and mixing on the abiotic hydrolysis of triazine and organophosphate pesticides. *J. Agr. Food Chem.*, **44**: 3685-3693.
242. Noertemann, B., Kuhm, A.E., Knackmuss, H.J. and Stolz, A. (1994) Conversion of substituted naphthalenesulfonates by Pseudomonas sp. BN6. *Arch. Microbiol.*, **161**: 320-327.
243. Ogutveren, U.B., Gonen, N. and Koparal, S. (1992) Removal of dye stuffs from waste water: Electrocoagulation of Acilan Blau using soluble anode. *J. Environ. Sci. Heal A*, **27**: 1237-1247.
244. Oliveros, E., Legrini, O., Hohl, M., Müiller, T. and Braun, A.M. (1997) Large scale development of a light-enhanced Fenton reaction by optimal experimental design. *Water Sci. Technol.*, **35**: 223-230.
245. Øllgaard, H., Frost, L., Galster, J. and Hansen, O.C., *Survey of azo-colorants in Denmark: Consumption, use, health and environmental aspects*. 1998, Ministry of Environment and Energy, Denmark.
246. O'Loughlin, E.J., Burris, D.R. and Delcomyn, C.A. (1999) Reductive dechlorination of trichloroethene mediated by humic-metal complexes. *Environ. Sci. Technol.*, **33**: 1145-1147.
247. O'Neill, C., Hawkes, F.R., Esteves, S.R.R., Hawkes, D.L. and Wilcox, S.J. (1999) Anaerobic and aerobic treatment of a simulated textile effluent. *J. Chem. Technol. Biotechnol.*, **74**: 993-999.
248. O'Neill, C., Hawkes, F.R., Hawkes, D.L., Lourenço, N.D., Pinheiro, H.M. and Delée, W. (1999) Colour in textile effluents - sources, measurement, discharge consents and simulation: a review. *J. Chem. Technol. Biotechnol.*, **74**: 10009-1018.
249. O'Neill, C., Hawkes, F.R., Hawkes, D.W., Esteves, S. and Wilcox, S.J. (2000) Anaerobic-aerobic biotreatment of simulated textile effluent containing varied ratios of starch and azo dye. *Water Res.*, **34**: 2355-2361.
250. O'Neill, C., Lopez, A., Esteves, S., Hawkes, F.R., Hawkes, D.L. and Wilcox, S. (2000) Azo-dye degradation in an anaerobic-aerobic treatment system operating on simulated textile effluent. *Appl. Microbiol. Biotechnol.*, **53**: 249-254.
251. Pagga, U. and Brown, D. (1986) The degradation of dyestuffs: Part II. Behaviour of dyestuffs in aerobic biodegradation tests. *Chemosphere*, **15**: 479-491.
252. Pagga, U. and Taeger, K. (1994) Development of a method for adsorption of dyestuffs on activated sludge. *Water Res.*, **28**: 1051-1057.
253. Palma, C., Moreira, M.T., Mielgo, I., Feijoo, G. and Lema, J.M. (1999) Use of a fungal bioreactor as a pretreatment or post-treatment step for continuous decolorisation of dyes. *Water Sci. Technol.*, **40**: 131-136.
254. Panswad, T. and Luangdilok, W. (2000) Decolorization of reactive dyes with different molecular structures under different environmental conditions. *Water Res.*, **34**: 4177-4184.
255. Panswad, T., Iamsamer, K. and Anotai, J. (2001) Decolorisation of azo-reactive dye by polyphosphate and glycogen-accumulating organisms in an anaeroboc-aerobic sequencing batch reactor. *Biores. Technol.*, **76**: 151-159.
256. Park, T.J., Lee, K.H., Jung, E.J. and Kim, C.W. (1999) Removal of refractory organics and color in pigment wastewater with Fenton oxidation. *Water Sci. Technol.*, **39**: 189-192.

257. Pasti, M.B. and Crawford, D.L. (1990) Isolation of microorganisms able to reductively transform aromatic compounds and their relevance to coal liquefaction. *Can. J. Microbiol.*, **37**: 902-907.
258. Peralta-Zamora, P., Kunz, A., de Moraes, S.G., Pelegrini, R., Moleiro, P.D., Reyes, J. and Duran, N. (1999) Degradation of reactive dyes - I. A comparative study of ozonation, enzymatic and photochemical processes. *Chemosphere*, **38**: 835-852.
259. Pereira, M.F.R., Órfão, J.M.M. and Figueiredo, J.L. (1999) Oxidative dehydrogenation of ethylbenzene on activated carbon catalysts. I. Influence of chemical groups. *Appl. Cat. A-Gen.*, **184**: 153-160.
260. Pereira, M.F.R., Órfão, J.M.M. and Figueiredo, J.L. (2000) Oxidative dehydrogenation of ethylbenzene on activated carbon catalysts. 2. Kinetic modelling. *Appl. Cat. A-Gen.*, **196**: 43-54.
261. Perkowski, J., Kos, L. and Ledakowicz, S. (1996) Application of ozone in textile wastewater treatment. *Ozone-Sci. Eng.*, **18**: 73-85.
262. Perlinger, J.A., Angst, W. and Schwarzenbach, R.P. (1996) Kinetics of the reduction of hexachloroethane by juglone in solutions containing hydrogen sulfide. *Environ. Sci. Technol.*, **30**: 3408-3417.
263. Perlinger, J.A., Buschmann, J., Angst, W. and Schwarzenbach, R.P. (1998) Iron porphyrin and mercaptojuglone mediated reduction of polyhalogenated methanes and ethanes in homogeneous aqueous solution. *Environ. Sci. Technol.*, **32**: 2431-2437.
264. Perlinger, J.A., Venkatapathy, R. and Harrison, J.F. (2000) Linear free energy relationships for polyhalogenated alkane transformation by electron-transfer mediators in model aqueous systems. *J. Phys. Chem. A*, **104**: 2752-2763.
265. Polman, J.K. and Breckenridge, C.R. (1996) Biomass-mediated binding and recovery of textile dyes from waste effluents. *Text. Chem. Color.*, **28**: 31-35.
266. Poots, V.J.P., McKay, G. and Healy, J.J. (1976) The removal of acid dye from effluent using natural adsorbents. I: Peat. *Water Res.*, **10**: 1061-1066.
267. Poots, V.J.P., McKay, G. and Healy, J.J. (1976) The removal of acid dye from effluent using natural adsorbents. II: Wood. *Water Res.*, **10**: 1067-1070.
268. Poulios, I. and Tsachpinis, I. (1999) Photodegradation of the textile dye Reactive Black 5 in the presence of semiconducting oxides. *J. Chem. Technol. Biotechnol.*, **74**: 349-357.
269. Prevot, A.B., Baiocchi, C., Brussino, M.C., Pramauro, E., Savarino, P., Augugliaro, V., Marci, G. and Palmisano, L. (2001) Photocatalytic degradation of acid Blue 80 in aqueous solutions containing TiO2 suspensions. *Environ. Sci. Technol.*, **35 (5)**: 971-976.
270. Prousek, J., Ivanova, E. and Kocmanikova, M. (1997) Utilization of Fenton reaction for the degradation of conventionally used dyes and coloured waste waters. *Chem. Listy*, **91**: 48-53.
271. Rafii, F., Franklin, W. and Cerniglia, C.E. (1990) Azoreductase activity of anaerobic bacteria isolated from human intestinal microflora. *Appl. Environ. Microbiol.*, **56**: 2146-2151.
272. Rafii, F., Smith, D.B., Benson, R.W. and Cerniglia, C.E. (1992) Immunological homology among azoreductases from Clostridium and Eubacterium strains isolated from human intestinal microflora. *J. Basic Microb.*, **32**: 99-105.
273. Rafii, F. and Cerniglia, C.E. (1993) Comparison of the azoreductase and nitroreductase from Clostridium perfringens. *Appl. Environ. Microbiol.*, **59**: 1731-1734.
274. Rafii, F. and Cerniglia, C.E. (1993) Localization of the azoreductase of *Clostridium perfringens* by immuno-electron microscopy. *Curr. Microbiol.*, **27**: 143-145.
275. Rafii, F. and Cerniglia, C.E. (1995) Reduction of azo dyes and nitroaromatic compounds by bacterial enzymes from the human intestinal tract. *Environ. Health Persp.*, **103**: 17-19.
276. Rajaguru, P., Kalaiselvi, K., Palanivel, M. and Subburam, V. (2000) Biodegradation of azo dyes in a sequential anaerobic-aerobic system. *Appl. Microbiol. Biotechnol.*, **54**: 268-273.
277. Ramakrishna, K.R. and Viraraghavan, T. (1997) Dye removal using low cost adsorbents. *Water Sci. Technol.*, **36**: 189-196.
278. Razo Flores, E., Luijten, M., Donlon, B.A., Lettinga, G. and Field, J.A. (1997) Complete biodegradation of the azo dye azodisalicylate under anaerobic conditions. *Environ. Sci. Technol.*, **31**: 2098-2103.

279. Reeves, P., Ohlhausen, R., Sloan, D., Pamplin, K., Scoggins, T., Clark, C., Hutchinson, B. and Green, D. (1992) Photocatalytic destruction of organic dyes in aqueous titanium oxide suspensions using concentrated simulated and natural solar energy. *Sol. Energy*, **48**: 413-420.
280. Reutergardh, L.B. and Iangphasuk, M. (1997) Photocatalytic decolourization of reactive azo dye: A comparison between TiO-2 and CdS photocatalysis. *Chemosphere*, **35**: 585-596.
281. 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. *Biores. Technol.*, **77**: 247-255.
282. Roxon, J.J., Ryan, A.J. and Wright, S.E. (1967) Enzymatic reduction of tartrazine by Proteus vulgaris from rats. *Food Cosmet. Toxicol.*, **5**: 645-656.
283. Rozzi, A., Malpei, F., Bonomo, L. and Bianchi, R. (1999) Textile wastewater reuse in Northern Italy (COMO). *Water Sci. Technol.*, **39**: 122-128.
284. Ruppert, G., Bauer, R. and Heisler, G. (1994) UV-O_3, UV-H_2O_2, UV-TiO_2 and photo-Fenton reaction - comparison of advanced oxidation processes for wastewater treatment. *Chemosphere*, **28**: 1447-1454.
285. Russ, R., Mueller, C., Knackmuss, H.J. and Stolz, A. (1994) Aerobic biodegradation of 3-aminobenzoate by Gram-negative bacteria involves intermediate formation of 5-aminosalicylate as ring-cleavage substrate. *FEMS Microbiol. Lett.*, **122**: 137-143.
286. Russ, R., Rau, J. and Stolz, A. (2000) The function of cytoplasmic flavin reductases in the reduction of azo dyes by bacteria. *Appl. Environ. Microbiol.*, **66**: 1429-1434.
287. Safarik, I., Nymburska, K. and Safarikova, M. (1997) Adsorption of water-soluble organic dyes on magnetic charcoal. *J. Chem. Technol. Biotechnol.*, **69**: 1-4.
288. Sani, R.K. and Banerjee, U.C. (1999) Decolorization of triphenylmethane dyes and textile and dye-stuff effluent by Kurthia sp. *Enzyme and Microbiol. Technol.*, **24**: 433-437.
289. Santoro, D. and Louw, R. (2001) Gas phase hydrogenolysis mediated by activated carbon: substituted chlorobenzenes. *Carbon*, **39**: 2091-2099.
290. Sarasa, J., Roche, M.P., Ormad, M.P., Gimeno, E., Puig, A. and J.l., O. (1998) Treatment of a wastewater resulting from dyes manufacturing with ozone and chemical coagulation. *Water Res.*, **32**: 2721-2727.
291. Sarnaik, S. and Kanekar, P. (1999) Biodegradation of methyl violet by *Pseudomonas mendocina* MCM B-402. *Appl. Microbiol. Biotechnol.*, **52**: 251-254.
292. Sarsour, J., Janitza, J. and Gähr, F. (2001) Biologische Abbau farbstoffhaltiger Abwässer. *WLB Wasser, Luft und Boden*,: 44-46.
293. Scheline, R.R. and Nygaard, R.T. (1970) Enzymatic reduction of the azo dye, Acid Yellow, by extracts of *Streptococcus faecalis* isolated from rat intestine. *Food Cosmet. Toxicol.*, **8**: 55-58.
294. Schwarzenbach, R.P., Stierli, R., Lanz, K. and Zeyer, J. (1990) Quinone and iron porphyrin mediated reduction of nitroaromatic compounds in homogeneous aqeous solution. *Environ. Sci. Technol.*, **24**.
295. Seignez, C., Adler, N., Suard, J.C. and Peringer, P. (1996) Aerobic and anaerobic biodegradability of 1-anthraquinone sulphonate. *Appl. Microbiol. Biotechnol.*, **45**: 719-722.
296. Semdé, R., Pierre, D., Geuskens, G., Devleeschouwer, M. and Moes, A.J. (1998) Study of some important factors involved in azo derivative reduction by Clostridium perfringens. *Int. J. Pharm.*, **161**: 45-54.
297. Semple, K.T., Cain, R.B. and Schmidt, S. (1999) Biodegradation of aromatic compounds by microalgae. *FEMS Microbiol. Lett.*, **170**: 291-300.
298. Seshadri, S., Bishop, P.L. and Agha, A.M. (1994) Anaerobic/aerobic treatment of selected azo dyes in wastewater. *Waste Manage.*, **14**: 127-137.
299. Shaul, G.M., Holdsworth, T.J., Dempsey, C.R. and Dostal, K.A. (1991) Fate of water soluble azo dyes in the activated sludge process. *Chemosphere*, **22**: 107-119.
300. Shi, W.X., Xu, X.J. and Sun, G. (1999) Chemically modified sunflower stalks as adsorbents for color removal from textile wastewater. *J. Appl. Polym. Sci.*, **71**: 1841-1850.
301. Shiang-Fu, Q., Barkovskii, A.L. and Adriaens, P. (1999) Reductive transformation of dioxins: an assessment of the contribution of dissolved organic matter to dechlorination reactions. *Environ. Sci. Technol.*, **33**: 3837-3842.

302. Smith, B., Koonce, T. and Hudson, S. (1993) Decolorizing dye wastewater using chitosan. *Am. Dyest. Rep.*, **82**: 18-35.
303. Sober, H.A., Harte, R.A. and Sober, E.K., *Handbook of biochemistry: selected data for molecular biology*. 1970, Cleveland, Ohio, USA: The Chemical Rubber Co.
304. Soeder, C.J., Hegewald, E. and Kneifel, H. (1987) Green microalgae can use naphthalenesulfonic acids as sources of sulfur. *Arch. Microbiol.*, **148**: 260-263.
305. Solozhenko, E.G., Soboleva, N.M. and Goncharuk, V.V. (1995) Decolourization of azodye solutions by Fenton's oxidation. *Water Res.*, **29**: 2206-2210.
306. Sosath, F., Libra, J. and Wiesmann, U., *Combined biological and chemical treatment of textile dye-house wastewater using rotating disc reactors*, in *Treatment of wastewaters from textile processing - Behandlung von Abwaessern der Textilverdlung*, A. Kornmueller, Editor. 1997: Berlin. p. 229-243.
307. Sosath, F. and Libra, J.A. (1997) Biologische Behandlung von synthetischen Abwaessern mit Azofarbstoffen (Purification of wastewaters containing azo dyes). *Acta Hydroch. Hydrob.*, **25**: 259-264.
308. Southern, T.G., *Technical solutions to the colour problem: a critical review*, in *Colour in dyehouse effluent*, P. Cooper, Editor. 1995, Society of Dyers and Colourists: Bradford, England. p. 73-91.
309. Specht, K. and Platzek, T. (1995) Textile dyes and finishes - Remarks to toxicological and analytical aspects. *Deut. Lebensm.-Rundsch.*, **91**: 352-359.
310. Stolz, A. (2001) Basic and applied aspects in the microbial degradation of azo dyes. *Appl. Microbiol. Biotechnol.*, **56**: 69-80.
311. Sublette, K.L., Snider, E.H. and Sylvester, N.D. (1982) A review of the mechanism of powdered activated carbon enhance ment of activated sludge treatment. *Water Res.*, **16**: 1075-1082.
312. Suidan, M.T., Flora, J.R.V., Boyer, T.K., Wuellner, A.M. and Narayanan, B. (1996) Anaerobic dechlorination using a fluidized-bed GAC reactor. *Water Res.*, **30**: 160-170.
313. Sumathi, S. and Manju, B.S. (2001) Fungal mediated decolorization of media containing procion dyes. *Water Sci. Technol.*, **43**: 285-290.
314. Suzuki, Y., Yoda, T., Ruhul, A. and Sugiura, W. (2001) Molecular cloning and characterization of the gene coding for azoreductase from Bacillus sp OY1-2 isolated from soil. *J. Biol. Chem.*, **276 (12)**: 9059-9065.
315. Swamy, J. and Ramsay, J.A. (1999) The evaluation of white rot fungi in the decoloration of textile dyes. *Enzyme and Microbiol. Technol.*, **24**: 130-137.
316. Tan, N.C.G., Opsteeg, J.L., Lettinga, G. and Field, J.A., *Integrated anaerobic/aerobic EGSB bioreactor for azo dye degradation*, in *Bioremediation of nitroaromatics and haloaromatic compounds*, B.C. Alleman and Leeson, A., Editors. 1999, Battelle Press: Columbus, Richland, USA. p. 253-258.
317. Tan, N.C.G. and Field, J.A., *Biodegradation of sulfonated aromatic compounds*, in *Environmental Technologies to Treat Sulfur Pollution. Principles and Engineering*, P. Lens and Hulshoff-Pol, L.W., Editors. 2000, IWA Publishing: London. p. 377-392.
318. Tan, N.C.G., Slenders, P., Svitelskaya, A., Lettinga, G. and Field, J.A. (2000) Degradation of azo dye Mordant Yellow 10 in a sequential anaerobic and bioaugmented aerobic bioreactor. *Water Sci. Technol.*, **42**: 337-344.
319. Tan, N.C.G., *Integrated and sequential anaerobic/aerobic biodegradation of azo dyes*, in *Agrotechnology and Food Sciences, Sub-department of Environmental Technology*. 2001, Wageningen University, The Netherlands: Wageningen, The Netherlands. p. 104.
320. Tang, W.Z. and An, H. (1995) Photocatalytic degradation kinetics and mechanism of Acid Blue 40 by TiO-2/UV in aqueous solution. *Chemosphere*, **31**: 4171-4183.
321. Tang, W.Z. and An, H. (1995) UV/TiO2 photodegradation of commercial dyes dyes in aqueous solutions. *Chemosphere*, **31**: 4157-4170.
322. Tang, W.Z. and Chen, R.Z. (1996) Decolorization Kinetics and Mechanisms of Commercial Dyes by H_2O_2/Iron Powder System. *Chemosphere*, **32**: 947-958.
323. Tang, W.Z., Zhang, Z., An, H., Quintana, M.O. and Torres, D.F. (1997) TiO2/UV photodegradation of azo dyes in aqueous solutions. *Environ. Technol.*, **18**: 1-12.

324. Tatsumi, H., Kano, K. and Ikeda, T. (2000) Kinetic aspects of fast hydrogenase reaction of *Desulfovibrio vulgaris* cells in the presence of exogenous electron acceptors. *J. Phys. Chem. B*, **104**: 12079-12083.
325. Timmons, A. and Ainsworth, J. (1994) New clarifying process solves textile effluent colour problem. *Water Wastew. Int.*, **9**: 54.
326. Townsend, R.B., Neytzell De Wilde, F.G., Buckley, C.A., Turpie, D.W.F. and Steenkamp, C. (1992) The use of dynamic membranes for the treatment of effluents arising from wool scouring and textile dyeing effluents. *Water SA*, **18**: 81-86.
327. Tratnyek, P.G. and Macalady, D.L. (1989) Abiotic reduction of nitro aromatic pesticides in anaerobic laboratory systems. *Journal of Agricultural and Food Chemistry*, **37**: 248-254.
328. Trüper, H.G. and Schlegel, H.G. (1964) Sulphur metabolism in *Thiorhodaceae*. 1: Quantitative measurements on growing cells of *Chromatium okenii*. *Anton. van Leeuw. J. M. S.*, **30**: 225-238.
329. Tunay, O., Kabdasli, I., Eremektar, G. and D., O. (1996) Color removal from textile wastewaters. *Water Sci. Technol.*, **34**: 9-16.
330. Vandevivere, P.C., Bianchi, R. and W., V. (1998) Treatment and reuse of wastewater from the textile wet-processing industry: Review of emerging technologies. *J. Chem. Technol. Biotechnol.*, **72**: 289-302.
331. Van't Hul, K.P., Racz, I.G. and Reith, T. (1997) The application of membrane technology for reuse of process water and minimization of waste water in textile washing range. *J. Soc. Dyers Colour.*, **113**: 287-295.
332. Vinodgopal, K., Wynkoop, D.E. and Kamat, P.V. (1996) Environmental photochemistry on semiconductor surfaces: Photosensitized degradation of a textile azo dye, acid orange 7, on TiO2 particles using visible light. *Environ. Sci. Technol.*, **30**: 1660-1666.
333. Vlyssides, A.G., Loizidou, M., Karlis, P.K., Zorpas, A.A. and Papaioannou, D. (1999) Electrochemical oxidation of a textile dye wastewater using a Pt/Ti electrode. *J. Hazard. Mater.*, **70**: 41-52.
334. Walker, G.M. and Weatherly, L.R. (1997) Adsorption of acid dyes on to granular activated carbon in fixed beds. *Water Res.*, **31**: 2093-2101.
335. Walker, G.M. and Weatherley, L.R. (1999) Biological activated carbon treatment of industrial wastewater in stirred tank reactors. *Chem. Eng. J.*, **75**: 201-206.
336. Walker, G.M. and Weatherley, L.R. (2000) Biodegradation and biosorption of acid anthraquinone dye. *Environmental pollution*, **108**: 219-223.
337. Walker, R. (1970) The metabolism of azo compounds: a review of the literature. *Food Cosmet. Toxicol.*, **8**: 659-676.
338. Walker, R., Gingell, R. and Murrells, D.F. (1971) Mechanisms of azo reduction by Streptococcus faecalis I. Optimization of assay conditions. *Xenobiotica*, **1**: 221-229.
339. Wang, Y.T. (1990) Methanogenic degradation of ozonation products of biorefractory or toxic aromatic compounds. *Water Res.*, **24**: 185-190.
340. Weber, E.J. and Wolfe, L.N. (1987) Kinetic studies of the reduction of aromatic azo compounds in anaerobic sediment/water systems. *Environ. Toxicol. Chem.*, **6**: 911-919.
341. Weber, E.J. (1991) Studies of benzidine-based dyes in sediment-water systems. *Environ. Toxicol. Chem.*, **10**: 609-618.
342. Weber, E.J. and Adams, R.L. (1995) Chemical- and sediment-mediated reduction of the Azo dye disperse blue 79. *Environ. Sci. Technol.*, **29**: 1163-1170.
343. Weber, E.J. (1996) Iron-mediated reductive transformation: Investigation of reaction mechanism. *Environ. Sci. Technol.*, **30**: 716-719.
344. Welham, A. (2000) The theory of dyeing (and the secret of life). *J. Soc. Dyers Colour.*, **116**: 140-143.
345. Wilcock, A. (1992) Spectrometric analysis of electrochemically treated simulated dispersed dyebath effluent. *Text. Chem. Color.*, **24**: 29-37.
346. Wu, J. and Wang, T. (2001) Ozonation of aqueous azo dye in a semi-batch reactor. *Water Res.*, **35**: 1093-1099.
347. Wu, J.N., Eiteman, M.A. and Law, S.E. (1998) Evaluation of membrane filtration and ozonation processes for treatment of reactive-dye wastewater. *J. Environ. Eng.-ASCE*, **124**: 272-277.

348. Wuhrmann, K., Mechsner, K. and Kappeler, T. (1980) Investigation on rate-determining factors in the microbial reduction of azo dyes. *Eur. J. Appl. Microbiol. Biotechnol.*, **9**: 325-338.
349. Yatome, C., Ogawa, T. and Matsui, M. (1991) Degradation of crystal violet by *Bacillus subtilis*. *J. Environ. Sci. Eng.*, **26**: 75-88.
350. Yatome, C., Yamada, S., Ogawa, T. and Matsui, M. (1993) Degradation of crystal violet by *Nocardia corallina*. *Appl. Microbiol. Biotechnol.*, **38**: 565-569.
351. Yoo, E.S., Libra, J. and Wiesmann, U. (2000) Reduction of azo dyes by *Desulfovibrio desulfuricans*. *Water Sci. Technol.*, **41**: 15-22.
352. Yoshida, H., Fukuda, S., Okamoto, A. and Kataoka, T. (1991) Recovery of direct dye and acid dye by adsorption on chitosan fiber - equilibria. *Water Sci. Technol.*, **23**: 1667-1676.
353. Yoshida, H., Okamoto, A. and Kataoka, T. (1993) Adsorption of acid dye on cross-linked chitosan fibres: equilibria. *Chem. Eng. Sci.*, **48**: 2267-2272.
354. Yoshida, H. and Takemori, T. (1997) Adsorption of direct dye on cross-linked chitosan fiber: Breakthrough curve. *Water Sci. Technol.*, **35**: 29-37.
355. Youssef, B.M. (1993) Adsorption of acid dyes by cellulose derivatives. *Am. Dyest. Rep.*, **82**: 30-33, 49.
356. Zaoyan, Y., Ke, S., Guangliang, S., Fan, Y., Jinshan, D. and Huanian, M. (1992) Anaerobic-aerobic treatment of a dye wastewater by combination of RBC with activated sludge. *Water Sci. Technol.*, **26**: 2093-2096.
357. Zbaida, S. and Levine, W.G. (1992) Role of electronic factors in binding and reduction of azo dyes by hepatic microsomes. *J. Pharmacol. Exp. Ther.*, **260**: 554-561.
358. Zhang, F. and Yu, J. (2000) Decolourisation of Acid Violet 7 with complex pellets of white rot fungus and activated carbon. *Bioprocess Eng.*, **23**: 295-301.
359. Zhang, T.Y., Oyama, T., Aoshima, A., Hidaka, H., Zhao, J.C. and Serpone, N. (2001) Photooxidative N-demethylation of methylene blue in aqueous TiO2 dispersions under UV irradiation. *J. Photoch. Photobio. A*, **140**: 163-172.
360. Zhou, W.C. and Zimmermann, W. (1993) Decolorization of industrial effluents containing reactive dyes by actinomycetes. *FEMS Microbiol. Lett.*, **107**: 157-162.
361. Zhu, C., Wang, L., Kong, L., Yang, X., Wang, L., Zheng, S., Chen, F., MaiZhi, F. and Zong, H. (2000) Photocatalytic degradation of AZO dyes by supported TiO2 + UV in aqueous solution. *Chemosphere*, **41**: 303-309.
362. Zimmermann, T., Kulla, H. and Leisinger, T. (1982) Properties of purified Orange II azoreductase, the enzyme initiating azo dye degradation by *Pseudomonas* KF46. *Eur. J. Biochem.*, **129**: 197-203.
363. Zimmermann, T., Kulla, H. and Leisinger, T. (1982) Purification and properties of orange II-azoreductase from Pseudomonas KF46. *Experientia*, **38**: 1380.
364. Zimmermann, T., Gasser, F., Kulla, H. and Leisinger, T. (1984) Comparison of two bacterial azoreductases acquired during adaptation to growth on azo dyes. *Arch. Microbiol.*, **138**: 37-43.
365. Zitomer, D.H. and Speece, R.H. (1993) Sequential environments for enhanced biotransformation of aqueous contaminants. *Environ. Sci. Technol.*, **27**: 227-244.

List of abbreviations

AC	Activated carbon
AO7	Acid Orange 7
AQDS	anthraquinone-2,6-disulphonate
AQS	anthraquinone-2-sulphonate
BCF	Bioconcentration factor
C.I.	Colour Index
COD	Chemical Oxygen Demand
E0	Redox potential at standard conditions
E0'	Redox potential at standard conditions and pH = 7
ED	Electron Donor
ECF	(Redox mediating) Enzyme cofactor
EGSB	Expanded Granular Sludge Blanket
FAD	Flavin adenide dinucleotide
FADH2	reduced FAD
FMN	Flavin adenide mononucleotide
FMNH2	reduced FMN
GC	Gas Chromatograph
HPLC	High Performance Liquid Chromatograph
HRT	Hydraulic Retention Time
ITD	Inclined Tubular Digester
K_{OW}	partition coefficient octanol/water
λ_{max}	wavelength of maximum absorbance
LAS	Linear Alkylsulphonate
LC_{50}	50% Lethal Concentration
LD_{50}	50% Lethal Dose
log P	log K_{OW}
M	molar
M_W	molecular weight
MWCO	Molecular Weight Cut-off
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced NAD
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced NADP
PVA	Polyvinylalcohol
RM	Redox mediator
RR2	Reactive Red 2
SA	Sulphanilic acid
SBR	Sequenced Batch Reactor
SRM	Sludge redox mediator
UASB	Upflow Anaerobic Sludge Blanket
VFA	Volatile Fatty Acids
VSS	Volatile Suspended Solids

Wetenschap bedrijven op het gebied van de milieutechnologie is een merkwaardige bezigheid. Er is een toepassing en er zijn duizend relevante vragen, vragen die tot op zekere hoogte vaak niet eens zo moeilijk te beantwoorden zijn, maar let wel: tot op zekere hoogte -en dat is niet voldoende. Je moet de diepte in, want het moet wel wat voorstellen, wetenschappelijk (consultant worden kan immers altijd nog). Dus maak je een sprong in de bodemloze poel van het onderzoek, kopje onder in de verstikkende vrijheid van de vele, vele mogelijkheden. Als het goed is heb je een beetje leren zwemmen, maar dat wil nog niet zeggen dat je dan ook een baron von Münchhausen bent: om jezelf op te kunnen richten heb je steunpunten nodig, de rest is een kwestie van smaak, intuïtie en geluk.

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Curriculum vitae

Frank Pieter van der Zee werd op 1 september 1969 te Middelburg geboren. In 1987 behaalde hij zijn diploma Gymnasium β aan de Stedelijke Scholengemeenschap Middelburg en begon hij milieuhygiëne te studeren aan de Landbouwuniversiteit Wageningen (LUW). Voor deze studie, die hij in augustus 1993 afrondde, deed hij afstudeervakken aan de vakgroepen Milieutechnologie (De invloed van beperkende hoeveelheden sulfaat op de omzetting van lagere vetzuren door syntrofe methanogene consortia) en Microbiologie (Fysiologische karakterisering van een thermofiele propionaatoxiderende sulfaatreducerende bacterie).

Na zijn afstuderen werd hij tijdelijk wetenschappelijk medewerker bij de vakgroep Milieutechnologie van de LUW. Onderbroken door zijn militaire diensttijd zou hij deze functie, waarbinnen hij werkte aan diverse projecten die alle verband hielden met anaërobe zuiveringstechnologie, blijven bekleden tot en met augustus 1997.

Vervolgens werkte hij als milieutechnoloog bij Biothane Systems International. Deze tijdelijke baan behelsde uitzending naar Athene, voor de opstart van de eerste Griekse 'high-rate' anaërobe zuiveringsreactor en de opleiding van het locale personeel.

In februari 1998 begon hij als assistent in opleiding (a.i.o.) bij de vakgroep (inmiddels sectie) Milieutechnologie van de LUW (inmiddels Wageningen Universiteit) aan de vier jaren promotieonderzoek waarvan dit proefschrift de weerslag is.

Sinds maart 2002 werkt hij in deeltijd bij de Lettinga Associates Foundation for Environmental Protection and Resource Conservation en is hij op zoek naar een baan buiten het Biotechnion.

Publications in scientific journals

as first author:

Van der Zee, F.P., Lettinga, G. and Field, J.A. (2000) The role of (auto)catalysis in the mechanism of anaerobic azo reduction. *Water Sci. Technol.*, **42**: 301-308.

Van der Zee, F.P., Lettinga, G. and Field, J.A. (2001) Azo dye decolourisation by anaerobic granular sludge. *Chemosphere*, **44**: 1169-1176.

Van der Zee, F.P., Bouwman, R.H.M., Strik, D.P.B.T.B., Lettinga, G. and Field, J.A. (2001) Application of redox mediators to accelerate the transformation of reactive azo dyes in anaerobic bioreactors. *Biotechnol. Bioeng.*, **75**: 691-701.

Van der Zee, F.P., Bisschops, I.A.E., Blanchard, V.G., Bouwman, R.H.M., Lettinga, G. and Field, J.A. (2002) Biotic and abiotic processes of azo dye reduction in anaerobic sludge. *submitted.*

Van der Zee, F.P., Bisschops, I.A.E., Lettinga, G. and Field, J.A. (2002) Activated carbon as an electron acceptor and redox mediator during the anaerobic biotransformation of azo dyes. *submitted.*

as co-author:

Cervantes, F.J., Van der Zee, F.P., Lettinga, G. and Field, J.A. (2001) Enhanced decolourisation of Acid Orange 7 in a continuous UASB reactor with quinones as redox mediators. *Water Sci. Technol.*, **44**: 123-128.

Field, J.A., Cervantes, F.J., Van der Zee, F.P. and Lettinga, G. (2000) Role of quinones in the biodegradation of priority pollutants: a review. *Water Sci. Technol.*, **42**: 215-222.

Puig-Grajales, L., Tan, N.C.G., Van der Zee, F., Razo-Flores, E. and Field, J.A. (2000) Anaerobic biodegradability of alkylphenols and fuel oxygenates in the presence of alternative electron acceptors. *Appl. Microbiol. Biotechnol.*, **54**: 692-697.

Van Lier, J.B., Van der Zee, F.P., Tan, N.C.G., Rebac, S. and Kleerebezem, R. (2001) Advances in high rate anaerobic treatment: staging of reactor systems. *Water Sci. Technol.*, **44**: 15-25.

Visser, A., Beeksma, I., Van der Zee, F., Stams, A.J.M. and Lettinga, G. (1993) Anaerobic degradation of volatile fatty acids at different sulfate concentrations. *Appl. Microbiol. Biotechnol.*, **40**: 549-56.

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