Integrated and sequential anaerobic/aerobic biodegradation of azo dyes

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. ir. L. Speelman,
in het openbaar te verdedigen
op dinsdag 27 maart 2001
des namiddags te vier uur in de Aula

Ontwerp omslag: Joke Peeters en Luc Tan

Author: Tan, N.C.G.
Title: Integrated and sequential anaerobic/aerobic biodegradation of azo dyes
ISBN: 90-5808-374-8
Publication year: 2001
Subject headings: azo dyes / anaerobic/aerobic / biodegradation / sulfonated aromatic amines
Thesis Wageningen University Research Center, Wageningen, The Netherlands – With references –With abstract – With summary in English and Dutch – 104 p.

Il est plus nécessaire d'étudier les hommes que les livres.

François la Rochefoucauld (1613-1680)

Voor Pa en Ma

Untuk Bapak dan Ibu

Tan, N.C.G., 2001. **Integrated and sequential anaerobic/aerobic biodegradation of azo dyes.** Doctoral Thesis. Wageningen University. Wageningen, The Netherlands, 104 pages.

Azo dyes constitute a major class of environmental pollutants accounting for 60 to 70% of all dyes and pigments used. These compounds are characterized by aromatic moieties linked together with azo groups (-N=N-). The release of azo dyes into the environment is a concern due to coloration of natural waters and due to the toxicity, mutagenicity and carcinogenicity of the dyes and their biotransformation products. Therefore, considerable attention has been given to evaluating the fate of azo dyes during wastewater treatment and in the natural environment. Azo dyes require an anaerobic and an aerobic phase for their complete biodegradation. Therefore, the aim of this thesis was to evaluate the mineralization of azo dyes under integrated and sequential anaerobic/aerobic conditions. These conditions were applied on three azo dyes, Mordant Orange 1 (MO1), 4-phenylazophenol (4-PAP) and Mordant Yellow 10 (MY10). Since many azo dyes are substituted with a sulfonic acid group, special attention was paid to the sulfonated azo dyes and their biodegradation products, the sulfonated aromatic amines.

The first step in the biodegradation of azo dyes is the azo dye reduction resulting in the formation of the aromatic amines. The influence of oxygen on the azo dye reduction of MO1 was tested in order to evaluate the integrated anaerobic/aerobic conditions. The co-substrate ethanol was successfully applied as electron donor for azo dye reduction and also created anaerobic microniches to facilitate anaerobic azo dye reduction in the presence of oxygen. Increasing oxygen concentrations showed decreasing azo dye-reduction rates with ethanol as co-substrate. These rates were higher when applying acetate as co-substrate. Moreover, Nedalco granular sludge could withstand better with the applied integrated anaerobic/aerobic conditions than Shell granular sludge.

Since none of the expected aromatic amines from MO1 was degraded, further research was conducted to evaluate the aerobic biodegradation of aromatic amines. MY10 and 4-PAP were tested under integrated anaerobic/aerobic conditions. All aromatic amines were removed if sufficient oxygen was present. Oxygen was primarily used to oxidize co-substrate and if sufficient oxygen was available the formed aromatic amines were further degraded. The removal of 4-aminobenzenesulfonic acid (4-ABS) was only possible after bioaugmentation of a 4-ABS-degrading enrichment culture. The results from the biodegradation study of 4-AP indicated that 4-aminophenol was removed due to autoxidation. Degradation of azo dyes is possible under integrated anaerobic/aerobic conditions if co-substrate and oxygen are in balance.

In the sequential anaerobic/aerobic bioreactor, the azo dye MY10 was successfully mineralized. Its constituent aromatic amines, 5-aminosalicylic acid (5-ASA) and 4-ABS, were both recovered in the anaerobic stage and degraded in the aerobic stage. Biodegradation of the 4-ABS was only possible after bioaugmentation with a 4-ABS-degrading enrichment culture. High recovery of sulfate indicated mineralization of 4-ABS. After long-term exposure to 5-ASA, the anaerobic granular sludge showed the ability to degrade this compound.

It was observed that the degradation of the sulfonated aromatic amine 4-ABS only proceeded after bioaugmentation of a specialized enrichment culture. Therefore, the ultimate biodegradability of the sulfonated aromatic amines is questionable. To investigate this matter, the fate of sulfonated aromatic amines with natural mixed cultures was evaluated in aerobic and anaerobic batch assays and bioreactor experiments. None of the ten tested compounds was degraded under anaerobic conditions and only 2-ABS and 4-ABS were aerobically mineralized. This was observed in batch as well as in bioreactor experiments. Degradation of the 2-ABS and 4-ABS was found with inoculum sources that were historically polluted with sulfonated aromatic compounds. At concentrations up to 1.0 g l^{-1}, none of the tested sulfonated aromatic amines showed any toxicity towards anaerobic and aerobic biomass.

The results of this research demonstrated that azo dyes are mineralized under integrated and sequential anaerobic/aerobic conditions. Due the difficulties with balancing the supply of co-substrate and oxygen in integrated anaerobic/aerobic systems, the sequential anaerobic/aerobic conditions are recommended for the mineralization of azo dyes. However, during the degradation of sulfonated azo dyes, many different sulfonated aromatic amines will be formed and these compounds are not likely to be degraded aerobically. Therefore, special attention should be paid on the removal of these compounds.

1

General introduction

Published in part as: Tan N.C.G. and Field J.A. (2000). Biodegradation of sulfonated aromatic compounds. In: Environmental Technologies to Treat Sulfur Pollution. Principles and Engineering. Lens P. and Hulshoff-Pol L. (Eds.). IWA Publishing, London. pp. 377-392.

1.1 Introduction

A few years ago some articles appeared in the Dutch newspapers dealing with the usage of forbidden azo dyes like benzidine analogue dyes. These were found in different cloths and still were used at manufacturing sites (Didde 1997). Since these publications, the interest in these compounds has increased significantly. This general introduction will provide more insight into the history, usage, toxicity and biodegradation of azo dyes and their precursors and biodegradation products.

The history of these compounds goes back over a hundred years. William Henry Perkin synthesized the first dye, mauve, by accident in 1856 from chemicals derived from coal. W.H. Perkin commercialized his innovation and developed the production processes for this new dye. This is the beginning of a chemical industry producing approximately 700,000 tons of colorants on yearly basis 131 years later (Zollinger 1987). Recent data on the world production are not available. However, it can be assumed that more colorants are produced in view of the economic prosperity and also because the productions of many colored goods increased and color printing and copying became normal by the end of the nineteenth century.

Azo dyes are by far the largest and most important group of dyes (Carliell et al. 1998) mainly due to the simple synthesis. The fundament of the production of azo dyes was laid in 1858 when P. Gries discovered the reaction mechanism, diazotization, for the production of azo compounds (Zollinger 1987). Presently, azo dyes are used for coloring many different materials such as textile, leather, plastics, food, and pharmaceuticals and for manufacturing paints and lacquers and for printings purposes as well.

Due to their chemical structure azo dyes absorb light in the visible spectrum. Their chemical structure is characterized by one or more azo groups (-N=N-). The azo group is substituted with benzene or naphthalene groups, which can contain many different substituents such as chloro (-Cl), methyl (-CH_3), nitro (-NO_2), amino (-NH_2), hydroxyl (-OH) and carboxyl (-COOH). A substituent often found in azo dyes is the sulfonic acid group (-SO_3H). The azo dyes containing this substituent are the so-called sulfonated azo dyes (Figure 1.1).

During manufacturing and usage of azo dyes an estimated amount of 10-15% is released into the environment (Vaidya and Datye 1982). Likely this figure is presently even higher since the usage of reactive dyes (which include azo dyes) increased lately and their fixation rate in dyeing processes can be as low as 50% (Easton 1995). Water-soluble azo dyes, like the sulfonated azo dyes, will enter the environment generally with wastewater discharges. Also these sulfonated azo dyes are widely used in different industries (Zollinger 1987). Sulfonated and unsulfonated azo dyes have a negative aesthetic effect on the wastewater and some of

these compounds and biodegradation products are also toxic, carcinogenic and mutagenic (Grover et al. 1996).

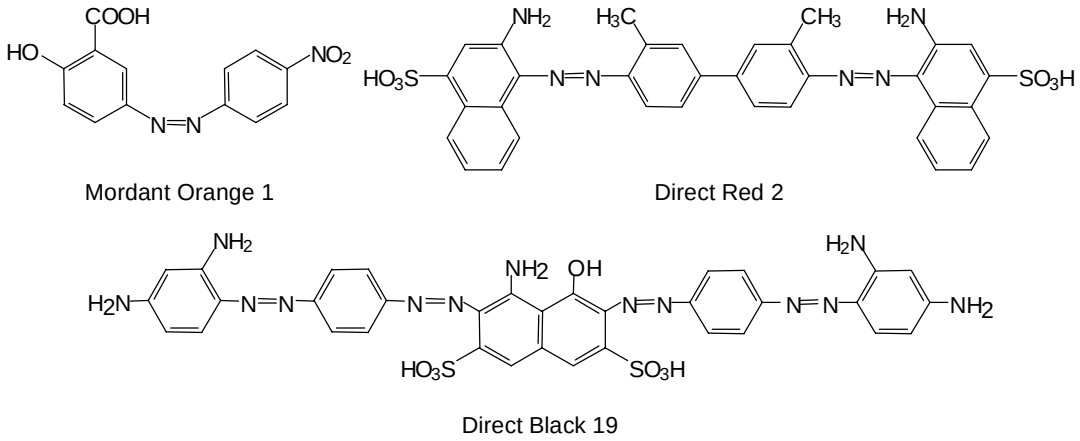

Figure 1.1 *Chemical structure of sulfonated and unsulfonated azo dyes with the one or more azo groups and the different substituents substituted to the aromatic rings.*

There exists a clear relationship between their chemical structure and their potential danger. All the azo dyes containing a nitro group were found to be mutagenic (Chung and Cerniglia 1992), and a high toxicity of these azo dyes was also observed for methanogenic granular sludge (Donlon et al. 1997). Furthermore, some azo dyes can produce toxic degradation products. Examples of such harmful moieties are 1,4-phenylenediamine, 1-amino-2-naphthol, benzidine and substituted benzidines, like o-tolidine (Figure 1.2) (Chung et al. 1981; Reid et al. 1984; Rosenkranz and Klopman 1989; Rosenkranz and Klopman 1990). The benzidine moieties in azo dyes are the already mentioned -presently prohibited- benzidine analogue dyes.

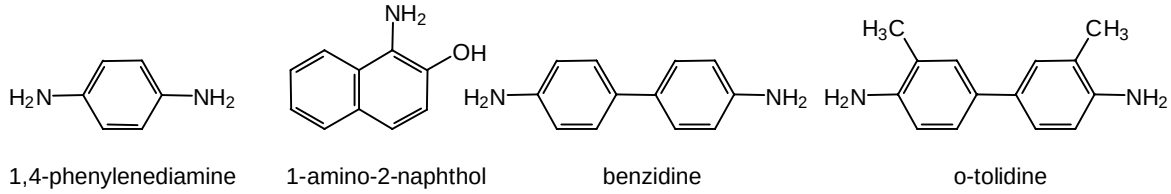

Figure 1.2 *Chemical structures that after metabolic activation of azo dyes show toxic*

There exists clear evidence that sulfonated azo dyes show decreased or no mutagenic effect compared to unsulfonated azo dyes due to their electric charge and low lipophilicity, which prevents them from uptake and metabolic activation (Chung and Cerniglia 1992; Jung et al. 1992; Levine 1991; Rosenkranz and Klopman 1990). Due to the above-mentioned effects, it is clear that azo dyes should not enter the environment. An attractive method to prevent this is to apply microbial treatment methods for their mineralization. Several other methods for azo

dye removal from wastewater are also available, but they mainly concern concentrating methods.

1.2 Biodegradation of azo dyes and aromatic amines

Microorganisms are known to play a crucial role in the mineralization of biopolymers and xenobiotic compounds, like azo dyes (Lie et al. 1998). The mineralization of azo dyes requires a integrated or sequential anaerobic and aerobic step (Field et al. 1995). The first step in the biodegradation of azo dyes concerns the azo dye reduction that readily proceeds under anaerobic conditions and results in the formation of aromatic amines (Carliell et al. 1995; Razo-Flores et al. 1997; Walker 1970; Weber and Wolfe 1987). Anaerobic consortia do generally not degrade the aromatic amines but most of the aromatic amines are readily biodegraded under aerobic conditions (Baird et al. 1977; Brown and Laboureur 1983a).

First, the anaerobic azo dye reduction will be reviewed briefly and next the aerobic biodegradation of azo dyes and aromatic amines. Special attention will be paid to the aerobic degradation of sulfonated aromatic amines because these compounds constitute the raw materials for the synthesis of sulfonated azo dyes and are also the main anaerobic degradation products of these widely used sulfonated azo dyes (Zollinger 1987). In addition some other biotechnological methods for the removal of sulfonated aromatic amines will be reviewed and furthermore the autoxidation of aromatic amines and the possibilities to degrade aromatic amines aerobically will be mentioned. Finally, the sequential and integrated anaerobic/aerobic biodegradation of azo dyes will be evaluated.

1.2.1 The anaerobic reduction and biodegradation of azo dyes

Under anaerobic conditions azo dyes are readily decolorized, as a result of the reductive transformation of the azo group. Due to this reduction two aromatic amines are formed (Figure 1.3). The aromatic amines do not absorb light in the visible spectrum and therefore azo dye reduction represents a decolorization process.

The anaerobic decolorization of azo dyes was first investigated using intestinal anaerobic bacteria (Allan and Roxon 1974; Brown 1981; Chung et al. 1978; Chung et al. 1992; Walker and Ryan 1971). Later these compounds were found to become also readily decolorized with various other anaerobic cultures (Beydilli et al. 1998; Brown and Laboureur 1983b; Carliell et al. 1994; Donlon et al. 1997; Razo-Flores et al. 1997). The exact mechanism of the anaerobic azo dye reduction is not clearly understood yet. Therefore, the term azo dye reduction may involve different mechanisms or locations like enzymatic (Haug et al. 1991; Rafii et al. 1990), non-ezymatic (Gingell and Walker 1971), mediated (Kudlich et al. 1997; Van der Zee et al. 2000a), intracellular (Mechsner and Wuhrmann 1982; Wuhrmann et al. 1980), extracellular (Carliell et al. 1995) and various combinations of these mechanisms and locations.

In one case even a complete anaerobic mineralization of the azo dye azodisalicylate was observed under methanogenic conditions. This azo dye was first reduced to the aromatic amine 5-aminosalicylic acid (5-ASA) and next 5-ASA was anaerobically mineralized (Razo-Flores et al. 1997).

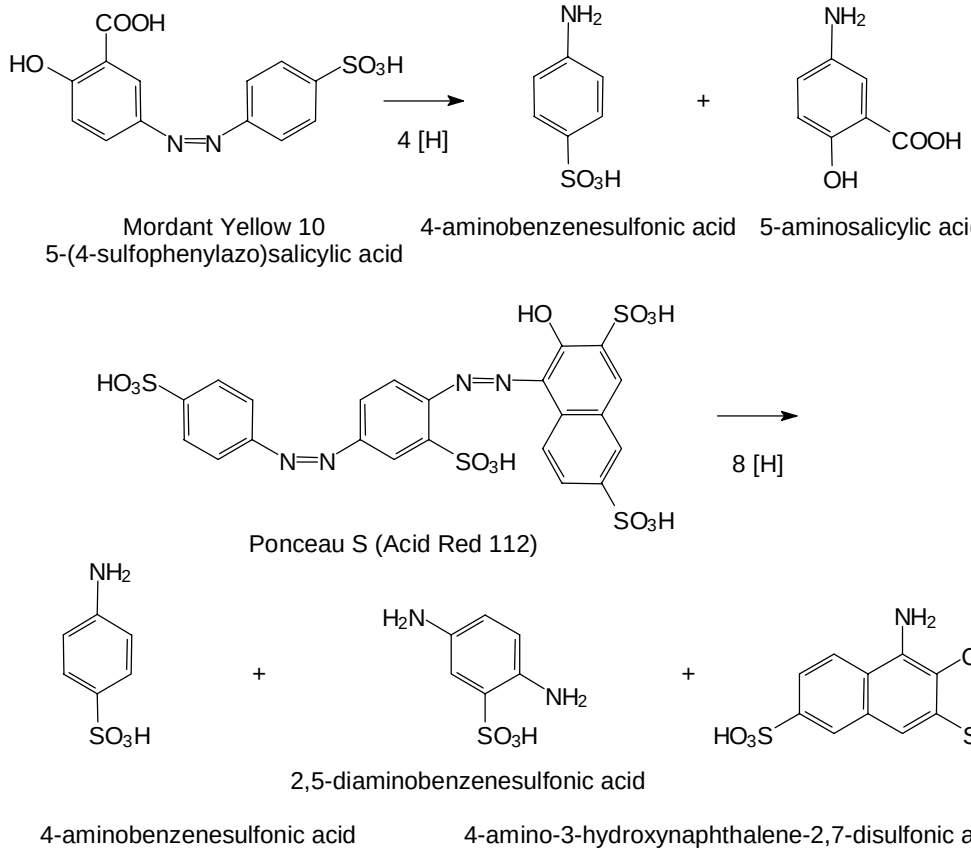

Figure 1.3 *Example azo reduction of sulfonated azo dye resulting in the formation of sulfonated and unsulfonated aromatic amines.*

A precondition for the reduction of azo dyes is the presence and availability of a co-substrate (Nigam et al. 1996b), because it acts as an electron donor for the azo dye reduction. Many different co-substrates were found to suite as electron donor, like glucose (Carliell et al. 1995; Nigam et al. 1996a), hydrolyzed starch (Willetts et al. 2000), tapioca (Chinwekitvanich et al. 2000), yeast extract (Nigam et al. 1996a), a mixture of acetate, butyrate and propionate (Donlon et al. 1997; Van der Zee et al. 2000c) and the azo dye reduction product 5-ASA as well (Razo-Flores et al. 1997). It also has been observed that the extend of decolorization of an azo dye like Remazol Black B varies depending on the co-substrates used, e.g. 82% with glucose, 71% for glycerol and lactose, 51% for starch and 39% for a distillery waste (Nigam et al. 1996b). Moreover, also the rate of azo-reduction process depends on the type of co-substrate used and/or on the chemical structure of the azo dyes (Van der Zee et al. 2000b; Van der Zee et al. 2000c). Furthermore, it has been observed that compounds that facilitate the transport of electrons, like mediators, considerably enhance the azo-reduction rate (Kudlich et

al. 1997; Van der Zee et al. 2000a). This suggests the prevalence of an extracellular process. Even some biodegradation products of azo dyes can act as a mediator (Keck et al. 1997; Van der Zee et al. 2000c). The reduction of azo dyes proceeds better under anaerobic thermophilic conditions than under mesophilic conditions, although the thermophilic process seems to be less stable compared to the mesophilic process (Willetts et al. 2000).

1.2.2 The aerobic biodegradation of (sulfonated) azo dyes

Although for a long time it was thought that azo dyes remained recalcitrant under aerobic conditions some specific aerobic bacterial cultures were found able to reduce the azo linkage via an enzymatic reaction. The azo reductases isolated from these organisms have a narrow substrate range (Kulla 1981; Kulla et al. 1983; Zimmermann et al. 1984; Zimmermann et al. 1982a; Zimmermann et al. 1982b).

The occurrence of aerobic conversions of sulfonated azo dyes were more recently reported by Heiss et al. (1992) and Shaul et al. (1991), and sometimes even a complete mineralization of a sulfonated azo dye was found under aerobic conditions. A bacterial strain S5, derived from *Hydrogenophaga palleronii* S1, was able to reduce the azo dye 4-carboxy-4'-sulfobenzene and to mineralize the azo dye reduction products 4-aminobenzenesulfonic acid (4-ABS) and 4-aminobenzoic acid. The sulfonated azo dye was used as carbon and energy source in this case (Blumel et al. 1998). Also a bacterial strain MI2, isolated from a biofilm reactor, was able to utilize Acid Orange 7 and 8 as sole carbon, nitrogen and energy source and the azo dye reduction product 4-ABS was also degraded (Coughlin et al. 1997). Furthermore, it was found that *Sphingomonas* sp. strain 1CX could use the sulfonated and unsulfonated azo dyes, Acid Orange 7, Acid Orange 8, Acid Orange 10, Acid Red 4 and Acid Red 88 as sole carbon and nitrogen source. All these dyes contained a hydroxyl group next to the azo bond on a naphthalene ring. However, a complete mineralization of Acid Orange 7 was not obtained because the azo dye reduction product 4-ABS accumulated (Coughlin et al. 1999).

In some studies, aerobic color removal of certain azo dyes was achieved, but all these stains required an additional energy and carbon source for their growth. Since the supply of this additional substrate could have easily led to the formation of anaerobic microniches, the occurrence of anaerobic azo dye reduction certainly can not be excluded (Govindaswami et al. 1993; Horitsu et al. 1977; Hu 1994; Hu 1998; Wong and Yuen 1996; Yatome et al. 1991; Zissi et al. 1997). Similarly, the degradation of azo dyes was also observed in aerobic biofilm reactors, but this also may have been a result of the presence of anaerobic microniches in the biofilm (Costerton et al. 1994; Harmer and Bishop 1992; Jiang and Bishop 1994). This certainly might prevail when additional substrate was supplied. In some cases the evidence for the occurrence of mineralization of the aromatic amines is poor (Harmer and Bishop 1992; Ogawa and Yatome 1990). Recently, the successful degradation of Acid Red 151 as sole

carbon source was described using an aerobic sequenced biofilm reactor and mineralization experiments showed that 73% of the carbon was transformed into carbon dioxide (Quezada et al. 2000).

1.2.3 The aerobic biodegradation of (sulfonated) aromatic amines

The aerobic biodegradation of many aromatic amines has been extensively studied. Many of these compounds were found to be degraded under aerobic conditions (Baird et al. 1977; Brown and Laboureur 1983a), e.g. compounds like aniline (Anson and MacKinnon 1984; Konopka 1993; Loidl et al. 1990; Lyons et al. 1984; Patil and Shinde 1988), carboxylated aromatic amines (Russ et al. 1994; Stolz et al. 1992), chlorinated aromatic amines (Hwang et al. 1987; Loidl et al. 1990; Reber et al. 1979), and (substituted) benzidines (Baird et al. 1977). However, a group of aromatic amines that remain difficult to degrade are the sulfonated aromatic amines (Bretscher 1981; Tan and Field 2000). These aromatic amines are released into the environment mainly via either the production or the biodegradation of sulfonated azo dyes. The aerobic and anaerobic biodegradation potential of the sulfonated aromatic amines, and their biodegradation pathways will be discussed in the following paragraphs. Along with some biotechnological applications for the removal of sulfonated aromatic amines and aromatic sulfonates from wastewater, and the occurrence of autoxidation, an abiotic process will be mentioned as well.

The ability of aerobic bacteria to mineralize sulfonated aromatic compounds was observed for the first time in the seventies. As a result of this biodegradation, the sulfur moiety of the sulfonic acid group can enter in the sulfur cycle (Cain and Farr 1968; Focht and Williams 1970; Ripin et al. 1971). Today, our knowledge of the biodegradation of sulfonated aromatic compounds is still rather limited and will be discussed below.

Sulfonated aromatic compounds can be divided in two main groups (Figure 1.4). The first group comprises the linear alkylbenzenesulfonic acids. Their fate when released in the environment has been extensively studied and reviewed (Berna et al. 1991; Federle and Ventullo 1990; Jimenez et al. 1991; Mampel et al. 1998; Thoumelin 1991). The second group of the sulfonated aromatic compounds comprises the aromatic sulfonates. There exists only limited information about the biodegradation of these compounds (Cook et al. 1998; Hooper 1991; Kertesz et al. 1994).

The group of aromatic sulfonates comprises the sulfonated azo dyes themselves and their precursors and biodegradation products the sulfonated aromatic amines and some additional sulfonated aromatic compounds. The aromatic sulfonates represent the raw materials for the production of azo dyes, drugs, detergents, optical brighteners, and artificial sweeteners (Hansen et al. 1992; Locher et al. 1989). Some aromatic sulfonates have a direct applicability,

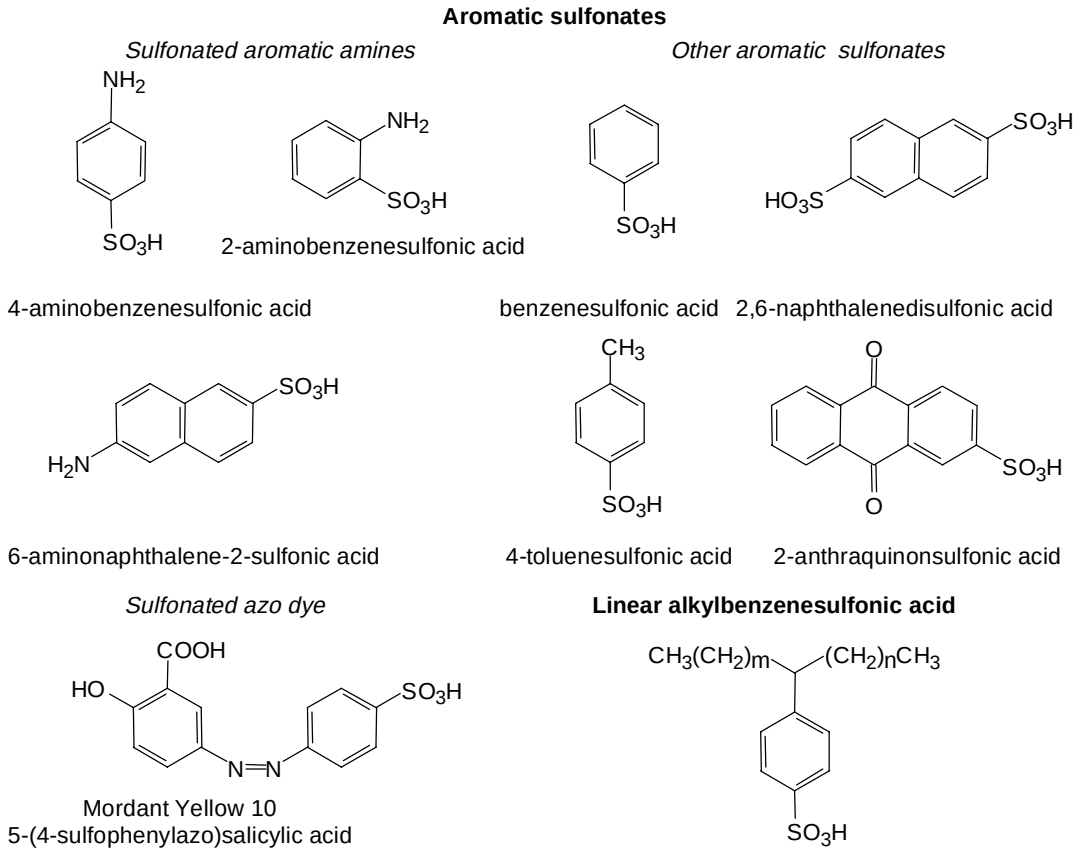

Figure 1.4 *Examples of the chemical structure of sulfonated aromatic compounds.*

e.g. 4-ABS is used as a preservative (Hooper 1991), 3-ABS as a mild oxidant (Locher et al. 1989) and 2-benzosulfonic acid (2-BOS) as a wetting agent in toothpaste (Hansen et al. 1992). Since many azo dyes contain the sulfonic acid group, attention will be paid to these compounds and -more especially- on their biodegradation products, the sulfonated aromatic amines. The sulfonated aromatic amines can be further sub-divided on the basis of their molecular structure into two groups: aminobenzenesulfonic acids and aminonaphthalenesulfonic acids.

Compounds containing a sulfonic acid group are highly soluble in the aqueous environment and consequently are abundant there, they do not accumulate in the sediment. Since the aromatic sulfonates are xenobiotics, they are mainly found in wastewater discharges from industries producing or processing these compounds, e.g. chemical, leather, textile, printing, paper, and pharmaceutical industries.

As mentioned above, mineralization of aromatic sulfonates results in the release of the sulfur moiety. The desulfonation process so far has mainly been studied under aerobic conditions. Under these conditions sulfite (HSO_3^-) will be formed during desulfonation, which can be further oxidized to sulfate (SO_4^{2-}). Three different patterns of the aerobic desulfonation are reported in the literature. Desulfonation occurs (a) before (b) during or (c) after ring cleavage (Tan and Field 2000) (Figure 1.5).

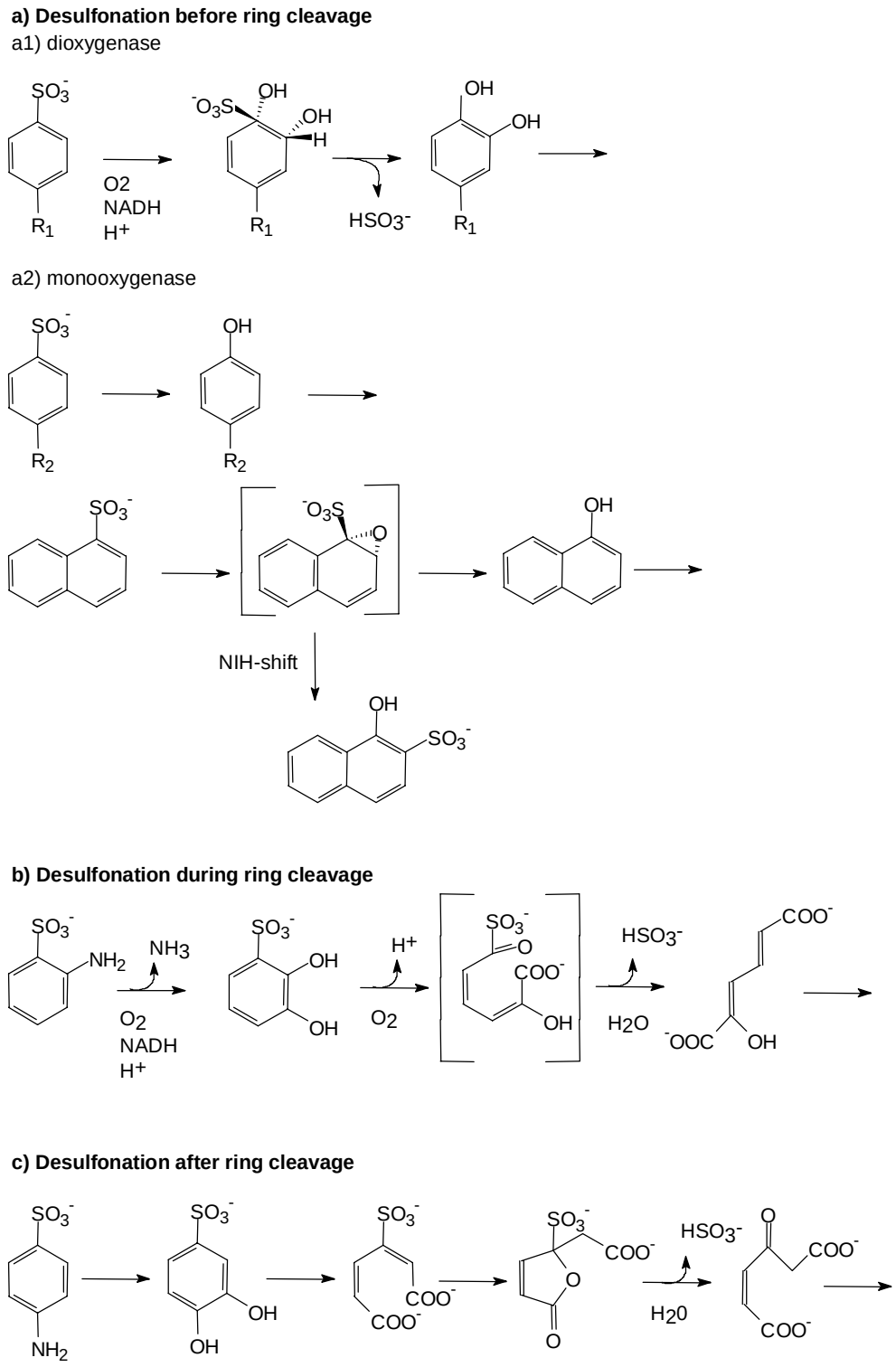

Figure 1.5 *Different patterns of aerobic desulfonation of sulfonated aromatic compounds (Feigel and Knackmuss 1988; Kertesz et al. 1994; Kneifel et al. 1997; Locher et al. 1991; Thurnheer et al. 1990; Zurrer et al. 1987).*

Results of the biodegradation experiments, using mixed natural cultures from sludges and sediments, have demonstrated that aromatic sulfonates are sometimes biodegradable. Aerobic biodegradation screening experiments conducted with activated sludge revealed that some sulfonated aromatic amines, although susceptible to biodegradation, could not completely be

mineralized (Brown and Hamburger 1987). Nevertheless, although difficult to degrade, some isolated aerobic bacterial strains were found able to degrade these compounds. Table 1.1 and 1.2 summarize results of the biodegradability experiments conducted with aminobenzenesulfonic acids and aminonaphthalenesulfonic acids.

Table 1.1 Bacterial strains able to degrade aminobenzenesulfonic acids (+ biodegradation; - no biodegradation; s compound used only as sulfur source; if not tested open; *Al. = Alcaligenes; A. = Arthrobacter; P. = Pseudomonas; P.a. = Pseudomonas acidovorans; H.p. = Hydrogenophaga palleronii*).

Bacteria	*Al.* sp.				*P.a.*	*P.* sp.		*A.* sp.	*P.* sp.	*H.p.*
Strain	O-1	O-2	S-1	S-3	M-1	S-313	S-832	DZ-6	DS1304	S1
Reference	a, b	a	a, b	a	b	c	c	c	d	e
2-ABS	+	+	-	-	-	+s	+s	+s		
3-ABS	-	-	-	-	+	+s	+s	+s		
4-ABS	-	-	+	+	-	+s	+s	+s	+	+

References: a (Thurnheer et al. 1986); b (Locher et al. 1989); c (Zurrer et al. 1987); d (Dubeikovskii et al. 1992); e (Feigel and Knackmuss 1988; Feigel and Knackmuss 1993).

As far as the aminobenzenesulfonic acids are concerned, only the simple substituted aminobenzenesulfonic acids (without any additional substituents) were found to be aerobically biodegradable. So far, bacteria able to degrade substituted aminobenzenesulfonic acids have not been isolated. Four different bacterial strains were isolated, which used aminobenzenesulfonic acids as sole energy and carbon sources. These bacterial isolates were obtained during enrichment experiments of inoculum from sewage plants treating sulfonate-containing industrial wastes (Junker et al. 1994; Locher et al. 1989; Thurnheer et al. 1986). One of these isolated bacteria, *Alcaligenes* sp. O-1, which degraded 2-ABS, was most extensively studied and the degradation pathway of 2-ABS was determined (Figure 1.5b) and enzymes were isolated and characterized.

Three other strains M-1, S-1 and S-3 were isolated, strain M-1 could degrade 3-ABS and strains S-1 and S-3 were capable of 4-ABS degradation. Strain S-1 was tested in our laboratory, but no degradation of 4-ABS was observed. This may indicate that the degradation capacity was located on a plasmid and was probably lost. A co-culture, composed of the two bacterial strains *Hydrogenophaga palleronii* S1[*] and *Agrobacterium radiobacter* S2, was found to be able to completely mineralize 4-ABS (Feigel and Knackmuss 1988; Feigel and Knackmuss 1993) and this compound was used as sole source of energy, carbon, nitrogen and sulfur. Strain S1 degrades 4-ABS via a dioxygenase to 5-sulfocatechol, which compound then

[*] This strain S1 is different to the strain S-1 described by Thurnheer *et al.* (1986).

is utilized by strain S2, herewith producing the essential vitamins for strain S1 (Dangmann et al. 1996). The degradation pathway of 4-ABS by this co-culture is depicted in Figure 1.5c.

Another bacterial strain was able to degrade 4-ABS as a pure culture. This *Pseudomonas* DS1304 attacks 4-ABS via an initial dioxygenase, which takes care of the desulfonation (Dubeikovskii et al. 1992). Furthermore, three strains have been described that use the sulfonic acid group of all three aminobenzenesulfonic acid isomers as a sulfur source for growth under sulfur-limiting growth conditions with glucose as the carbon source. These bacteria utilize a monooxygenase for the desulfonation (Zurrer et al. 1987).

The bacterial strains B-1, P-2, P-3, *Comamonas testosteroni* T-2, *C. testosteroni* PSB-4 (Thurnheer et al. 1986), N-1 (Locher et al. 1989), L-1 (Khlebnikov et al. 1997), *P. maltophila* BSA56 (Lee and Clark 1993), *Alcaligenes* sp. GA-1 (Takeo et al. 1997) and *Agrobacterium radiobacter* S2 (Feigel and Knackmuss 1988; Feigel and Knackmuss 1993) have been tested on one or more aminobenzenesulfonic acid compounds. None of them were able to degrade any of these compounds. However, these strains were able to degrade other sulfonated aromatic compounds.

Table 1.2 Bacterial strains able to degrade aminonaphthalenesulfonic acids (+ biodegradation; - no biodegradation; s compound used only as sulfur source; if not tested open; *S.* = *Sphigomonas*; *M.* = *Moraxella*; *A.* = *Arthrobacter*; *P.* = *Pseudomonas*; Cc11. = co-culture of 11 bacterial strains).

Bacteria	*S.* sp.	Cc11	*P.* sp	*P.* sp.	*M.* sp	*A.* sp.	*P.* sp.		
Strain	BN6		TA-1	TA-2	ASL4	DZ-6	S-313	S-832	Z-63
Reference	a	b	c	d	e	f	f	f	f
2-A-1-NS			+	+					
5-A-1-NS						+s	+s	-	+s
6-A-1-NS	-								
8-A-1-NS						+s	+s	+s	+s
5-A-2-NS	+		-	-					
6-A-2-NS	+	+		-	+				
7-A-2-NS	+								
8-A-2-NS	+								
6-A-4-H-2-NS	+					+s	+s	+s	-
7-A-4-H-2-NS	-					+s	+s	+s	+s
3-A-1,5-NDS						+s	+s	+s	-
2-A-4,8-NDS	-								
4-A-5-H-2,7-NDS						+s	+s	+s	-

References a (Nortemann et al. 1986; Nortemann et al. 1994); b (Rozgaj and Glancer 1992); c (Ohe and Watanabe 1986); d (Ohe et al. 1990); e (Wittich et al. 1988); f (Zurrer et al. 1987).

Various specialized aerobic bacteria were found to degrade several aminonaphthalenesulfonic acids. The isolated bacterial strains and the compounds degraded are summarized in Table 1.2. From the table it follows that 6-A-2-NS is one of the most studied aminonaphthalenesulfonic acids and can be completely degraded by two co-cultures and one bacterial strain ASL-4 (Nortemann et al. 1986; Rozgaj and Glancer 1992; Wittich et al. 1988).
Nortemann et al. (1986) isolated *Sphigomonas* sp. BN6 responsible for the initial degradation of 6-A-2-NS out of the co-cultures. This strain BN6 also was able to transform 5-, 6-, 7- or 8-A-2-NS. These compounds were degraded via an initial dioxygenase taking care of the desulfonation. After ring cleavage, the corresponding aminosalicylic acids accumulated in the growth medium (Figure 1.6). Aminosalicylic acids can be degraded aerobically by another member of the co-culture (Nortemann et al. 1986; Nortemann et al. 1994). The biodegradation of 5-A-2-NS by strain BN6 resulted in the formation of a dead-end product 5-hydroxy-quinoline-2-carboxylic acid (Nortemann et al. 1993).

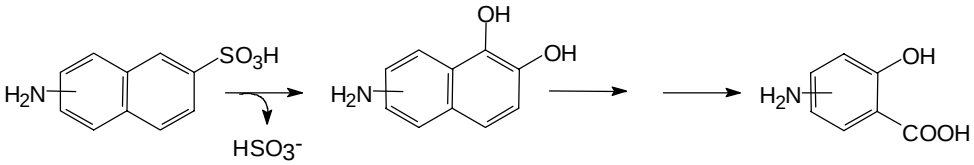

Figure 1.6 *Degradation of A-2-NS by Sphigomonas sp. BN6 (Nortemann et al. 1986).*

Another co-culture consisting of 11 bacterial strains, was able to degrade 6-A-2-NS. It formed a stable co-culture and was enriched from a treatment plant treating industrial wastewater. None of the single strains could degrade the 6-A-2-NS individually, apparently each strain participates in the degradation process. Therefore, this co-culture consisted of highly cooperating consortia (Rozgaj and Glancer 1992).
The strain ASL-4, originally isolated on 2,6- and 1,6-naphthalenedisulfonic acid (NDS), also showed activity on 6-A-2-NS. According to Wittich et al. (1998) the biodegradation of 6-A-2-NS may proceed via an initial dioxygenase, which deaminates the compound. After ring cleavage, 5-sulfosalicylic acid is formed, which is further degraded to gentisate via an oxygenolitic elimination of the sulfonic acid group (Wittich et al. 1988). Two *Pseudomonas* strains, isolated from soil samples by a Japanese group, were able to degrade 2-A-1-NS and used this compound as the sole carbon and nitrogen source. Both bacteria degraded the compound via an initial 1,2-dioxygenase, which lead to the desulfonation and deamination of the molecule (Ohe et al. 1990; Ohe and Watanabe 1986).
The same strains, which used the sulfonic acid group from aminobenzenesulfonic acids under sulfur-limitation, were also able to use the sulfonic acid group of aminonaphthalenesulfonic acids as a sulfur source under sulfur-limited conditions. Under these conditions, these strains

used the sulfonic acid group of aminohydroxynaphthalenesulfonic acid (AHNS), aminohydroxynaphthalenedisulfonic acid (AHNDS) and aminonaphthalenedisulfonic acid (ANDS) compounds (Zurrer et al. 1987).

1.2.4 Biotechnological applications for removal of sulfonated aromatic amines

Only a few applications have been reported so far dealing with the biotechnological removal of aromatic sulfonates. The results are summarized in Table 1.3. Aromatic sulfonates tested, like 4-toluenesulfonic acid (4-TS), 1-, 2-naphthalenesulfonic acid (1-NS, 2-NS) and some NDSs, were degraded in biotechnological systems developed for the removal of these compounds (Tan and Field 2000). Only two reports dealt with biotechnological systems developed for the removal sulfonated aromatic amines.

Table 1.3 Summary of literature dealing with biotechnological systems for the removal of aromatic sulfonates from wastewater (*Al. = Alcaligenes*; *C.t. = Comamonas testosteroni*; *P.t.= Pseudomonas testosteroni*; *S. = Sphigomonas*).

Compounds treated	Reactor type (volume (l))	Inoculum used	Organic loading rate (g COD l^{-1} d^{-1})	Efficiency (%)	Reference
2- ,3- ,4-ABS 4-BOS 4-TS BS, 4-HBS 4-CBS	Chemostat (2.0)	*C.t.* T-2 *C.t.* PBS-4 *Al.* O-1 strain M-1 strain S-1	0.3 – 10.1	100	a
6-AN-2-S	airlift-loop reactor (2.5)	*S.* BN6 BN9 BN11	8.7	99	b
2-NS	airlift-loop reactor (2.5)	*P.t.* A3	13.1	99	b
4-TS	fixed bed biofilm reactor (0.066)	*C.t.* T-2	6.2 – 12.4	70	c
4-TS	fixed bed biofilm reactor (0.066)	*C.t.* L-1	6.2 – 12.4	59	c
3-NBS 3-ABS	trickling filter (0.7)	6 activated sludges	not given	removal with industrial sludge	d
1-, 2-NS 1,5-NDS 1,6-NDS 2,6-NDS	two stage airlift-loop reactors low salt (2.5)	*P.t.* A3 strain RK3 mixed culture	2.2 – 69.3 first stage 1.0 – 8.1 second stage	84, except no removal of 1,5-NDS	e
1-, 2-NS 1,5-NDS 1,6-NDS 2,6-NDS	two stage airlift-loop reactors high salt (2.5)	*P.t.* A3 strain RK3	2.2 – 69.3 first stage 1.0 – 8.1 second stage	71, except no removal of 1,5-NDS	e
2-NS	airlift (2.0)	*P.t.* A3	11.9	100	f

References: a (Thurnheer et al. 1988); b (Diekmann et al. 1990); c (Khlebnikov et al. 1997); d (Kolbener et al. 1994); e (Krull and Hempel 1994); f (Pack and Hempel 1997).

In one report, Thurnheer et al. (1988) used a chemostat with a co-culture of five different bacteria to degrade seven sulfonated aromatic compounds. The bacterial strains used were *Comamonas testosteroni* T-2, *C. testosteroni* PSB-4, *Alcaligenes* sp. O-1, strain M-1, and strain S-1. A mixtures of these strains could degrade 2-, 3-, 4-ABS, 4-BOS, 4-TS, benzenesulfonic acid (BS), and 4-hydroxybenzenesulfonic acid (4-HBS) in a continuous stirred tank reactor. A maximum degradation rate of 138 mg C h^{-1} l^{-1} was observed for all compounds together. At the end of the experiment after 903 days 4-chlorobenzenesulfonic acid (4-CBS) was supplied to the reactor and also this compounds was degraded (Thurnheer et al. 1988).

In another report, Kolbener et al. (1994) investigated the degradation of 3-nitrobenzenesulfonic acid (3-NBS) and 3-ABS trickling filter. As inoculum for the filter, six activated sludge samples of different origin were tested. Four sludges originated from domestic wastewater and two from an industrial wastewater treatment plants. Interestingly, only the last two sludges were able to degrade both compounds (Kolbener et al. 1994).

1.2.5 The autoxidation of (sulfonated) aromatic amines

Some aromatic amines like phenylenediamines, aminophenols, aminonaphthol and o-amino-hydroxynaphthalenesulfonic acid tend to autoxidize under aerobic conditions (Haller 1978; Jensen et al. 1993; Jensen et al. 1992; Kudlich et al. 1999). Autoxidation implies a process in which oxygen reacts with the aromatic products via free radical reactions. This process results in the formation of colored oligomers and polymers, which is obviously undesirable. Moreover, the initially formed oligomers may have toxic and mutagenic effects (Field et al. 1995). Consequently, the autoxidation process eliminates the aromatic amines and the compounds produced are although more recalcitrant biological degradation. Unless autoxidation would result in insoluble polymers the occurrence of this process should be prevented.

1.2.6 The anaerobic degradation of (sulfonated) aromatic amines

Aromatic amines are commonly not degraded under anaerobic conditions. Of the many different aromatic amines tested only a few were degraded. Some aromatic amines, substituted with hydroxyl or carboxyl group were degraded under methanogenic and sulfate reducing conditions (Kalyuzhnyi et al. 2000; Kuhn and Suflita 1989; Razo-Flores et al. 1996; Razo-Flores et al. 1997; Razo-Flores et al. 1999). Some evidence has been obtained that under sulfate and nitrate reducing conditions aniline was degraded (De et al. 1994; Schnell and Schink 1991).

So far there is no clear evidence for the biodegradation of aromatic sulfonates under anaerobic conditions and desulfonation of under anaerobic conditions is seldom found. Fermentative bacteria used some specific aromatic sulfonates as a sulfur source under anaerobic conditions.

Also one of the sulfonic acid groups of the sulfonated azo dye Acid Red 1 (disulfonated azo dye) can be removed by *Clostridium* strains. (Chien et al. 1995; Denger and Cook 1997; Denger et al. 1996). But how the process proceeds, i.e. the pathway and the enzymes involved, is obscure so far. Potentially, the sulfonic acid group could be used as alternative electron acceptors under anaerobic conditions, but any sulfonated aromatic compound so far was shown to serve as an electron acceptor (Cook et al. 1998; Lie et al. 1998; Lie et al. 1996).
There exists no evidence for mineralization of sulfonated aromatic amines under anaerobic conditions. Some reports deal with research on this matter, but none of the sulfonated aromatic amines tested could serve as carbon and energy source under the applied methanogenic and sulfate-reducing conditions (Brown and Hamburger 1987; Kuhn and Suflita 1989; Razo-Flores et al. 1996). Therefore, anaerobic degradation of sulfonated aromatic amines is not likely to occur.

1.3 Combined anaerobic/aerobic biodegradation of azo dyes

As pointed out above the biodegradation of azo dyes requires an anaerobic and aerobic phase for the complete mineralization. The required condition can be implemented by spatial separation of the two sludges using a sequential anaerobic/aerobic reactor system (Zitomer and Speece 1993). But these conditions can also be imposed in one reactor in the so-called integrated anaerobic/aerobic reactor system (Field et al. 1995).

1.3.1 The sequential anaerobic/aerobic reactor system

Sequential anaerobic/aerobic conditions can readily be imposed to wastewater by first exposing it to anaerobic conditions and next aerobic conditions by using an anaerobic reactor followed by an aerobic reactor. The sequential anaerobic and aerobic degradation has been studied for the conversion of azo dyes by numerous researchers (Basibuyuk and Forster 1997; FitzGerald and Bishop 1995; Lourenço et al. 2000; O'Neill et al. 1999a; O'Neill et al. 2000a; O'Neill et al. 2000b; Rajaguru et al. 2000). However, in various cases clear evidence for a complete biodegradation was not found, mainly due to a lack of proof for mineralization of the aromatic amines. In most experiments only the decolorization and organic load were measured while additional examinations, like the mineralization of the aromatic amines, were omitted. Only in one case there exists real proof of mineralization of an azo dye by a bacterial co-culture under sequential anaerobic/aerobic batch conditions (Haug et al. 1991).
The major part of the organic load (co-substrate) will be consumed anaerobically in a sequential anaerobic/aerobic treatment system, and consequently the aromatic amines serve as main substrate for the organisms in the aerobic bioreactor. Considering the extensive application of many sulfonated azo dyes, many different sulfonated and unsulfonated aromatic amines will be formed that have to be treated aerobically. As already mentioned,

aromatic amines are generally degraded aerobically, but for sulfonated aromatic amines this only is the case for a few of these compounds (Tan and Field 2000).

1.3.2 The integrated anaerobic/aerobic reactor system

Anaerobic and aerobic microorganisms can cooperate beneficially in one single biofilm (Knackmuss 1997; Zitomer and Shrout 1998). The proper conditions can be created by keeping the oxygen concentrations and diffusion of oxygen low, so that anaerobic and aerobic microniches are created (Costerton et al. 1994). Supplying oxygen into an anaerobic system together with co-substrate can also create such integrated anaerobic/aerobic conditions. Due to the limited diffusion of the oxygen compared to that of the co-substrate a part of the co-substrate will be consumed via oxidative processes in the periphery of the biofilm, while the inner layers of the biofilm will remain anaerobic. In this system, the reduction of the azo dyes and the aerobic mineralization of the aromatic amines will proceed side by side in the same biofilm. In this way occurrence of the unwanted process of autoxidation of the aromatic amines is not likely (Field et al. 1995).

One of the problems prevailing in integrated anaerobic/aerobic systems is the imbalance between oxygen and co-substrate. Due to the oxidation of co-substrate insufficient co-substrate can be available for the reduction processes, or -in the reversed situation- insufficient oxygen for the oxidation processes. A good balance between co-substrate and oxygen is required but this is not easy to accomplish. Moreover, also the kind of co-substrate can be important fact for the creating proper conditions.

The suggested integrated anaerobic/aerobic conditions can be created by using an anaerobic granular sludge with a high tolerance for oxygen as a carrier material for aerobic biofilm (Kato et al. 1993a; Kato et al. 1993b; Shen and Guiot 1995; Shen et al. 1996; Zitomer 1998; Zitomer and Shrout 1998). These integrated conditions are in practice also possible by usage a support material. Because anaerobic bacteria will develop inside the support material and in the outer layer of the support material aerobic bacteria will grow, which in this way form a protection barrier for the anaerobic bacteria (Gerritse and Gottschal 1992; John et al. 1996; Kudlich et al. 1996; Muller et al. 1994).

The same co-culture that degraded an azo dye under the sequential anaerobic/aerobic batch conditions was used for degradation of azo dyes under integrated anaerobic/aerobic conditions. As a support material calcium alginate beats were used to create the integrated anaerobic/aerobic conditions (Kudlich et al. 1996).

1.4 Objective of this Ph.D. research and thesis outline

The objective of this dissertation is to explore methods of obtaining the complete biodegradation of sulfonated and unsulfonated azo dyes in bioreactor systems that combine anaerobic and aerobic phases. Therefore, two different approaches are considered. The first approach, known as integrated anaerobic/aerobic treatment, evaluates the potential of combining the anaerobic and aerobic phases in one bioreactor concurrently. The second approach, known as sequential anaerobic/aerobic treatment, is achieved by using an anaerobic reactor subsequently followed by an aerobic reactor. The azo dye reduction and the sulfonated and unsulfonated aromatic amine degradation will be evaluated in both treatment systems.

Chapter 2 will focus on the influence of oxygen on the anaerobic azo dye reduction and Chapter 3 on the biodegradation of sulfonated and unsulfonated azo dyes using integrated anaerobic/aerobic conditions. Chapter 4 deals with the problems of complete degradation of sulfonated azo dyes using a sequential anaerobic/aerobic reactor system. The biodegradation and fate of sulfonated aromatic amines under both aerobic and anaerobic conditions are the main subject of Chapter 5 and Chapter 6 presents discussion and a summary of the results of this dissertation.

2

Reduction of the azo dye Mordant Orange 1 by methanogenic granular sludge exposed to oxygen

Abstract Integration of anaerobic and aerobic conditions in a single bioreactor is a good strategy for the complete mineralization of azo dyes. In order for this strategy to work, azo dye reduction should occur in biofilms exposed to oxygen. Therefore, the effect of oxygen on the azo dye reduction by methanogenic granular sludge was studied using Mordant Orange 1 (MO1) as a model. Azo dye-reduction rates by two different granular sludges were determined in batch assays with various concentrations of oxygen in the headspace. Azo dye reduction occurred in the presence of oxygen if co-substrates, either ethanol or acetate were added. The rate of dye reduction was highly positively correlated with the oxygen-consuming activity of the sludge. The results suggest that co-substrates stimulate oxygen respiration, which lowers oxygen penetration into the biofilm and thereby creates anaerobic microniches where azo dye reduction can occur.

Tan N.C.G., Lettinga G. and Field J.A. (1999). Bioresource Technology 67(1): 35-42.

2.1 Introduction

Azo dyes are one of the oldest man-made chemicals and they are still widely used in textile, printing and the food industries. The annual production worldwide is approximately 700,000 tons (Zollinger 1987). Of this amount, approximately 10-15% of the dyes are released into the environment during manufacturing and usage (Vaidya and Datye 1982). Some dyes and some of their N-substituted aromatic biotransformation products are toxic and/or carcinogenic and, therefore, the dyes are considered as important environmental pollutants (Chung and Cerniglia 1992).

During conventional aerobic wastewater treatment, azo dyes are not degraded (Pagga and Brown 1986; Shaul et al. 1991). However, azo linkages are easily reduced under anaerobic conditions (Brown and Laboureur 1983b), with digester sludge (Carliell et al. 1995; Carliell et al. 1994), anaerobic granular sludge (Donlon et al. 1997; Razo-Flores et al. 1997) or sediments (Weber 1991). The azo dyes act as electron acceptors for reduced flavin nucleotides and reduction is enhanced by redox mediators (Gingell and Walker 1971; Keck et al. 1997; Kudlich et al. 1997). The reduction of azo dyes results in the formation of aromatic amines.

Most aromatic amines, which accumulate after azo cleavage, are not mineralized anaerobically (Brown and Hamburger 1987), with the exception of a few aromatic amines substituted with hydroxyl and carboxyl groups which were fully degraded under methanogenic conditions (Razo-Flores et al. 1996). However, the aromatic amines are readily degraded aerobically (Brown and Laboureur 1983a; Konopka 1993; Loidl et al. 1990). A combination of anaerobic and aerobic conditions is therefore proposed as a feasible biological treatment strategy for azo compounds (Field et al. 1995).

Anaerobic and aerobic conditions can be applied in a sequential anaerobic/aerobic bioreactor system but also in a single reactor in which anaerobic and aerobic microniches occur side by side. Sequential anaerobic/aerobic treatment for azo dyes and textile industry wastewater has been evaluated in several studies (An et al. 1996; Brown and Hamburger 1987; FitzGerald and Bishop 1995; Seshadri et al. 1994; Zaoyan et al. 1992). Also a simultaneous anaerobic/aerobic treatment methodology for an azo dye was recently described. Two different cultures were immobilized in calcium alginate beads. One bacterial strain caused the reduction of Mordant Yellow 3 in the anaerobic zones leading to the formation of 6-aminonaphthalene-2-sulfonate (6-ANS) and 5-aminosalicylic acid (5-ASA). Subsequently, the same strain could degrade 6-ANS to 5-ASA and the second strain could mineralize 5-ASA in the aerobic zones (Kudlich et al. 1996). Instead of calcium alginate beads, anaerobic granular sludge can be used as a carrier material for both anaerobic and aerobic microniches

(Kato et al. 1993b; Shen and Guiot 1995; Shen et al. 1996). In this present study, the influence of oxygen on azo dye reduction in anaerobic granular sludge was tested. The goal was to determine if anaerobic microniches are created in granular sludge exposed to oxygen where azo dye reduction can occur. Both the effects of the amount of oxygen and the type of co-substrate on the rate of azo dye reduction were considered. For this study azo dye Mordant Orange 1 (MO1) was used as a model.

2.2 Materials and Methods

2.2.1 Biomass

Methanogenic granular sludge from a full-scale upflow anaerobic sludge blanket (UASB) reactor treating wet oxidized industrial effluent of Shell Nederland Chemie (Moerdijk, The Netherlands) and from a full-scale UASB reactor treating effluent of an alcohol distillery at Nedalco (Bergen op Zoom, The Netherlands) were used for the experiments. Both granular sludge sources were stored at 4 °C and washed and sieved to remove the fine particles before use in the batch tests.

2.2.2 Basal medium

The basal medium used in all batch experiments contained (mg l^{-1}): $NaHCO_3$ (5000), NH_4Cl (280), $CaCl_2 \cdot 2H_2O$ (10), K_2HPO_4 (250), $MgSO_4 \cdot 7H_2O$ (100), yeast extract (100), H_3BO_3 (0.05), $FeCl_2 \cdot 4H_2O$ (2), $ZnCl_2$ (0.05), $MnCl_2 \cdot 4H_2O$ (0.05), $CuCl_2 \cdot 2H_2O$ (0.03), $NH_4SeO_3 \cdot 5H_2O$ (0.05), $AlCl_3 \cdot 6H_2O$ (2), $NiCl \cdot 6H_2O$ (0.05), $NaSeO_3 \cdot 5H_2O$ (0.1), EDTA (1), resazurin (0.2); and 36% HCl (0.001 ml l^{-1}).

2.2.3 Analyses

The methane content in the headspace of the serum flasks was determined by gas chromatography. A 100 µl gas sample was injected in a Chrompack Packard model 438 S (Delft, The Netherlands) gas chromatograph, equipped with 2 m × 2 mm steel column packed with Poropack Q (mesh 80-100). The temperatures of the column, injection port and the flame ionization detector were 60°, 200° and 220°C, respectively. The carrier gas nitrogen was used at a flow rate of 20 ml min^{-1}.

Headspace contents of oxygen, carbon dioxide and nitrogen were measured by another gas chromatographic technique. A 100 µl sample was injected in a Packard Becker 433 (Delft, The Netherlands) gas chromatograph, equipped with two parallel-connected columns (split 1:1), one with a 1.2 m × 2 mm steel column packed with molecular sieve 5A (mesh 60-80) and the other with Teflon packed Chromsorb (1.5 m × 2 mm) column. The temperatures of the column, injection port and the flame ionization detector were 40, 110, 125°C, respectively. Helium was used as the carrier gas (20 ml min^{-1}). All gas samples analyses were

conducted after calibration with standards of known amounts of the respective gasses using a pressure-locked gas syringe (Dynatech Precision Sampling Corporation, Louisiana, USA).

Ethanol and acetate concentrations were determined with a Hewlett Packard 5980 GC gas chromatograph (Palo Alto, USA). The glass column (2 m × 2 mm) was packed with Supelcoport 100-200 mesh coated with 10% Fluorad FC 431. The temperature of the column was 70°C for ethanol and 130°C for acetate, respectively. The temperatures of the injector and the flame ionization detector were 220 and 240°C, respectively. The carrier gas, nitrogen gas saturated with formic acid, was used at a flow rate of 40 ml min^{-1} Before use the gas chromatograph was calibrated with standard solutions of ethanol and acetate. A 1.0 µl aqueous sample was injected and samples were diluted in a saturated formic acid solution.

The azo dye MO1 was measured spectrophotometrically with a Spectronic 60 spectrophotometer (Milton Roy Analytical Products Division, Oostende, Belgium) at its absorbance maximum of 373 nm. The extinction coefficient of MO1 in aqueous solution was 19.5 mM^{-1} cm^{-1} and there was no absorbance at 373 nm of the reductively formed aromatic amines. The samples were centrifuged (7833 x *g*, 10 minutes) and diluted in a 0.10 M sodium phosphate buffer (pH 7.0) solution and measured in a 1.0 cm 100-QS (Hellma Benelux The Hague, The Netherlands) quartz cuvet.

The aromatic amines formed were analyzed with a colorometric amine assay (Oren et al. 1991). A calibration curve for this assay was made by determining the absorbance of a solution containing equal concentration of 5-ASA and 1,4-phenylenediamine on molar basis. The mixture was treated via the assay. These two aromatic amines are the known cleavage products of MO1 azo dye reduction (Donlon et al. 1997).

Total suspended solids and volatile suspended solids (VSS) were determined according to Standard Methods for Examination of Water and Wastewater (APHA 1985). The concentrations of ethanol and acetate are expressed in chemical oxygen demand (COD), commonly used in wastewater treatment. Conversion factors used were 2.087 g COD g^{-1} ethanol and 1.067 g COD g^{-1}acetate.

2.2.4 Assays

The oxygen toxicity was measured with the acetoclastic methanogenic activity assay. This assay was performed in 120 ml glass serum vials. The bottles contained 25 ml of basal medium, granular sludge (2.0 g VSS l^{-1}) and acetate (2.5 g COD l^{-1}). Acetate was added from a neutralized stock solution containing 62.5 g COD l^{-1} acetate. The assay serum flasks were then flushed with N_2 / CO_2 (70% / 30%) gas for 5 minutes and incubated overnight in a temperature-controlled room at 30 ± 2°C while being shaken (50 rpm). The following day oxygen was added by first removing gas from the bottle and replenishing it with the same amount of oxygen. Oxygen headspace percentages are reported as the initial levels. However,

oxygen was consumed throughout the incubation period of three days. After three days, the headspace was reflushed with N_2 / CO_2 (70% / 30%) gas for 5 minutes and acetate (1.0 g COD l^{-1}) was added from the stock solution. The bottles were reincubated and methane production was measured hourly for a period of 6 to 8 hours. The maximum specific methanogenic activity was calculated from the slope of the methane production versus time.

Azo dye-reduction rate experiments were performed in 120 ml and 309 ml serum bottles. These bottles were filled with 25 ml or 64 ml of basal medium, granular sludge (0.4 g VSS l^{-1}), co-substrate (2.0 g COD l^{-1}, ethanol or acetate) and 50 mg l^{-1} (0.174 mM) MO1 and closed with a butyl rubber septum (Rubber B.V., Hilversum, The Netherlands) and a crimp-seal aluminum cap. The headspace of the bottles was flushed with N_2 / CO_2 (70% / 30%) gas for 5 minutes. The N_2 / CO_2 (70% / 30%) gas was removed and replenished with same amount of oxygen gas to arrange an initial headspace oxygen percentage (IHOP). The assays were performed in either duplicate or triplicate. The assay bottles were incubated in a temperature-controlled room at 30 ± 2°C on a orbital-motion shaker at 50 strokes min^{-1} (Gerhardt, Bonn, Germany). Methane, oxygen and co-substrates were measured gas chromatographically and MO1 was measured spectrophotometrically.

2.2.5 Chemicals

All chemicals were purchased from either Jansen Chimica (Tilburg, The Netherlands), Merck (Darmstadt, Germany) or Sigma (Bornem, Belgium). All chemicals were of the highest purity commercially available and not purified further.

2.3 Results

2.3.1 Oxygen toxicity

Both types of granular sludge were tested for their susceptibility to oxygen toxicity. Acetoclastic methanogenic activities were measured immediately after the sludge had been exposed for 3 days to different initial amounts of oxygen in the headspace. Table 2.1 gives the inhibitory oxygen concentrations (IC_{50}) causing 50% loss of acetoclastic activity and the acetoclastic methanogenic activities of the controls (not exposed to oxygen). Nedalco granular sludge was more tolerant to oxygen and this sludge had a higher acetoclastic activity than Shell granular sludge.

Table 2.1 The acetoclastic methanogenic activity and oxygen toxicity of Nedalco and Shell granular sludges.

Biomass source	Acetoclastic activity of control (g COD-CH_4 g^{-1} VSS day^{-1})	IC_{50} (initial headspace oxygen percentage)
Shell granular sludge	0.53	17.7
Nedalco granular sludge	1.10	25.8

2.3.2 Effect of oxygen on azo dye reduction

The maximum azo dye-reduction rates were calculated using time-courses of the batch experiments. Figure 2.1 shows typical time-courses of MO1 exposed to 10 IHOP with Nedalco granular sludge and compares the addition of ethanol, acetate and no co-substrates. The reduction of MO1 was mediated biologically since the MO1 concentration remained constant in assays with autoclaved sludge (data not shown). As can be seen in Figure 2.1, both oxygen uptake and azo dye reduction were most rapid with ethanol as co-substrate. With both co-substrates, azo dye reduction occurred while oxygen was present in the headspace. Neither was the dye decolorized nor was oxygen consumed if no co-substrate was added. Similar results were obtained with Shell granular sludge (data not shown).

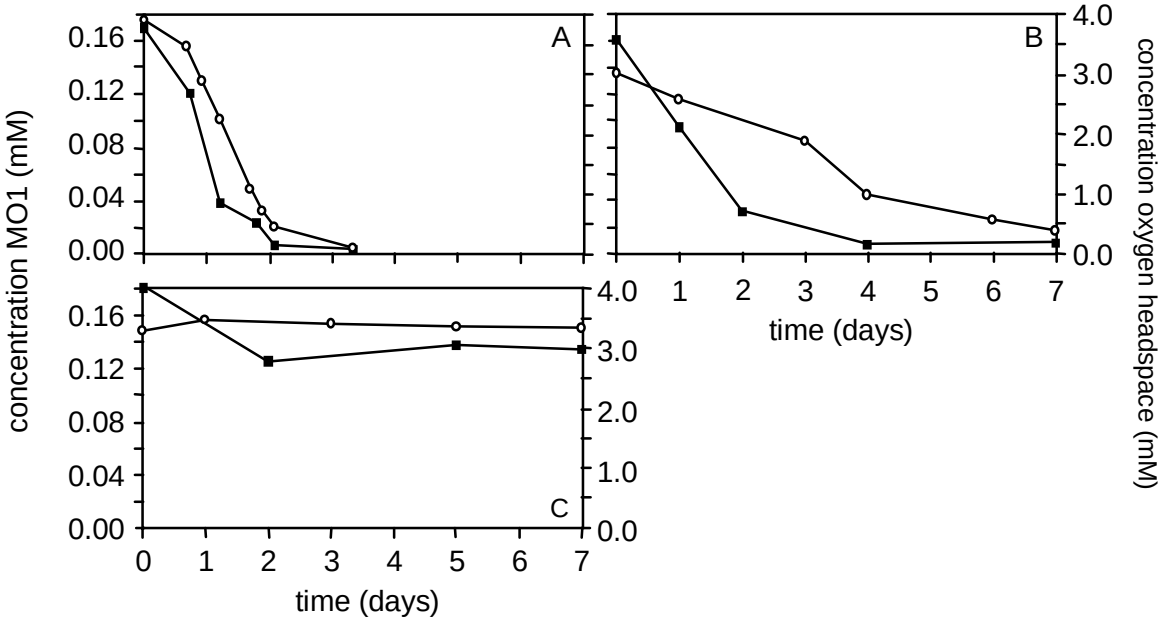

Figure 2.1 *Time courses of MO1 (○) and oxygen (■) at 10 IHOP with ethanol (A), or acetate (B) as co-substrate, and no co-substrate (C) and Nedalco granular sludge as biomass source.*

Figure 2.2 shows the azo dye-reduction rate as a function of the IHOP for Nedalco granular sludge. The azo dye reduction was most rapid when ethanol was used as co-substrate. In the absence of oxygen, this rate was 125 mg MO1 g^{-1} VSS d^{-1} and the rate decreased

incrementally with increasing IHOP to 48 mg MO1 g^{-1} VSS d^{-1} at 15 IHOP and remained more or less constant thereafter at higher oxygen concentrations.

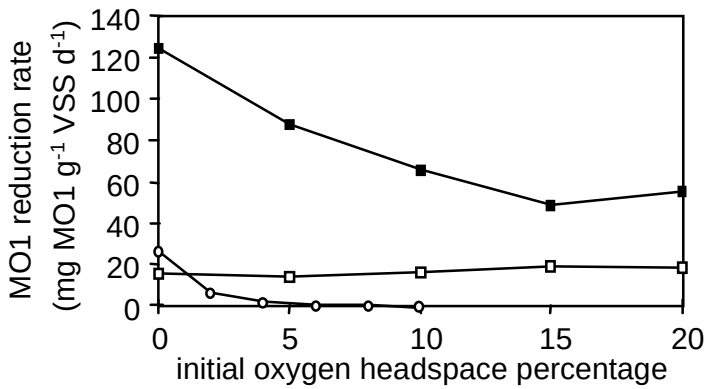

Figure 2.2 *Reduction rates of MO1 in the presence of oxygen with Nedalco granular sludge as biomass source with acetate (□) or ethanol (■) as co-substrate, or no co-substrate (○).*

In the absence of oxygen, acetate and no co-substrate supported much lower azo dye-reduction rates of approximately 20 mg MO1 g^{-1} VSS d^{-1}. The rate dropped to zero at 4 IHOP when no co-substrate was used. However, when acetate was used as co-substrate, the rate was not affected by the presence of oxygen up to 20 IHOP. Similar results were also obtained with Shell granular sludge (Figure 2.3), except that the absolute rate with ethanol as co-substrate of 40 mg MO1 g^{-1} VSS d^{-1} was clearly lower than that of the Nedalco granular sludge. Exposure to oxygen up to 20 IHOP had no effect on the rate. When co-substrate was lacking, Shell granular sludge had reducing activity only in the complete absence of oxygen.

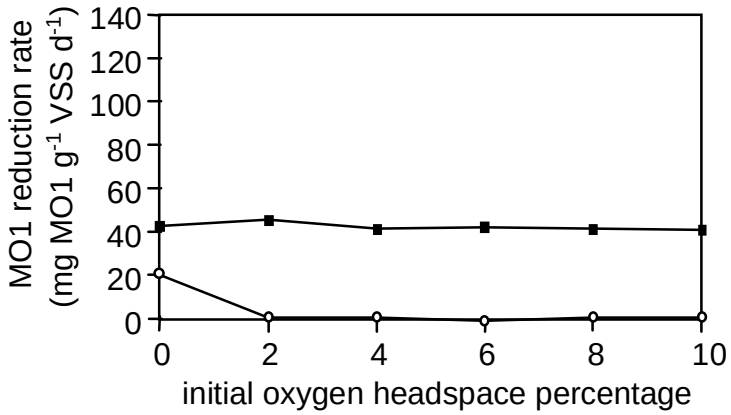

Figure 2.3 *Reduction rates of MO1 in the presence of oxygen with Shell granular sludge as biomass source with ethanol (■) as co-substrate, or no co-substrate (○).*

During all of the experiments it was observed that methane production occurred only after almost all the azo dye had been reduced. An example is shown for Nedalco granular sludge

with 0 and 10 IHOP and ethanol as co-substrate (Figure 2.4). If no azo dye was added, methane production occurred when oxygen was present.

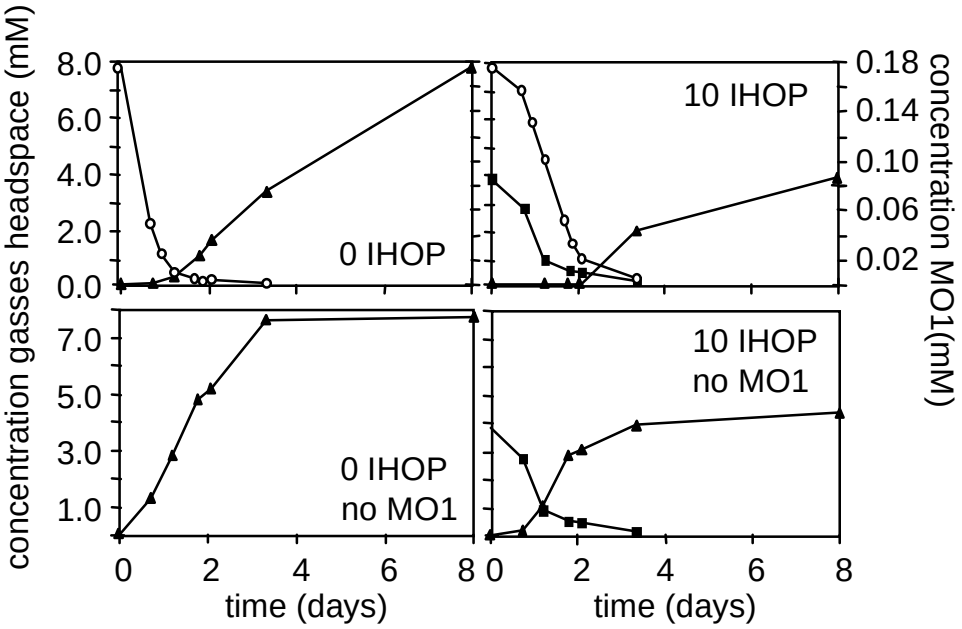

Figure 2.4 *Methane (▲) production and oxygen (■) consumption in the presence of 0 and 10 IHOP, and with or without MO1 (○), with Nedalco granular sludge plus ethanol as co-substrate (IHOP = initial headspace oxygen percentage).*

2.3.3 Chemical oxygen demand balance

The recovery of COD after a two-day batch assay (for methane after eight days) as a function of the IHOP is shown in Figure 2.5. Under completely anaerobic conditions almost all of the COD was recovered as methane. The fraction recovered as methane decreased incrementally with increasing oxygen concentrations. While the fraction of COD, due to oxygen uptake by facultative microorganisms, progressively increased. At 20 IHOP the methane production accounted for approximately 15% of the COD supplied, while the oxygen uptake accounted for about half of the COD supplied and around 90% of the oxygen supplied was consumed. The total recovered COD (sum of oxygen uptake plus methane production) only accounted for a little more than half of the COD supplied as substrate, probably due to the high cell yield of facultative anaerobes grown aerobically. The fraction of COD consumed to reduce the azo dye was relatively small compared with methane production and the oxygen uptake by aerobic respiration of the substrate. The maximal COD consumption due to five moles of H_2 needed to reduce one mole of MO1 (azo and nitro group) would be only 14 mg COD l^{-1}, which would not be enough to show up on the graph.

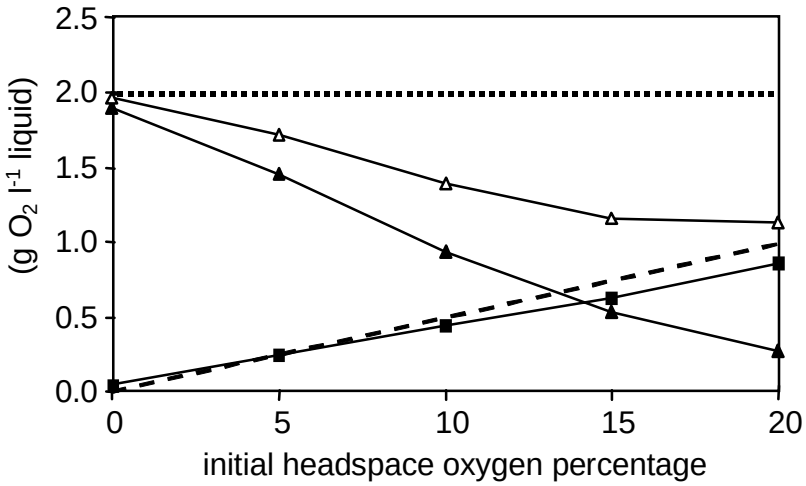

Figure 2.5 *COD mass balance as a function of the IHOP for Nedalco granular sludge plus ethanol as co-substrate after two days (COD expressed as g O_2 l^{-1}liquid; oxygen uptake (■), methane production (▲), sum methane production + oxygen uptake (Δ), ethanol supplied (▪▪▪) and oxygen supplied (----), methane values used were calculated from measurements after eight days).*

2.3.4 Aromatic amine recovery

The recoveries of aromatic amines at the end of the incubations were measured in order to determine if they were oxidized with the residual oxygen (Table 2.2). Table 2.2 indicates that recovery of the aromatic amines as a percentage of the amount of MO1 reduced was very high. These results indicate that there was no further metabolism of the aromatic amines, irrespective of the initial or final oxygen concentrations. At least at the highest IHOP tested oxygen was not consumed completely suggesting that lack of aromatic amine degradation was not due to the lack of oxygen.

Table 2.2 Percentage recovery of aromatic amines which were reductively formed from MO1; Nedalco granular sludge as biomass source.

Initial headspace oxygen percentage	Final headspace oxygen percentage		Aromatic amine recovery [#]	
	Co-substrates		Co-substrates	
	Ethanol	Acetate	Ethanol	Acetate
0	0.00	0.00	92.3	89.3
5	0.59	0.88	94.4	94.8
10	0.39	0.47	96.1	97.0
15	1.54	0.67	94.5	92.0
20	1.17	0.96	93.9	91.3

[#] molar yields in percentage = 100 × ([aromatic amines] / 2) / [initial MO1])

2.4 Discussion

2.4.1 Role of co-substrate

The results presented clearly illustrate that azo dye reduction occurs in granular sludge exposed to oxygen if co-substrates are available. The co-substrates are preferably metabolized aerobically by facultative anaerobes naturally present in granular sludge, and this results in consumption of oxygen. Ethanol was more rapidly respired aerobically than acetate in Nedalco granular sludge. Also, Nedalco granular sludge had a higher oxygen consuming activity than Shell granular sludge, indicating that the former granular sludge contained a higher concentration of facultative anaerobes, as had been observed previously (Kato et al. 1993a).

The rate of oxygen consumption was correlated highly with the rate of azo dye reduction in the assays exposed to a given concentration of oxygen (Figure 2.6). This correlation can be explained on the basis of oxygen penetration into a biofilm as a function of the oxygen respiration rates. It is well established in the literature that the depth of oxygen penetration into a biofilm is lowered by increasing oxygen-consumption rates (Kudlich et al. 1996; Kurosawa and Tanaka 1990). In actively respiring aerobic biofilms, the depth of oxygen penetration is limited to a few hundred μm (Costerton et al. 1994; De Beer et al. 1993; Hooijmans et al. 1990; Muller et al. 1994; Nielsen et al. 1990). Consequently, it can be expected that the volume of anaerobic microniches, which can support azo dye reduction

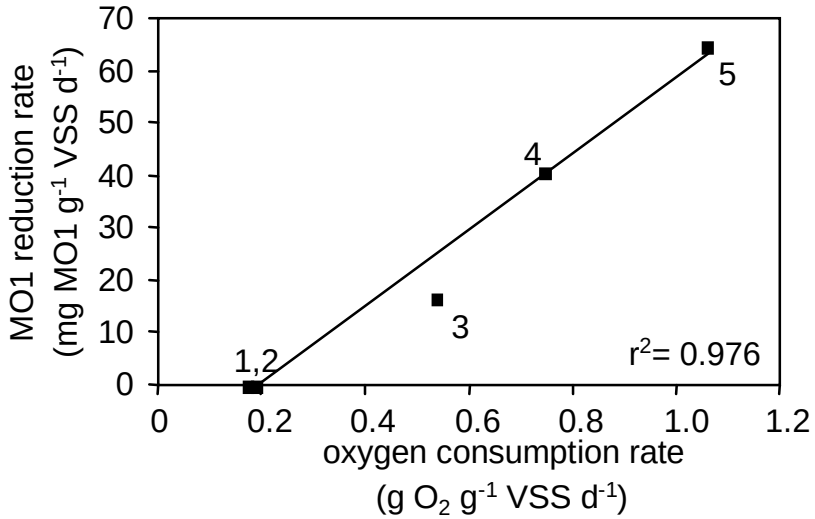

Figure 2.6 *Correlation between oxygen consumption rate and azo dye-reduction rate at 10 IHOP (1 = Shell granular sludge with no co-substrate; 2 = Nedalco granular sludge with no co-substrate; 3 = Nedalco granular sludge with acetate as co-substrate; 4 = Shell granular sludge with ethanol as co-substrate; 5 = Nedalco granular sludge with ethanol as co-substrate).*

would increase with greater oxygen-consuming activity of the biofilm. This hypothesis can also explain the occurrence of methane production in granules exposed to oxygen (Kato et al. 1993b) and the unusually high tolerance of methanogens in granular sludges to oxygen toxicity (Kato et al. 1993a).

A second role of co-substrates is that they provide reducing equivalents to anaerobic microorganisms for azo dye reduction. The present hypothesis of azo dye reduction is the involvement of reduced cofactors, such as flavin adenine dinucleotide (Gingell and Walker 1971; Kudlich et al. 1997). By comparing azo dye-reduction rates in the assay under completely anaerobic conditions, it was observed that ethanol greatly stimulated the rates beyond that supported by the meager supply of endogenous substrates in the granular sludge. The same increase in azo dye reduction was reported when glucose was added as readily metabolizable carbon source (Carliell et al. 1995). Acetate, however, did not cause any stimulation. These findings are similar to the ability of ethanol, and the inability of acetate, to support nitro aromatic reduction in granular sludge (Donlon et al. 1996). During the degradation of ethanol interspecies hydrogen is produced and this is apparently used by anaerobic bacteria to reduce azo dyes and nitro aromatics. Acetate, however, is a direct substrate of methanogens and consequently during its degradation no reduced interspecies intermediates are released. Nonetheless, acetate did stimulate azo dye reduction in the presence of oxygen, and this could be explained solely on the basis of its role in stimulating oxygen consumption by facultative microorganisms.

2.4.2 Products of azo dye reduction

The main products of MO1 azo dye reduction are the aromatic amines, 5-aminosalicylic acid (5-ASA) and 1,4-phenylenediamine (Donlon et al. 1997). In this study the aromatic amines were recovered in high stoichiometric yields from the reduction of MO1 both in the presence and absence of oxygen. Under anaerobic conditions it is well established that aromatic amines are poorly biodegradable (Blum et al. 1986; Kuhn and Suflita 1989; Razo-Flores et al. 1996). Although 5-ASA was reported to be mineralized in the absence of oxygen after long adaptation periods of several hundred days (Donlon et al. 1997), this would not be expected with the unadapted granular sludge used in this study. The absence of aromatic amine degradation in the presence of oxygen is probably due to the lack of a suitable population of aerobic microorganisms in the anaerobic granular sludge which are capable of metabolizing these compounds. For the development of an integrated anaerobic/aerobic bioreactor system, addition of adapted aerobic biomass will therefore most likely be required for the mineralization of aromatic amines. However, it should be noted that aerobic aniline-degrading enrichment cultures have been developed from granular sludge within several weeks (unpublished).

2.4.3 Azo dye inhibition of methanogens

Under both anaerobic and aerobic conditions, we observed that the presence of the azo dye MO1 was very inhibiting of methanogenesis. This finding is consistent with the previously reported 50% inhibiting concentration of MO1 towards acetoclastic methanogenesis of 4.0 mg l^{-1} (0.014 mM), which is approximately twelve-fold lower than concentrations used in this study. The methanogenic inhibition was reversed as soon as the MO1 was completely reduced (Figure 2.4) as would be expected from the much lower toxicity of the aromatic amines (Donlon et al. 1997). The sudden reversibility of the methanogenic toxicity indicates that MO1 does not cause lasting damage to the methanogens; rather, a competitive inhibition is inferred. One possible explanation is the reduction of MO1 by electrons diverted away from methanogenesis. However, this explanation is not likely since the concentration of MO1 used, of 50 mg l^{-1} (0.174 mM), could maximally consume five mole equivalents of H_2 which would only account for only 0.63% of the COD supplied as ethanol.

3

Biodegradation of azo dyes in co-cultures of anaerobic granular sludge with aerobic aromatic amine degrading enrichment cultures

Abstract A prerequisite for the mineralization (complete biodegradation) of many azo dyes is a combination of reductive and oxidative steps. In this study, the biodegradation of two azo dyes, 4-phenylazophenol (4-PAP) and Mordant Yellow 10 (4-sulfophenylazosalicylic acid; MY10), was evaluated in batch experiments where anaerobic and aerobic conditions were integrated by exposing anaerobic granular sludge to oxygen. Under these conditions, the azo dyes were reduced, resulting in a temporal accumulation of aromatic amines. 4-Aminophenol (4-AP) and aniline were detected from the reduction of 4-PAP. 5-Aminosalicylic acid (5-ASA) and 4-aminobenzenesulfonic acid (4-ABS) were detected from the reduction of MY10. Subsequently, aniline was degraded further in the presence of oxygen by the facultative aerobic bacteria present in the anaerobic granular sludge. 5-ASA and 4-ABS were also degraded, if inoculum from aerobic enrichment cultures were added to the batch experiments. Due to rapid autoxidation of 4-AP, no enrichment culture could be established for this compound. The results of this study indicate that aerobic enrichment cultures developed on aromatic amines combined with oxygen tolerant anaerobic granular sludge can potentially be used to completely biodegrade azo dyes under integrated anaerobic/aerobic conditions.

Tan N.C.G., Prenafeta-Boldú F.X., Opsteeg J.L., Lettinga G. and Field J.A. (1999). Applied Microbiology and Biotechnology 51(6): 865-871.

3.1 Introduction

Azo dyes are aromatic structures characterized by one or more azo linkages (R_1-N=N-R_2). An estimated amount of 700,000 tons of dyes are produced annually worldwide (Zollinger 1987), of which 60-70% are azo dyes (Carliell et al. 1995). During manufacturing and usage, an estimated amount of 10-15% is released into the environment (Anliker 1979). Even at very low concentrations, water-soluble azo dyes can cause waste streams to become highly colored. Aside from their negative aesthetic effects certain azo dyes and their biotransformation products have been shown to be toxic and in some case these compounds are carcinogenic and mutagenic (Chung and Cerniglia 1992). Therefore, emission of these pollutants should be avoided. One interesting approach is to promote the microbiological degradation of these man-made compounds in wastewater treatment systems.

Biodegradation of azo dyes is generally only feasible if the azo linkage is first reduced. Some specialized strains of aerobic bacteria have developed the ability to reduce the azo group by special oxygen tolerant azo reductases (Kulla 1981). However, these azo reductases have a narrow substrate range (Zimmermann et al. 1982a). On the other hand, azo dye reduction is a ubiquitous capacity of many microorganisms under anaerobic conditions (Carliell et al. 1995; Donlon et al. 1997; Razo-Flores et al. 1997; Walker 1970). Therefore, anaerobic conditions are preferable for azo dye reduction.

Azo dye reduction leads to the formation of aromatic amines. Aromatic amines are generally not degraded and accumulate under anaerobic conditions (Brown and Hamburger 1987; Field et al. 1995), with the exception of a few aromatic amines characterized by the presence of hydroxyl and/or carboxyl groups (Heider and Fuchs 1997; Kuhn and Suflita 1989; Razo-Flores et al. 1996). Mineralization of the aromatic amines by aerobic bacteria and aerobic sludge in treatment plants is more common and, therefore, aerobic conditions are preferable to degrade the accumulated aromatic (Brown and Laboureur 1983a; Manalney 1960). However, it should be noted that some aromatic amines are readily autoxidized in the presence of oxygen to humic like oligomeric and polymeric structures (Parris 1980).

A possible means of integrating anaerobic and aerobic processes is to use granular methanogenic sludge exposed to oxygen as suggested by Kato et al. (1993a). Aerobic processes occur in the outer regions of the biofilm, while deep inside the biofilm anaerobic processes prevail. Methanogenic activity of granular sludge could be maintained after prolonged exposure to oxygen (Kato et al. 1993a; Kato et al. 1993b; Shen and Guiot 1995; Shen et al. 1996). Recently, we demonstrated azo dye reduction occurs in anaerobic microniches created inside granular sludge exposed to oxygen. The formation of anaerobic microniches depends on the supply of co-substrate for oxygen consumption. Additionally,

some co-substrates donate interspecies electrons (e.g. H_2) stimulating the dye reduction further (Chapter 2).

The integration of anaerobic and aerobic conditions in a single biofilm has been proposed and proven to be a good strategy for the mineralization of azo dyes (Field et al. 1995; Haug et al. 1991; Kudlich et al. 1996). Inside the biofilm, anaerobic conditions will result in azo dye reduction yielding aromatic amines. The aromatic amines are susceptible for mineralization in the periphery of the biofilm, if the right bacteria are present.

The objective of this study is to investigate if azo dyes are biodegraded under integrated anaerobic/aerobic conditions by creating co-cultures with anaerobic granular sludge and aerobic aromatic amine degrading bacterial enrichment cultures.

3.2 Materials and methods

3.2.1 Biomass sources and basal medium

Methanogenic granular sludge from a full-scale upflow anaerobic sludge blanket (UASB) reactor treating wet oxidized industrial effluent of Shell Nederland Chemie (Moerdijk, The Netherlands) and from a full-scale UASB reactor treating effluent of an alcohol distillery of Nedalco (Bergen op Zoom, The Netherlands), were used for the batch experiments. Both granular sludge sources were washed and sieved to remove the fine particles before use in the batch tests. Aerobic Rhine sediment was collected along side the banks of the river near Lexkesveer in Wageningen (January 1997), The Netherlands. The sediment was suspended in 500 ml water and sieved (pore size 1.5 mm). All biomass sources were stored at 4°C before usage.

The basal medium used in all batch experiments contained (mg l^{-1}): $NaHCO_3$ (5000), NH_4Cl (280), $CaCl_2 \cdot 2H_2O$ (10), K_2HPO_4 (250), $MgSO_4 \cdot 7H_2O$ (100), yeast extract (100), H_3BO_3 (0.05), $FeCl_2 \cdot 4H_2O$ (2), $ZnCl_2$ (0.05), $MnCl_2 \cdot 4H_2O$ (0.05), $CuCl_2 \cdot 2H_2O$ (0.03), $NH_4SeO_3 \cdot 5H_2O$ (0.05), $AlCl_3 \cdot 6H_2O$ (2), $NiCl \cdot 6H_2O$ (0.05), $NaSeO_3 \cdot 5H_2O$ (0.1), EDTA (1), resazurin (0.2); and 36% HCl (0.001 ml l^{-1}).

3.2.2 Analyses

The biogases (oxygen, carbon dioxide, nitrogen and methane) in the headspace of the batches and ethanol in the liquid phase were analyzed via gas chromatographic methods described previously (Chapter 2). The volatile suspended solids (VSS) were measured according to Standard Methods for Examination of Water and Wastewater (APHA 1985). The concentration of ethanol is expressed in terms of chemical oxygen demand (COD), commonly used in wastewater treatment. Conversion factor used was 2.087 g COD g^{-1} ethanol.

The aromatic amines used in the aerobic biodegradation assays were analyzed spectrophotometrically with a Spectronic 60 spectrophotometer (Milton Roy Analytical

Products Division, Oostende, Belgium) at their absorbance maximums; aniline at 230 nm; 4-ABS at 248 nm; 4-AP at 230 nm; 5-ASA at 212 nm. Liquid batch samples were centrifuged (7833 x *g*, 10 minutes) and diluted in a 0.10 M sodium phosphate buffer solution (pH 7.0) and measured in a 1 cm 100-QS (Hellma Benelux, The Hague, The Netherlands) quartz cuvette.

The azo dyes and their corresponding amines in the integrated anaerobic/aerobic degradation experiments were analyzed with high performance liquid chromatography (HPLC). Samples from the batch experiment were centrifuged (7833 x *g*, 10 minutes) and diluted in demineralized water and 10 µl samples were injected with a Marathon autosampler (Separations, Hendrik Ido Ambacht, The Netherlands). The azo dyes and their corresponding aromatic amines were detected spectrophotometrically with a Spectroflow 783 UV detector (Kratos Analytical, Hendrik Ido Ambacht, The Netherlands) at their absorbance maximum (355 nm for MY10, 347 nm for 4-PAP and the previously stated wavelengths for the aromatic amines).

Methanol with 2% demineralized water (A) and 0.5% acetic acid in demineralized water adjusted to pH 5.9 (B) were used as liquid phase and were pumped (Separations High Precision Pump Model 104, Seperations, Hendrik Ido Ambacht, The Netherlands) at a flow rate of 300 µl min^{-1} first through a Separations GT-103 degaser (Hendrik Ido Ambacht, The Netherlands) and afterwards through two reverse phase C18 columns (200 mm x 3 mm, Chromosphere C18, Chrompack, Bergen op Zoom, The Netherlands). The following gradient program was used for the detection of MY10 and its corresponding aromatic amines, 5-ASA and 4-ABS: 0 minutes 2% A : 98% B; 5 minutes 2% A : 98% B; 7.5 minutes 80% A : 20%B; 15 minutes 80% A : 20%; 20 minutes 2% A : 98% B. The following gradient program was used for the detection of 4-PAP and its corresponding aromatic amines, aniline and 4-AP: 0 minutes 100% B; 5 minutes 100% B; 15 minutes 80% A : 20% B; 20 minutes 80% A : 20% ; 25 minutes 100% B.

3.2.3 Aerobic biodegradation assay

The aerobic biodegradation of the aromatic amines was studied in experiments with 117 ml glass bottles filled with 22.5 ml of basal medium and inoculum (10% of liquid volume for the Rhine sediment or 2.0 g VSS l^{-1} of Shell granular sludge). The assay serum flasks were flushed with N_2 / CO_2 (70% / 30%) gas for 5 minutes. Variable initial headspace oxygen percentages (IHOPs) were arranged by first removing a given amount of gas from the bottles headspace and replenishing it with the same amount of oxygen. In order to correct for background endogenous oxygen uptake by the inoculum source, control batches were incubated without aromatic amines. Sterilized controls and control batches without inoculum source were used to distinguish between biotic and abiotic degradation mechanisms of the aromatic amines. The concentration of aromatic amine applied in the aerobic biodegradation

experiments was 200 mg l^{-1} for all the aromatic amines tested. Aerobic aromatic amine degrading enrichments were established by replenishing aromatic amines and oxygen when they were consumed. The assays were also preformed without addition of oxygen (Nedalco granular sludge 1.0 g VSS l^{-1} for aniline, 4-AP and 5-ASA; 2.0 g VSS l^{-1} for 4-ABS; the aromatic amine concentration was 100 mg l^{-1}), in order to confirm that Nedalco granular sludge could not degrade aromatic amines anaerobically. All batch experiments were either preformed in duplicate or triplicate. Batches were incubated in a temperature-controlled room at 30 ± 2°C and were shaken on an orbital-motion shaker at 50 strokes per minute. The aromatic amine concentrations were monitored based on UV absorbance from the spectrophotometric measurements and oxygen headspace content was measured via the gas chromatographic method. The lack of anaerobic biodegradation was also checked via methane measurements (Razo-Flores et al. 1996).

3.2.4 Integrated anaerobic/aerobic degradation of the azo dyes

Mordant Yellow 10 (MY10) Integrated anaerobic/aerobic MY10 degradation assays were performed in 117 ml serum bottles batches in triplicate. Bottles were filled with 24 ml basal medium and granular sludge (4.0 g VSS l^{-1}), active aerobic aromatic amines degrading enrichments (5% v/v), ethanol (2.5 g COD l^{-1}) and 180 mg MY10 l^{-1} (491 µM) and closed with a butyl rubber septum (Rubber B.V., Hilversum, The Netherlands) and a crimp-seal aluminum cap.

4-Phenylazophenol (4-PAP) Integrated anaerobic/aerobic 4-PAP degradation assays were performed in 309 ml serum bottles batches in duplicate. Bottles were filled with 64 ml basal medium, granular sludge (1.0 g VSS l^{-1} from an integrated anaerobic/aerobic reactor treating 4-PAP-containing-artificial wastewater), ethanol (2.0 g COD l^{-1}) and 60 mg 4-PAP l^{-1} (300 µM) and closed with a butyl rubber septum (Rubber B.V., Hilversum, The Netherlands) and a crimp-seal aluminum cap.

Afterwards, all assay serum flasks were flushed with N_2 / CO_2 (70% / 30%) gas for 5 minutes. Variable IHOPs were arranged by first removing a given amount of gas from the bottle its headspace and replenishing it with the same amount of oxygen. Sterilized controls and control batches without inoculum source were used to distinguish between biotic and abiotic degradation mechanisms of the azo dyes. The assay bottles were incubated in a temperature-controlled room at 30 ± 2°C and were shaken on an orbital-motion shaker at 50 strokes per minute. Methane, oxygen and ethanol were measured by gas chromatographic methods. The azo dyes and their corresponding aromatic amines were measured with the HPLC methods.

3.3 Results

3.3.1 Aerobic aromatic amine degradation

Four different aromatic amines are potentially formed by the anaerobic reduction of MY10 and 4-PAP. The aromatic amines are 4-ABS and 5-ASA from MY10; 4-AP and aniline from 4-PAP and these were tested as to whether they could be degraded aerobically. 4-ABS, 5-ASA and aniline were biodegraded aerobically as confirmed by biologically mediated removal of the compounds and corresponding oxygen uptake measurements corrected for background respiration in controls without compound. Assuming that approximately 40% of the compound's COD was used for biomass yield, the observed oxygen uptake corresponding to 60-69% of the COD of the aromatic amines indicates that complete mineralization took place for these three aromatic amines.

The aerobic degradation of 4-ABS was only observed with aerobic Rhine sediment (Figure 3.1A). Several other inoculum sources were tested (activated sludge, forest humus soil and anaerobic granular sludge form Shell and Nedalco) but none of the others supported degradation of 4-ABS.

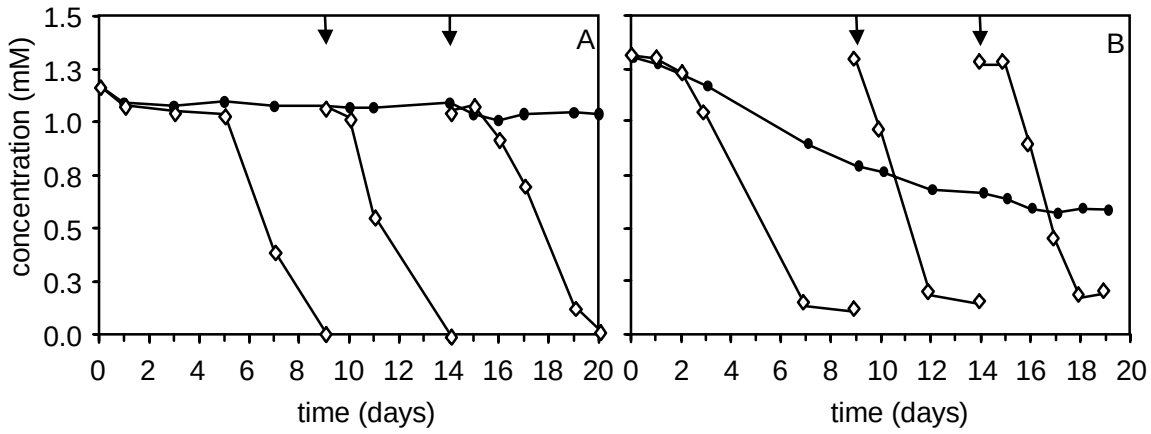

Figure 3.1A-B *Time courses of the aerobic biodegradation of 4-ABS by aerobic Rhine sediment (A) and 5-ASA by a 4-ABS enrichment culture (B); legend: compound + inoculum (◇), sterile control (●); arrows indicate new additions of compound in the inoculated batches.*

The enrichment culture developed from the Rhine sediment after three feedings of 4-ABS was also able to degrade 5-ASA (Figure 3.1B). The sterilized control and control lacking inoculum showed some decreases in the 5-ASA concentration due to slow autoxidation of 5-ASA.

Two different inoculum sources, the aerobic Rhine sediment and the Shell anaerobic granular sludge, were able to aerobically degrade aniline. The fact that anaerobic granular sludge could be used as an inoculum source for aerobic aniline degradation was surprising.

The aerobic degradation experiments for 4-AP indicates that this compound was rapidly autoxidized. Fast disappearance rates were observed in the sterile control, indicating that the degradation mechanism was principally due to autoxidation.

The anaerobic biodegradation of all four aromatic amines with granular sludge was also tested. However, none of the tested compounds were degraded under the anaerobic conditions during the test period of approximately 100 days. Therefore, an aerobic step is required for the mineralization of the aromatic amines formed during azo dye reduction.

3.3.2 Integrated anaerobic/aerobic degradation of MY10

Integrated anaerobic/aerobic conditions were created after addition of oxygen to co-cultures of anaerobic granular sludge with aerobic aromatic amine degrading enrichment cultures. Figure 3.2 shows the results of the integrated anaerobic/aerobic MY10 experiment. The reduction of MY10 was observed at all IHOPs applied. Even in presence of oxygen, azo dye reduction occurred due to addition of ethanol as co-substrate. In the sterile controls no reduction of MY10 occurred.

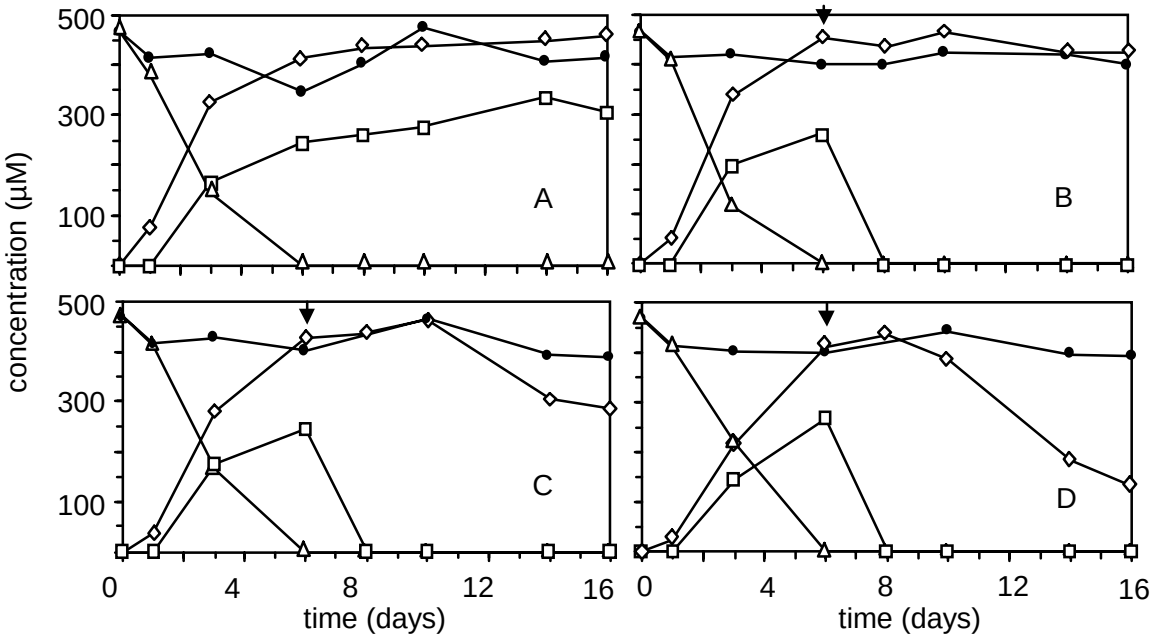

Figure 3.2A-D *Concentrations of MY10 (Δ), 5-ASA (□) and 4-ABS (◇) during the integrated anaerobic/aerobic MY10 degradation experiment at different IHOPs: 0 (A), 5 (B), 10 (C), and 20 (D). Concentration of MY10 in the sterile control (●); arrows indicate a second addition of oxygen corresponding to the IHOP.*

Both aromatic amines, 4-ABS and 5-ASA, accumulated in the integrated anaerobic/aerobic MY10 biodegradation experiment (Figure 3.2). 4-ABS was formed in stoichiometric amounts; by contrast, 5-ASA was not completely recovered in stoichiometric amounts at all applied IHOPs. Neither of the formed aromatic amines were degraded at 0 IHOP (Figure 3.2A). Due to fast respiration of the added ethanol at higher IHOPs (Figure 3.3), no oxygen was available

for the degradation of the aromatic amines. Therefore, in the assays with 5-20 IHOP, the initial amount of oxygen was replenished on day 6 (Figure 3.3B-D). After this additional supplement of oxygen, 5-ASA was readily degraded in the assays with 5-20 IHOP. 4-ABS was only degraded in the batches with 10-20 IHOP (Figure 3.2C-D).

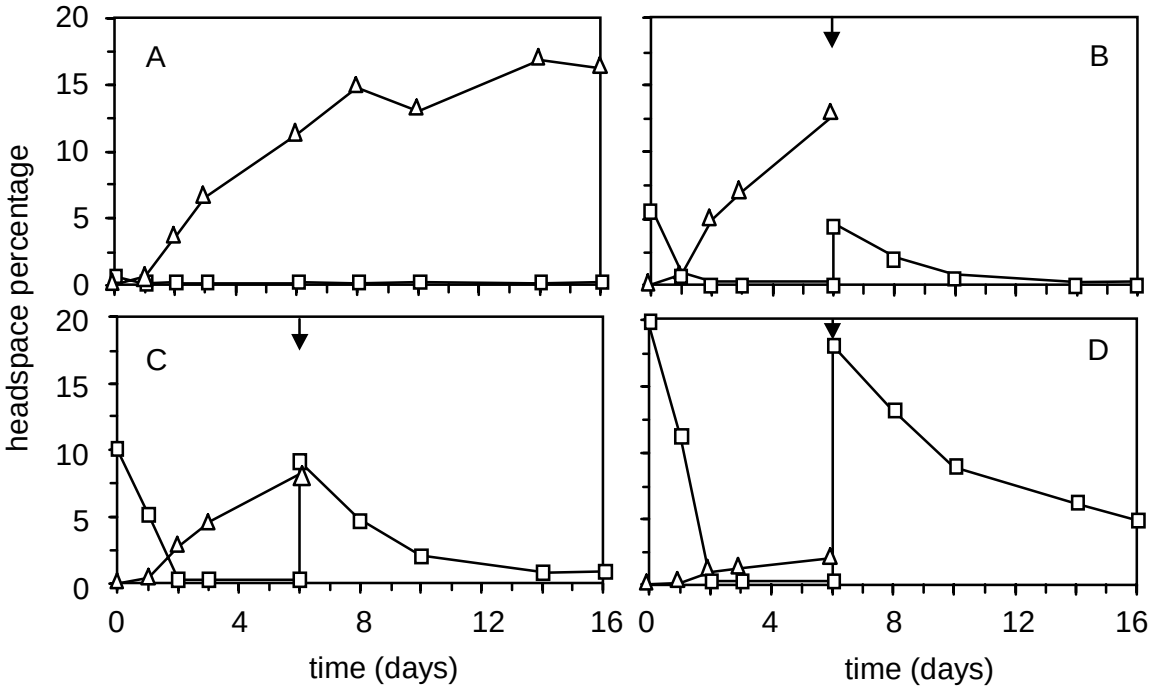

Figure 3.3A-D *Percentages of the methane (Δ) and oxygen (□) in the headspace of the assays during the integrated anaerobic/aerobic MY10 degradation experiment at different IHOPs: 0 (A), 5 (B), 10 (C), and 20 (D); arrows indicate a second addition of oxygen corresponding to the IHOP after flushing the batches with N_2/ CO_2 (70% / 30%).*

The formation of methane even in the presence of oxygen was also observed (Figure 3.3). This observation confirms the presence of anaerobic microniches in which methanogenic conversions and azo dye reduction can occur. Figure 3.3B-D also shows that the oxygen added on day 6 was taken up for the degradation of the aromatic amines.

3.3.3 Integrated anaerobic/aerobic degradation of 4-PAP

Figure 3.4 shows the results for the integrated anaerobic/aerobic 4-PAP biodegradation experiment at different IHOPs applied. The figure clearly shows a rapid reduction of 4-PAP at all IHOPs applied. As was observed with MY10, the reduction of 4-PAP even occurred in presence of oxygen due to the addition of ethanol. Again it was observed that in sterile controls no degradation of 4-PAP occurred.

The formation of both aromatic amines, 4-AP and aniline, is also clearly shown in Figure 3.4. As expected, the anaerobic degradation of both of these compounds did not occur (Figure 3.4A). In contrast to aniline, 4-AP was not recovered stoichiometrically in any of the incubations. The results for 10-30 IHOP clearly show that aniline and 4-AP which accumulate

were subsequently degraded. Only a small amount of both compounds were degraded at 10 IHOP (Figure 3.4B) due to limited amounts of oxygen remaining (Figure 3.5B). The main degradation mechanism for 4-AP was probably autoxidation, as was indicated from the aromatic amine degradation studies.

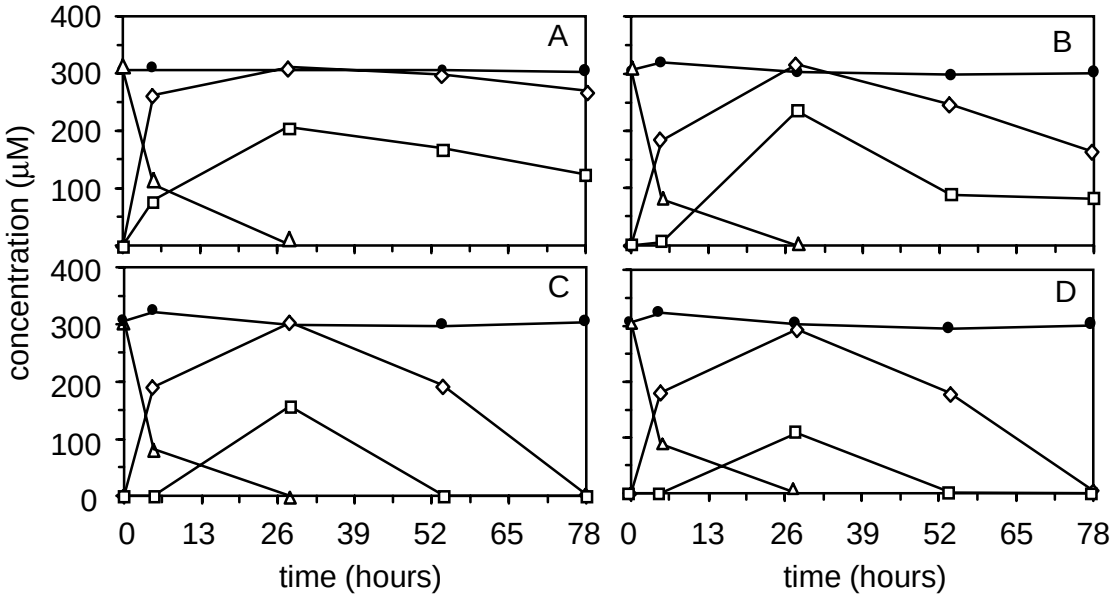

Figure 3.4A-D *Concentrations of 4-PAP) (Δ), 4-AP (□) and aniline (◇) during the integrated anaerobic/aerobic 4-PAP degradation experiment at different IHOPs: 0 (A), 10 (B), 20 (C), and 30 (D). Concentration of 4-PAP in the sterile control (●) at 10 IHOP.*

In both integrated anaerobic/aerobic azo dye degradation experiments it was noted that the recovery of 5-ASA and 4-AP from azo dye reduction were less than stoichiometric. The substoichiometric recovery could be attributed to autoxidation of these susceptible compounds between sampling and analysis.

The formation of methane in the presence of oxygen even up to 30 IHOP was also observed (Figure 3.5). This fact confirms the formation of anaerobic microniches where azo dye reduction can take place. Due to the higher oxygen/ethanol ratio applied (range 0.24-0.72 oxygen/ethanol COD ratios) in this experiment compared to the integrated anaerobic/aerobic MY10 degradation experiment (range 0.09-0.37 oxygen/ethanol COD ratios), no extra additions of oxygen were required to obtain degradation of the aromatic amines.

3.4 Discussion

3.4.1 Aerobic aromatic amine degradation

The aerobic biodegradation experiments revealed that aniline, 4-ABS, and 5-ASA were readily degraded aerobically. The aerobic biodegradation of aniline is ubiquitous as evidenced by numerous reports (Gheewala and Annachhatre 1997; Lyons et al. 1984; Manalney 1960). However, microorganisms capable of biodegradation of 5-ASA are less numerous (Stolz et al. 1992). 4-ABS biodegradation was only reported in a few cases. In one case, a co-culture of *Hydrogenophaga palleronii* S1and *Agrobacterium radiobacter* S2 was found to completely degrade 4-ABS (Feigel and Knackmuss 1993). In an other report, an undefined bacterial strain S1 was shown to degrade 4-ABS (Thurnheer et al. 1986). In this study, only bacteria from aerobic Rhine sediment were able to degrade 4-ABS, although other sources were tested. It is known that the river Rhine was and is still used as sewer for many industrial effluents, and an estimated amount of 7-15% of the organic pollutants in the Rhine are sulfonated aromatic compounds (Malle 1978). It was therefore, not surprising that Rhine sediment was a source of 4-ABS-degrading bacteria. Furthermore, the developed enrichment was also able to degrade 5-ASA.

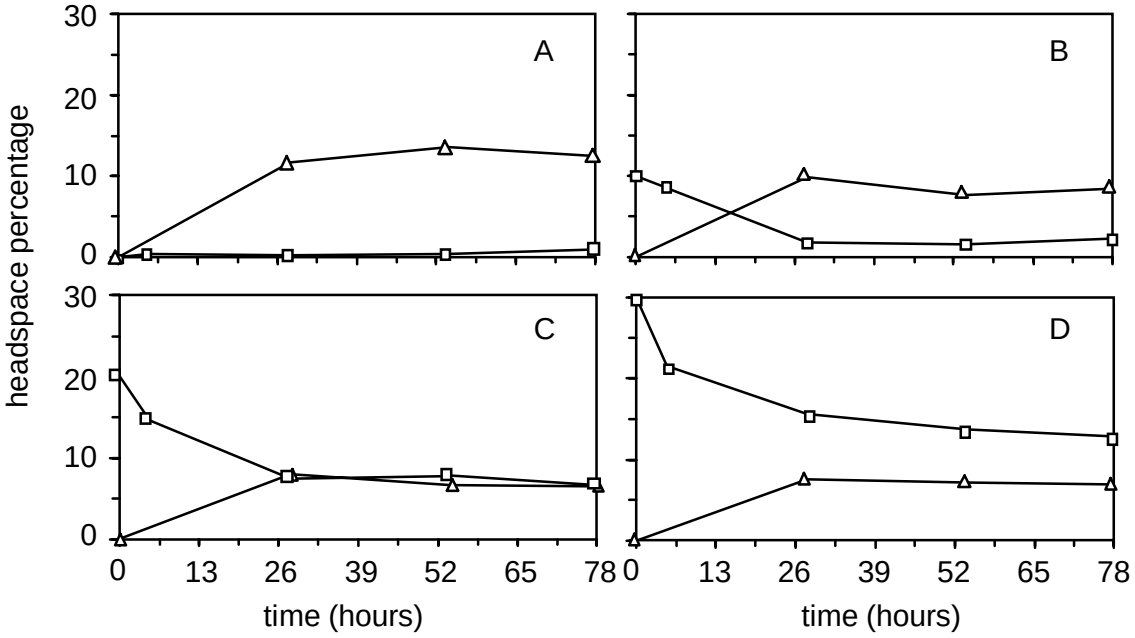

Figure 3.5A-D *Percentages of the methane (Δ) and oxygen (□) in the headspace of the batches during the integrated anaerobic/aerobic 4-PAP degradation experiment at different IHOPs: 0 (A), 10 (B), 20 (C), and 30 (D).*

Aerobic aniline degradation was as well observed using anaerobic granular sludge as an inoculum source. The fact that anaerobic granular sludge posses some facultative bacteria capable of quickly growing and carrying out aerobic metabolism was shown previously for the oxidation of methane (Kato et al. 1993b).

Aromatic amines with one or more hydroxyl group tend to autoxidize in presence of oxygen. This autoxidation process was observed for 4-AP and as well for 5-ASA (Jensen et al. 1992). Autoxidation rates for 4-AP were orders of magnitude faster compared to 5-ASA. Due to its slower autoxidation rates, biodegradation of 5-ASA was possible whereas autoxidation rates appeared to outcompete biodegradation rates in the case of 4-AP. The autoxidation products such as oligomeric and polymeric humic substances are not very susceptible for biodegradation.

3.4.2 Integrated anaerobic/aerobic azo dye degradation

Azo dyes generally need an anaerobic and an aerobic step to become completely mineralized (Field et al. 1995). A sequential anaerobic/aerobic degradation was described for azo dyes Acid Orange 10, Acid Red 14 and Acid Red 18 (FitzGerald and Bishop 1995). Alternatively, an integrated anaerobic/aerobic defined bacterial co-culture was shown to successfully biodegrade the azo dye Mordant Yellow 3 (Kudlich et al. 1996). The results described here demonstrate that undefined mixed cultures, realistic of the situation in wastewater treatment plants, can be used to completely mineralize azo dyes. Co-cultures of anaerobic granular sludge and aerobic aromatic amine degrading enrichment cultures mineralized the azo dyes MY10 and 4-PAP under the integrated anaerobic/aerobic conditions.

For azo dye reduction under integrated anaerobic/aerobic conditions, a co-substrate is required, in this case, ethanol was used, first, to create anaerobic microniches in which azo dye reduction can occur and second, to donate reducing equivalencies for the azo dye reduction (Chapter 2). However, under integrated anaerobic/aerobic conditions ethanol is also readily metabolized aerobically. The oxidation of co-substrate has two negative effects. First, oxygen is consumed and therefore becomes unavailable for mineralization of the aromatic amines as was observed in the MY10 experiment: an extra supplement of oxygen was needed to degrade the accumulated aromatic amines. The aromatic amines formed during anaerobic reduction of the azo dyes were easily degraded if oxygen was present. In the case of the integrated anaerobic/aerobic MY10 degradation experiment, residual oxygen was first utilized for the degradation of 5-ASA, which was more rapidly degraded than 4-ABS. Only when high IHOPs were applied enough oxygen was available to support the degradation of 4-ABS. Second, ethanol is consumed aerobically and therefore becomes less available for donating reducing equivalents for azo dye reduction. However, azo dye reduction still occurred, because only small amounts of ethanol are needed for the reduction of the azo dyes. The

electrons required to reduce the azo dyes only account for 0.12-0.16% of ethanol on COD basis. Furthermore, due to the poor water solubility of oxygen, diffusion and penetration into granular sludge are highly favoring ethanol compared to oxygen. Inside the granule, ethanol can donate electrons for anaerobic processes, as was evidenced not only by dye reduction but also by methane production even when 30% oxygen was initially present in the headspace.

4

Degradation of azo dye Mordant Yellow 10 in a sequential anaerobic and bioaugmented aerobic bioreactor

Abstract Complete biodegradation of azo dyes requires an anaerobic and aerobic step, in the anaerobic step sulfonated azo dyes are reduced, yielding (sulfonated) aromatic amines which can be degraded aerobically. The complete biodegradation of the sulfonated azo dye Mordant Yellow 10 (MY10) was studied in a sequential anaerobic and aerobic bioreactor. Anaerobically, MY10 was reductively cleaved and the resulting aromatic amines, 5-aminosalicylic acid (5-ASA) and 4-aminobenzenesulfonic acid (4-ABS), were both recovered in high stoichiometric yields. One of the aromatic amines, 5-ASA, was readily degraded under aerobic conditions. However, 4-ABS was not degraded aerobically in the continuous experiment because no 4-ABS-degrading bacterial activity was present in the system. Therefore, a 4-ABS-degrading enrichment culture derived from Rhine sediment was used as an inoculum source. This enrichment culture was bioaugmented into the aerobic reactor by increasing the hydraulic retention time (HRT), thus enabling 4-ABS-degrading activity to develop and be maintained in the aerobic reactor. After decreasing the HRT, the 4-ABS-degrading activity remained in the bioreactor and the stoichiometric recovery of sulfate (a 4-ABS biodegradation product) indicated mineralization of 4-ABS after bioaugmentation. Batch experiments with aerobic reactor sludge confirmed the biodegradation of 4-ABS and 5-ASA. The sequential anaerobic/aerobic bioreactor was able to completely remove the sulfonated azo dye MY10 at a maximum loading rate of 210 mg MY10 l_r^{-1} d^{-1} after the appropriate microorganisms for aerobic degradation of 4-ABS were bioaugmented into the aerobic bioreactor.

Tan N.C.G., Borger A., Slender P., Svitelskaya A.V., Lettinga G. and Field J.A. (2000). Water Science and Technology 45(5-6): 337-344.

4.1 Introduction

The production of dyes is extensive: up to 10^8 kg were manufactured in 1987 in the United States (Zollinger 1987). During production and usage of dyes and pigments, an estimated amount of 10-15% is released into the environment mainly via wastewater (Vaidya and Datye 1982). If dyes are divided in groups by their chemical structure than azo dyes are the largest (60-70%) group (Carliell et al. 1995). These compounds are mainly used in textile, printing, pharmaceutical and food industry for coloration. Many azo dyes are substituted with a sulfonate group. These sulfonated azo dyes absorb visible light due to the conjugated system of one or more azo groups (R_1-N=N-R_2) substituted with aromatic groups. Owing to the toxic and carcinogenic character of some azo dyes (Chung and Cerniglia 1992) as well as their breakdown products, the emission of these compounds should be minimized. A good way to minimize the emission of azo dyes is via biological degradation.

The initial step in the biodegradation of azo dyes is the reduction of the azo linkage, which generally results in the decolorization of the compound. Azo dye reduction is a ubiquitous process under anaerobic conditions if co-substrate is present to donate electrons (Carliell et al. 1995; Razo-Flores et al. 1996; Walker 1970). The azo dye reduction products, the (sulfonated) aromatic amines, are not very likely to be degraded under anaerobic conditions (Kuhn and Suflita 1989). Therefore, these compounds are excreted after the anaerobic treatment step.

Under aerobic conditions, many aromatic amines are readily degraded (Brown and Laboureur 1983a). In contrast, sulfonated aromatic amines are not very likely to be degraded under aerobic conditions. Only some reports describe complete degradation of sulfonated aromatic amines when used as carbon and energy source (Feigel and Knackmuss 1988; Hooper 1991; Nortemann et al. 1986; Thurnheer et al. 1986). One advantage of sulfonated aromatic amines compared to unsulfonated analogues is that the sulfonated aromatic amines are less toxic than their unsulfonated counterpart (Jung et al. 1992). Experiments at our laboratory revealed that only a two of the seven sulfonated aromatic amines tested were degraded in aerobic batch experiments using activated sludge or Rhine sediment (Chapter 5). Thus, a combination of anaerobic and aerobic treatment for the degradation of sulfonated azo dyes is only possible if the microorganisms are present to degrade the sulfonated aromatic amines. A way to stimulate the biodegradation of these sulfonated aromatic amines is to bioaugment the aerobic reactor with strains or enrichment cultures capable of degrading these compounds.

The aim of this study is to assess if MY10 can be completely mineralized under continuous conditions in a sequential anaerobic and aerobic bioreactor. The major problem with regard to

this aim is the aerobic biodegradation of the reduction product 4-ABS. Therefore, special attention was given to the optimization of biodegradation efficiency of the aerobic reactor.

4.2 Materials and methods

4.2.1 Sequential Anaerobic/Aerobic Reactor Set-up

In this study, MY10 was continuously treated in a sequence of an anaerobic and an aerobic lab-scale bioreactor (Figure 4.1). The anaerobic reactor, a 2.5 l expanded granular sludge blanket (EGSB, diameter 0.05 m and height 1.0 m), was connected to a 1.0 l aerobic activated sludge reactor (diameter 0.06 m and height 0.30 m). The upflow velocity of the anaerobic EGSB reactor was kept 2.0 m h^{-1} by recycling the EGSB effluent with a recycle factor of 40. The flow rate of the compressed air to aerate the bioreactor was 75 l h^{-1}. The washed-out aerobic activated sludge could settle in a conical settler (0.3 l) from which a sludge recycle stream was led back into the aerobic reactor.

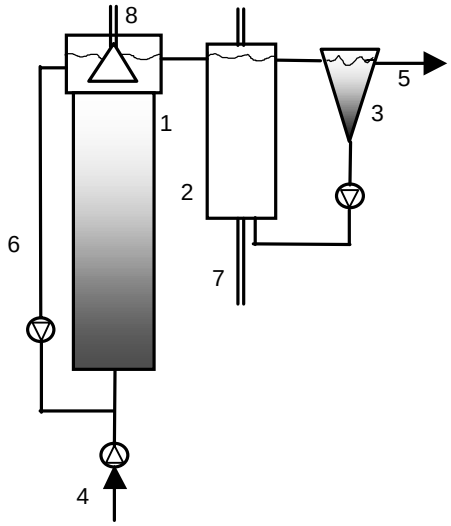

Figure 4.1 *Schematic draws of the sequential anaerobic EGSB and aerobic reactor (1. anaerobic EGSB reactor; 2. aerobic reactor; 3. settler; 4. influent; 5. effluent; 6. EGSB recycle stream; 7. air supply; 8. gas liquid separator).*

The influent consisted of a synthetic wastewater containing MY10 (100 - 200 mg l^{-1}), $NaHCO_3$ (1,0 g l^{-1}), and mineral medium (Chapter 2). Ethanol (EtOH, 1.0 - 2.0 g chemical oxygen demand (COD) l^{-1}) was added as the co-substrate for the reduction of the azo dye.

4.2.2 Biomass

Methanogenic granular sludge from a full-scale upflow anaerobic sludge blanket (UASB) reactor treating effluent of an alcohol distillery of Nedalco (Bergen op Zoom, The Netherlands), was used for the experiments. The granular sludge was washed and sieved to remove the fine particles before use in the reactor and batch tests. Both the aerobic and

anaerobic reactors were seeded with the Nedalco biomass with an amount of 20.0 g volatile suspended sludge (VSS) (APHA 1985) per liter reactor volume.
Aerobic Rhine sediment was collected along side the banks of the river near Lexkesveer in Wageningen (The Netherlands). The sediment (100 g) was suspended in 500 ml water and sieved (pore size 1.5 mm). This diluted sediment was used for the aerobic enrichment experiment (see below). All biomass sources were stored at 4°C before usage.

4.2.3 Batch Assays

Anaerobic activity, toxicity and biodegradation experiments The anaerobic activity, toxicity and biodegradation assays experiments were conducted via the method described before (Donlon et al. 1995; Razo-Flores et al. 1996).
Aerobic biodegradation experiments The aerobic biodegradation assays of 5-ASA and 4-ABS were performed as described before (Chapter 2). Enrichments were obtained the same way as the biodegradation assays. After degradation occurred, the batches were repetitively fed with compound and supplied with sufficient oxygen. The degrading biomass was frequently transferred in fresh and sterilized mineral medium.

4.2.4 Analyses

UV method and High Pressure Liquid Chromatography (HPLC) for MY10, 4-ABS and 5-ASA The UV and HPLC method for the determination of 4-ABS, 5-ASA and MY10 were described before (Chapter 3).
Gaschromatography (GC) for EtOH and for methane and biogasses Gas composition (O_2, N_2, CO_2 and CH_4) and ethanol concentrations were measured via the GC methods described before (Chapter 2).
HPLC for sulfate The mobile-phase (10.8 mM, potassiumbiphthalate) for the sulfate HPLC method was pumped with a high-pressure precision pump model 480 (Separations, Hendrik Ido Ambacht, The Netherlands) and a flow rate of 1.2 ml min^{-1} through the HPLC system. A 20 µl sample was injected with a Midas autosampler (Separation, Hendrik Ido Ambacht, The Netherlands) and anions were separated on a reverse-phase Vydac 302 IC405 anion column (Vydac, Hesperia CA, USA; length 250 mm x ID 4.6 mm). The anions were detected with a Waters 431 conductivity detector (Millipore, Etten-Leur, The Netherlands).
Chemical Oxygen Demand (COD) The soluble COD was measured colormetrically. Firstly, the samples were centrifuged 5.0 minutes at 10,000 rpm. Secondly, 5.0 ml volume samples were treated with 3.0 ml 0.083 N $K_2Cr_2O_7$ and 7.0 ml 18M H_2SO_4 which contains 0.034 M Ag^+. Thirdly, the closed sample tubes were stored in a 150 °C oven for two hours. Finally, after cooling the samples were measured at 600 nm with a Cecil CE 1011 spectrophotometer (Cecil Instruments Limited Technical Center, Cambridge, United Kingdom).

4.3 Results and Discussion

The results of the experiments will be presented and discussed separately for both reactors. First, the results of the anaerobic reactor will be presented. Second, the results of the aerobic reactor will be shown and discussed. At the end, the complete sequential anaerobic and aerobic reactor will be evaluated.

4.3.1 Anaerobic EGSB Bioreactor

Table 4.1 Operational parameters and results of the anaerobic EGSB bioreactor (average values).

		Anaerobic										
Parameter	Unit	Operational parameters										
Period		I	II	III	IV	V	VI	VII	VIII	IX	X	XI
End period	day	21	42	68	88	188	245	279	295	335	405	425
EtOH *	g COD l^{-1}	1.70	1.63	1.60	1.83	1.16	1.00	0.89	1.59	1.05	0.92	0.77
MY10 *	mg l^{-1}	0	57	100	192	116	153	137	147	131	159	133
MY10 load	mg $(l_r\ d)^{-1}$	0	44	73	157	92	165	143	210	164	164	109
HRT	day	1.34	1.30	1.38	1.22	1.25	0.93	0.96	0.70	0.80	0.97	1.22
		Efficiencies **										
EtOH	% removal	100	100	100	100	100	100	100	100	100	100	100
MY10	% removal	-	94	98	97	100	100	87	100	98	98	98
4-ABS	% recovery	-	99	80	88	104	99	100	100	87	95	106
5-ASA	% recovery	-	78	63	61	79	82	79	57	39	47	67
Activity ***	g COD CH_4 g VSS^{-1} d^{-1}	0.70	0.75	0.93	0.92	0.75	0.85	-	-	1.97	-	-

* Influent concentrations of the compounds. ** Removal and recovery based on the influent concentrations. *** Measured at the beginning of the period. Period V started at day 125; between days 89 and 124 no measurements were done.

The main results of the anaerobic EGSB bioreactor are summarized in Table 4.1. These results clearly show that the anaerobic EGSB bioreactor performed well, as it achieved complete decolorization of MY10 with increasing loads of MY10. The co-substrate ethanol was completely degraded in the anaerobic reactor and converted into methane and carbon dioxide. No inhibitory effects of the applied azo dye MY10 and its biodegradation products 4-ABS and 5-ASA to the anaerobic granular sludge were observed. The acetoclastic methanogenic activity remained high and even increased at the end of the experiment (Table 4.1) where azo dye concentrations up to 159 mg MY10 l^{-1} were applied. No inhibition was expected since the IC_{50} values for these compounds were 952 and 444 mg l^{-1} for MY10 and 5-ASA, respectively (Donlon et al. 1997). Batch experiments with 4-ABS even did not show any inhibition of the acetoclastic methanogenic activity up to concentration of 1.0 g 4-ABS l^{-1} (Chapter 5).

Both aromatic amines were recovered in high yields after the anaerobic reduction of MY10. 4-ABS was recovered in high stoichiometrically percentages (Table 4.1). Anaerobic degradation of 4-ABS is not likely since this compound was not degraded in anaerobic biodegradation experiments after long test periods (Kuhn and Suflita 1989; Razo-Flores et al. 1996). The other aromatic amine, 5-ASA, was only recovered for 60-80% which was probably due to autoxidation of this compound prior to measurements or to partial anaerobic degradation of 5-ASA. Indeed, the presence of bacteria capable of degrading 5-ASA anaerobically was demonstrated in an anaerobic biodegradability experiment in the period X. The results of the biodegradation experiment clearly show the anaerobic biodegradation of 5-ASA (Figure 4.2). The amount of methane produced during biodegradation of 5-ASA was 101% of the theoretical methane production, calculated via the Buswell equation (Tarvin and Buswell 1934). Biodegradation was also confirmed by stoichiometric recovery of ammonium (results not shown). The fact that 5-ASA was biodegraded by anaerobic granular sludge is not surprising because this was already reported (Razo-Flores et al. 1996). After anaerobic degradation of 5-ASA was proven, the HRT was increased from 0.97 to 1.22 d between days 405 to 423 in order to stimulate the anaerobic bacteria capable of degrading 5-ASA. However, no positive effect on the anaerobic degradation of 5-ASA was observed. Probably, this was because of the too slow growth of the anaerobic 5-ASA-degrading biomass.

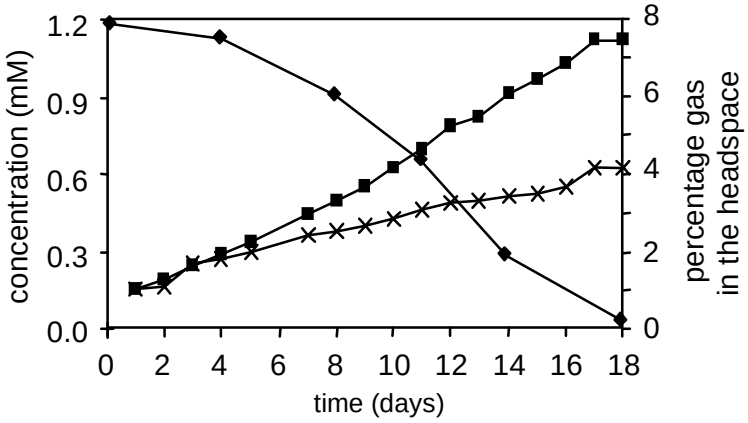

Figure 4.2 *Anaerobic biodegradation of 5-ASA with 8.0 g volatile suspended solids (bioreactor granular sludge) per litre. 5-ASA biodegradation (◆); methane (■); methane (x) production control without 5-ASA.*

4.3.2 Aerobic Bioreactor

A summary of the operational parameters and the results of the aerobic bioreactor performance are summarized in Table 4.2. For a period of more than 245 days, no 4-ABS degradation took place as it was recovered at nearly stoichiometric amounts in the effluent of the aerobic reactor. Nevertheless, in batch degradation experiments using Rhine sediment as inoculum source 4-ABS biodegradation could be obtained and an aerobic enrichment culture was developed. This enrichment was already successfully used to bioaugment batches in which MY10 was degraded under integrated anaerobic/aerobic conditions (Chapter 3). Therefore, this further enriched culture was also used to bioaugment the aerobic continuous reactor in period VII. In order to establish good retention of the 4-ABS-degradation culture, the HRT of the aerobic reactor was increased to from 0.37 to 3.3 d on day 261. Afterwards, the HRT was slowly decreased to its original value. The 4-ABS-degrading biomass still maintained its ability to degrade 4-ABS for more than 146 days. Degradation of 4-ABS was also confirmed in the stoichiometric recovery of sulfate during period IX (Figure 4.3).

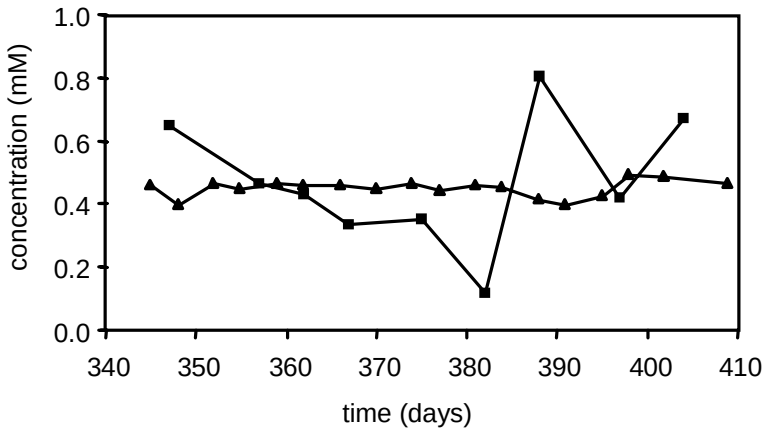

Figure 4.3 *Sulfate (■) produced during biodegradation of 4-ABS and the theoretical amount of sulfate (▲) produced calculated from the amount of 4-ABS degraded during period IX.*

The aerobic reactor was originally inoculated with granular sludge. Granular sludge showed to ability to degrade aniline (Tan et al. 1999) the unsulfonated analogue of 4-ABS. During the reactor experiment the granular sludge developed more like activated sludge. Since the aerobic bioreactor was only fed with 4-ABS and 5-ASA, the biomass, which developed, was specialized to degrade these compounds. No further research was done to locate on micro scale the fate of the 4-ABS-degrading bioaugmented biomass. Aerobic batch experiments with the aerobic reactor sludge (sample at the end of the reactor experiments) clearly revealed the mineralization of 4-ABS and 5-ASA (Figure 4.4). Oxygen uptake and stoichiometric sulfate production associated with 4-ABS degradation also confirmed these results. Figure 4.4

also shows that during 5-ASA biodegradation, also autoxidation occurs in the sterile control. The formation of all kinds of 5-ASA autoxidation products in aqueous solutions was already reported (Jensen et al. 1992). However, the active batches show that biodegradation is faster than autoxidation and is the dominant route for removal for 5-ASA (Figure 4.4).

Table 4.2 Operational parameters and results of the aerobic reactor (average values).

		Aerobic										
Parameter	Unit	Operational parameters										
Period		I	II	III	IV	V	VI	VII	VIII	IX	X	XI
End period	day	21	42	68	88	188	245	279	295	335	405	425
4-ABS*	mg l^{-1}	-	25	37	78	57	72	56	70	53	70	65
5-ASA*	mg l^{-1}	-	17	26	48	38	52	39	35	21	31	37
HRT	day	0.54	0.52	0.55	0.49	0.59	0.37	2.10	0.28	0.28	0.38	0.67
		Efficiencies **										
4-ABS	% removal	-	1	1	1	3	7	93	93	70	97	98
5-ASA	% removal	-	91	93	100	97	100	88	100	92	100	100

* Influent concentrations are due to the anaerobic reduction of MY10. ** Removal efficiencies based on the influent concentrations. Period V started at day 125 between 89 and 124 no measurements were done.

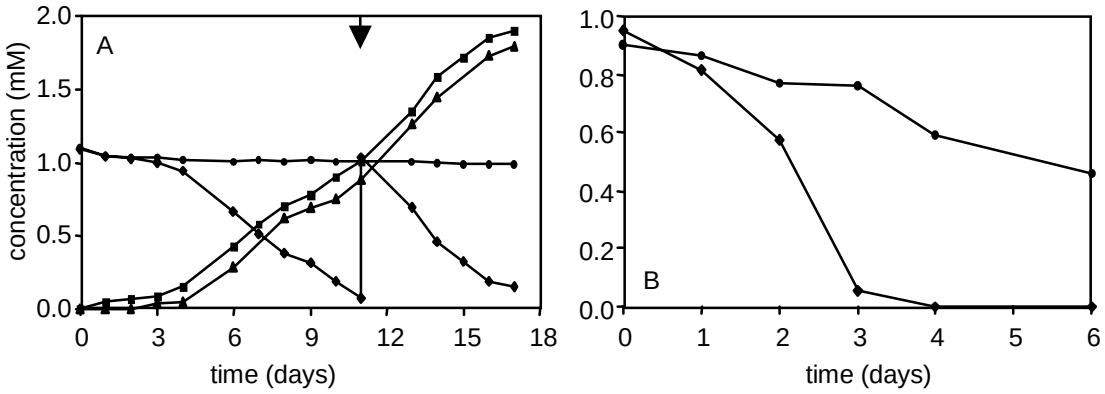

Figure 4.4 *Aerobic degradation of 4-ABS (A) and 5-ASA (B) in batch experiment with aerobic reactor sludge; arrow indicates readdition of compound, compound (◆) in active batches, compound in sterilized batches (●), sulfate (▲) concentration, cumulative amount of compound (■) degraded.*

4.3.3 Combined sequential anaerobic/aerobic reactor

In general, the sequential system showed a good removal of the sulfonated azo dye MY10, co-substrate and its constituent products 4-ABS and 5-ASA. Both aromatic amines were produced in the anaerobic phase and degraded in the aerobic phase. The aerobic biodegradation of 4-ABS had to be stimulated via bioaugmentation of a 4-ABS-enrichment culture. Bioaugmentation was successful and retention of the 4-ABS-degrading biomass was obtained.

The treatment of industrial wastewater polluted with sulfonated azo dyes in a sequential anaerobic/aerobic system will be more difficult since many different aromatic intermediates will be formed which also need to be degraded. Of these, the aerobic biodegradation of the sulfonated aromatic amines can be considered a major problem. Therefore, further research is needed on how the sulfonated aromatic amine degradation can be improved and efficiently incorporated in the total reactor system.

4.4 Conclusions

The complete degradation of MY10 was clearly demonstrated in a sequential anaerobic (EGSB) and aerobic (activated sludge) continuous bioreactor at a maximum loading rate 210 mg MY10 l^{-1} d^{-1}. The aerobic reactor was successfully bioaugmented with an aerobic 4-ABS-degrading enrichment culture. The degradation of 4-ABS was also confirmed by stoichiometric production of sulfate in batch as well as in continuous experiments. Furthermore, this research showed that bioaugmentation of xenobiotic degrading microorganisms is a useful tool for the removal of recalcitrant compounds such as sulfonated azo dyes and sulfonated aromatic amines.

5

Biodegradability of sulfonated aromatic amines, an important class of azo dye biotransformation products

Abstract Ten sulfonated aromatic amines were tested for their aerobic and anaerobic biodegradability and toxicity potential in sediments and sludges. None of the tested compounds were degraded under anaerobic conditions. In aerobic studies, only two aminobenzenesulfonic acid (ABS) isomers, 2- and 4-ABS, were degraded by inoculum sources that were historically polluted with sulfonated aromatic amines. Bioreactor experiments confirmed the results from the batch degradation experiments. Both ABS isomers were degraded in long term continuous experiment. The maximum degradation rate in the aerobic bioreactor was 1.6-1.8 g l^{-1} d^{-1} for 2-ABS and a somewhat lower value for 4-ABS at hydraulic retention times (HRT) of 2.8-3.3 hours. Evidence for extensive mineralization of 2- and 4-ABS was based on oxygen uptake and carbon dioxide production during the batch experiments and the high levels of chemical oxygen demand (COD) removal in the bioreactor. Furthermore, mineralization of the sulfonic acid group was demonstrated by high sulfate recovery percentages. Kinetic parameters describing biodegradation of 2-ABS were obtained via respirometric batch experiments with biomass from the reactor and resulted in a net biomass yield of 0.17 mg VSS mg^{-1} COD converted and a maximum growth rate of 0.077 h^{-1}.

Tan N.C.G., Van Leeuwen A., Van Voorthuizen E.M., Slenders P., Prenafeta-Boldú F.X., Temmink H., Lettinga G. and Field J.A. (2001). Water Research (submitted).

5.1 Introduction

Sulfonated aromatic amines are used as precursors for sulfonated azo dyes, optical brighteners, pesticides, ion-exchange resins, pharmaceuticals, and concrete plasticisers. The presence of a sulfonated group makes these compounds highly water-soluble. Therefore, during production processes sulfonated aromatic amines are easily discarded via the water system. Sulfonated aromatic amines are found in river and surface waters (Zerbinati et al. 1997) and it is estimated that sulfonated aromatic compounds represent 10% of the dissolved organic present in the river Rhine (Malle 1978).

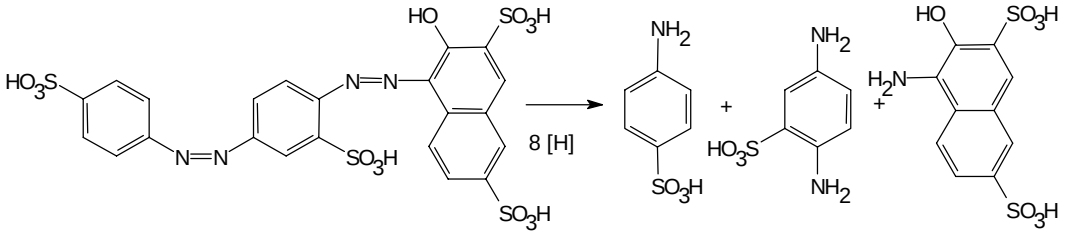

Figure 5.1 *Anaerobic reduction of sulfonated azo dye Ponceau S (Acid Red 112, C.I. 27915) and the formation of sulfonated aromatic amines.*

One of the important pollution sources of sulfonated aromatic amines are sulfonated azo dyes. Sulfonated aromatic amines are formed during the reduction of sulfonated azo dyes under anaerobic conditions (Figure 5.1). It is estimated that during the dyeing process, sulfonated reactive dyes are fixed for 50 to 90 percent and the remaining 10 to 50 will end up in the wastewater (O'Neill et al. 1999b). The discharge of these sulfonated azo dyes does not only a negative aesthetic effect but also some azo dyes and their degradation products, sulfonated and unsulfonated aromatic amines, are toxic or even carcinogenic (Chung and Cerniglia 1992; Oh et al. 1997). However, due to their poor lipophilicity, sulfonated aromatic amines are considered less dangerous than their unsulfonated analogues (Jung et al. 1992).

Concerning the anaerobic biodegradability of sulfonated aromatic amines, only limited data is available in literature indicating that none of these compounds tested were degraded (Kuhn and Suflita 1989; Razo-Flores et al. 1996). Under aerobic conditions, some sulfonated aromatic amines are degraded and used as carbon and energy source by specific strains. However, these specified strains only have a narrow substrate range and therefore only a limited number of different compound are degraded (Cook et al. 1998; Hooper 1991; Kertesz et al. 1994; Tan and Field 2000). Some sulfonated aromatic amines can be used as a sulfur source under sulfur poor conditions, which are unrealistic for natural and wastewater treatment environments (Ruff et al. 1999; Zurrer et al. 1987). Additionally, an other non-biological process for the removal of sulfonated aromatic compounds is autoxidation. Some sulfonated aromatic amines are susceptible to autoxidation reactions, resulting in the

transformation to colored polymers (Kudlich et al. 1999; Zerbinati et al. 1997). In practice, sulfonated aromatic amines are not readily degraded. Drinking water-producing facilities have problems in removing the sulfonated aromatic amine via their treatment systems (Lange et al. 1995).

The aim of this study was to investigate which sulfonated aromatic amines could be readily mineralized by natural mixed cultures present in wastewater treatment plants and sediments under aerobic or anaerobic conditions. The biodegradation of the sulfonated aromatic amines was first tested in batch experiments using sediment and activated sludge as inoculum sources. The biodegradability was then confirmed in long-term laboratory-scale bioreactors. Additionally, the toxicity of these compounds towards microbial activity in sludges was examined.

5.2 Materials and methods

5.2.1 Media

The basal medium used in the batch experiments contained (mg l^{-1}); $NaHCO_3$ (5000); NH_4Cl (280); $CaCl_2 \cdot 2H_2O$ (10); K_2HPO_4 (250); $MgSO_4 \cdot 7H_2O$ (100); yeast extract (100); H_3BO_3 (0.05); $FeCl_2 \cdot 4H_2O$ (2); $ZnCl_2$ (0.05); $MnCl_2 \cdot 4H_2O$ (0.05); $CuCl_2 \cdot 2H_2O$ (0.03); $NH_4SeO_3 \cdot 5H_2O$ (0.05); $AlCl_3 \cdot 6H_2O$ (2); $NiCl \cdot 6H_2O$ (0.05); $NaSeO_3 \cdot 5H_2O$ (0.1); EDTA (1); resazurin (0.2); and 36% HCl (0.001 ml l^{-1}). The concentration of trace metals and $NaHCO_3$ was ten- and five-time lower for the bioreactor experiments.

For the aerobic biodegradation experiments in which carbon dioxide was measured as mineralization product, a medium modified from (Feigel and Knackmuss 1988) was used. This medium contained (mg l^{-1}) K_2HPO_4 (3000); $NaH_2PO_4 \cdot H_2O$ (1000); $MgCl_2 \cdot 6H_2O$ (100); NH_4NO_3 (500); K_2SO_4 (100); 10 ml $FeCl_2 \cdot 4H_2O$ (concentration 6.27 $g \cdot l^{-1}$, pH 2); H_3BO_3 (1.0); $CoCl_2 \cdot 6H_2O$ (0.5), $ZnCl_2$ (0.2); $MnCl_2 \cdot 4H_2O$ (0.14); $NaMoO_4 \cdot 2H_2O$ (0.06), $CuCl_2 \cdot 2H_2O$ (0.02); $NaSeO_3 \cdot 5H_2O$ (0.06), $NiCl_2 \cdot H_2O$ (0.02). For the batch experiments, the sulfonated aromatic amines were added from sterilized and neutralized (pH = 7.0) stock solutions.

5.2.2 Inoculum sources

Activated sludge was obtained from a wastewater treatment plant in Aarle-Rixtel (The Netherlands). This plant treated domestic and industrial wastewater, including effluents from several textile industries. Aerobic Rhine sediment was collected along side the banks of the river near Lexkesveer in Wageningen (The Netherlands). The sediment was suspended in 500 ml water and sieved (pore size 1.5 mm). Anaerobic granular sludges, from a full-scale upflow anaerobic sludge blanket (UASB) reactor treating wet oxidized industrial effluent of Shell Nederland Chemie (Moerdijk, The Netherlands) and from a full-scale UASB reactor treating

effluent of an alcohol distillery at Nedalco (Bergen op Zoom, The Netherlands), were used for the experiments. All biomass sources were stored at 4°C before usage.

5.2.3 Chemicals

Sulfonated aromatic amines 2-ABS, 3-ABS, 4-ABS, 2,4-diaminobenzenesulfonic acid (2,4-DABS), 3-amino-4-hydroxybenzenesulfonic acid (3-A-4-HBS), 1-aminonaphthalene-4-sulfonic acid (1-AN-4-S), 1-aminonaphthalene-5-sulfonic acid (1-AN-5-S), 2-aminonaphthalene-1,5-disulfonic acid (2-AN-1,5-DS) 3-aminonaphthalene-2,7-disulfonic acid (3-AN-2,7-DS), and 8-aminonaphthol-3,6-disulfonic acid (8-ANOH-3,6-DS) were purchased from Aldrich (Milwaukee, USA), Sigma (St. Louis, USA), Fluka (Neu-Ulm, Switzerland) and Riedel-de Haën (Seelze, Germany) at the highest purity commercially available.

5.2.4 Analyses

The aromatic amines, used in the biodegradation assays, were analyzed spectrophotometrically with a Spectronic 60 spectrophotometer (Milton Roy Analytical Products Division, Oostende, Belgium) at their absorbance maximums; 2-ABS, 3-ABS and 4-ABS at 236, 236 and 248 nm and for 2,4-DABS, 1-AN-4-S, 2-AN-1,5-DS and 3-AN-2,7-DS the values were 220, 317, 247, and 247 nm, respectively. Liquid samples were centrifuged (7833 x *g*, 5.0 minutes) and diluted in a 0.10 M sodium phosphate buffer solution (pH 7.0) and measured in a 1.0 cm 100-QS (Hellma Benelux, The Hague, The Netherlands) quartz cuvette.

The three ABS isomers, used in the bioreactor experiment, were analyzed with high performance liquid chromatography (HPLC). Influent and effluent samples were centrifuged (7833 x *g*, 5.0 minutes) and 10 µl samples were injected with a Marathon autosampler (Separations, Henderik Ido Ambacht, The Netherlands). A 0.5% acetic acid solution (pH 5.9) was used as liquid phase and was pumped (Separations High Precision Pump Model 104, Seperations, Hendrik Ido Ambacht, The Netherlands) at a flow rate of 300 µl min^{-1} through the system. Samples and liquid phase were first degassed (Separations GT-103, Hendrik Ido Ambacht, The Netherlands) and separation of the compounds occurred in two reverse phase C18 temperature-controlled (20 °C) columns (200 mm x 3 mm, Chromosphere C18, Chrompack, Bergen op Zoom, The Netherlands). The ABS isomers were detected spectrophotometrically with a Spectroflow 783 UV detector (Kratos Analytical, Hendrik Ido Ambacht, The Netherlands) at 248 nm.

Sulfate was measured by HPLC using a Vydac-anion exchange column (302IC405, Mojave, USA) 250 mm x 4.6 mm inner diameter, which was temperature-controlled at 20 °C. Sulfate was detected with a Waters 431 conductivity detector (Waters Millipore, The Netherlands).

As liquid phase a potassium biphthalate solution (0.018 M) was used and a flow of 1.2 ml min^{-1} was applied.

Headspace contents of oxygen, carbon dioxide and nitrogen were measured by gas chromatographic technique. A sample (100 µl) was injected, with a pressure-locked gas syringe (Dynatech Precision Sampling Corporation, Louisiana, USA), in a Packard Becker 433 chromatograph (Delft, The Netherlands) gas chromatograph. Two parallel-connected columns (split 1:1), one with a 1.2 m × 2 mm steel column packed with molecular sieve 5A (mesh 60-80) and the other with Teflon packed Chromsorb (1.5 m × 2 mm) column were used to separate the gasses. The temperatures of the column, injection port and the flame ionization detector were 40, 110, 125°C, respectively. Helium was used as the carrier gas (20 ml min^{-1}). All gas samples analyses were conducted after calibration with standards of known amounts. Chemical oxygen demand (COD) and volatile suspended solids (VSS) were determined according to the Standard Methods (APHA 1985).

5.2.5 Bioreactor

The bioreactor was originally used as the aerobic post treatment to degrade aromatic amines formed from the azo dye reduction of the sulfonated azo dye Mordant Yellow (MY10). This azo dye was treated in a sequential anaerobic/aerobic setup. The aerobic reactor was successfully bioaugmented with a 4-ABS-degrading enrichment culture derived from Rhine sediment (Chapter 4). For further investigation the aerobic reactor was disconnected from the anaerobic reactor 146 days after bioaugmentation occurred and further fed with the three ABS isomers. The aerobic reactor had a volume of 1.0 l and aerated with compressed air with a flow of 75 l h^{-1}. Resulting in oxygen concentration in the reactor of 3.0-3.5 mg l^{-1}. For the sludge retention the aerobic reactor was connected to a settler (0.3 l) in order to return the sludge to the reactor. The flow from this settler varied during the experiment due to incidental failures in the sludge return. The operational parameters of the aerobic reactor are shown in Table 5.1.

Table 5.1 Operational parameters of the aerobic bioreactor fed with ABS isomers (values are averages during the period).

Period	(days)	2-ABS (mg l^{-1})	3-ABS (mg l^{-1})	4-ABS (mg l^{-1})	ABS (mg l^{-1})	HRT (hours)
I	0-104	-	-	150	150	8.8
II	105-155	96	-	110	206	10.7
III	156-189	76	51	79	206	14.9
IV	190-294	150	-	111	261	7.7
V	295-380	204	-	160	364	3.3

5.2.6 Assays

Aerobic biodegradation Aerobic biodegradation experiments were described previously (Chapter 3). As inoculum for the aerobic degradation experiment, Rhine sediment (5-10% w/v) or activated sludge from Aarle-Rixtel (5-10% v/v) were used. The following sulfonated aromatic amines, 2-ABS, 3-ABS, 4-ABS, 2,4-DABS, 1-AN-4-S, 1-AN-5-S, and 2-AN-1,5-DS, were tested. The target concentrations applied in the biodegradation experiments were 100 mg l^{-1} of sulfonated aromatic compound and were daily analyzed via UV measurements. Mineralization was followed in time by measuring oxygen uptake, carbon dioxide production and sulfate production. For these experiments the modified Feigel medium (Feigel and Knackmuss 1988) was used and experiments were conducted in duplicate.

Anaerobic biodegradation Anaerobic biodegradation experiments were described previously (Chapter 3). Granular sludge was added to a 117 ml batch bottle together with basal medium (25 ml) containing the sulfonated aromatic amine (100-200 mg l^{-1}). The bottles were flushed with N_2 / CO_2 (70% / 30%) and were stored on a shaker (50 strokes per minute) in a temperature controlled room (30 °C). The sulfonated aromatic amines, 2-ABS, 3-ABS, 4-ABS, 2,4-DABS, and 3-A-4-HBS, were tested with Shell granular sludge (1.0-2.0 g VSS l^{-1}) as inoculum source. The sulfonated aromatic amines, 2-ABS, 3-ABS, 4-ABS, 2,4-DABS, 1-AN-4-S 1-AN-5-S, and 3-A-4-HBS were tested with Nedalco granular sludge (1.0-2.0 g VSS l^{-1}). Methane was measured as parameter for biodegradation and compared with methane production in control batches without addition of sulfonated aromatic amine. The anaerobic biodegradation experiments were conducted in triplicate.

Anaerobic toxicity The anaerobic toxicity assay was described previously (Chapter 2). Similar batches were used as in the anaerobic biodegradation assay. Nedalco granular sludge (2.0 g VSS l^{-1}) was incubated with different concentrations of sulfonated aromatic amines. The highest concentration applied was 1.0 g l^{-1} of sulfonated aromatic amine. After three days the batches were flushed again with N_2 / CO_2 (70% / 30%) and acetate (2.0 g COD l^{-1}) was added. The acetoclastic methanogenic activity was measured and compared with incubations without addition of sulfonated aromatic amines. The sulfonated aromatic amines tested were 4-ABS, 1-AN-4-S, 1-AN-5-S, and 8-ANOH-3,6-DS. The anaerobic toxicity experiments were conducted in triplicate.

Aerobic toxicity Aerobic toxicity of sulfonated aromatic amines to activated sludge from Aarle-Rixtel was measured with oxygen uptake experiments. Prior to the measurements, the sludge was aerated overnight to oxidize as much as possible of the readily available substrate. Bottles of 294 ml were filled with 275 ml of sludge. The concentration of dissolved oxygen (DO) was followed in time with an oxygen electrode (WTW, Ochten, The Netherlands) and measured with an Microporcessor Oximeter 196 (Weilheim, Germany). The closed bottles

were mixed with a magnetic stirrer and maintained at a temperature of 20 ± 2 °C. To correct for the endogenous respiration, the DO initially was first measured without any substrate available. Afterward, 1.5 ml acetate solution (10.0 g l^{-1}, pH 7.0) and 12.0 ml of a sulfonated aromatic amine solution (25.0 g l^{-1}, pH 7.0) were added. The final concentration of the sulfonated aromatic amine was 1.0 g l^{-1}. Subsequently, the oxygen uptake in the bottle was monitored for 20 minutes and compared with the oxygen uptake rate without addition of the sulfonated aromatic amine. The sulfonated aromatic amines tested were 2-ABS, 3-ABS, 4-ABS, 2,4-DABS, 1-AN-4-S, and 3-AN-2,7-DS. All the experiments were conducted in triplicate.

5.2.7 Kinetic parameters

The kinetic parameters of the sludge in the aerobic reactor were determined with a respirometer. In a 1.2 l bottle oxygen concentrations were measured with an oxygen probe (WTW, Ochten, The Netherlands). The oxygen concentration was computer controlled, if the concentration became below 3.0 mg l^{-1} compressed air was blown (500 ml min^{-1}) into the bottle until a concentration of 6.0 mg l^{-1} was reached. The pH was controlled between 6.0 and 8.0. The respiration bottle was filled with 950 ml of effluent of the reactor and 2.0 ml NH_4Cl solution (48.0 g l^{-1}) as a nitrogen source. Furthermore, 1.0 ml of a micro nutrient solution was added and 30 ml of sludge (4.12 gr VSS l^{-1}). As sole organic carbon source 2-ABS was supplied as an aliquot of 100 ml at with a concentration of 5.0 g l^{-1}. The respiration rate was calculated from the course of the DO, which was logged onto a computer. Periodically, samples were taken to determine the concentration of the sulfonated aromatic amines and sulfate.

The oxygen consumption rate (OUR), 2-ABS and sulfate analyses were used to estimate the parameters of a (growth) Monod model, i.e. the biomass yield (Y), the substrate affinity constant (K_s) and the maximum specific growth rate (μ_{max}). In this model additional oxygen consumption by chemical oxidation of the mineralization sulfate was taken into account. This experiment was conducted four-times and average values were of the parameters were calculated.

5.3 Results

5.3.1 Biodegradation experiments

Under anaerobic conditions none of the tested compounds (2-ABS, 3-ABS, 4-ABS, 2,4-DABS, 1-AN-4-S, 1-AN-5-S and 3-A-4-HBS) were degraded by the two inoculum sources tested after periods ranging from 100 to 180 days. Additionally, 3-A-4-HBS was studied in a batch anaerobic re-circulation reactor and no degradation was observed after 117 days based on HPLC measurements (unpublished).

Under aerobic conditions, of all the sulfonated aromatic amines tested (2-ABS, 3-ABS, 4-ABS, 2,4-DABS, 1-AN-4-S, 1-AN-5-S, and 2-AN-1,5-DS) as is shown in Table 5.2 only 2- and 4-ABS were readily degraded. Decrease of UV at the absorbance maximum, high sulfate recovery, good carbon dioxide recovery, and significant uptake of oxygen clearly demonstrated the mineralization of 2- and 4-ABS. Aerobic degradation was observed with the two inoculum sources tested (Rhine sediment and Aarle-Rixtel sludge). Aerobic biodegradation of 4-ABS was also tested with no pre-adapted inoculum sources such as humus soil layer, polluted Harbor sludge, and activated sludge from a plant treating domestic wastewater but no degradation was observed.

In one replicate of a duplicate batch experiment, degradation of 3-ABS was observed. This was proven by high sulfate and carbon dioxide recovery and oxygen uptake (Table 5.2). Further readdition of substrate and enrichments did not show any continued degradation of 3-ABS and therefore, degradation of this compound remains uncertain.

Table 5.2 Aerobic biodegradability of selected sulfonated aromatic amines.

Inoculum	Rhine sediment			Aarle-Rixtel sludge		
	Recovery	Uptake O_2	Recovery CO_2	Recovery	Uptake O_2	Recovery CO_2
Compound	$S\text{-}SO_4^{2-}$ (% S)	(% COD)*	(% COD)*	$S\text{-}SO_4^{2-}$ (% S)	(% COD)*	(% COD)*
2-ABS	120 ± 13	62	48	108 ± 3	68 ± 19	61 ± 8
3-ABS$^+$	98	52	52		-	
4-ABS	104 ± 13	45 ± 25	34 ± 14	125 ± 6	65 ± 20	52 ± 25
2,4-DABS		-			-	
1-AN-4-S		-			-	
3-AN-2,7-DS		-			-	
2-AN-1,5-DS		-			-	

- No degradation observed. * Corrected for the oxygen uptake and carbon dioxide production due to endogenous substrate respiration. $^+$ Degradation occurred only in one replicate of a duplicate batch experiment.

5.3.2 Toxicity experiments

The anaerobic and aerobic toxicity experiments did not show any toxicity of the sulfonated aromatic amines tested (anaerobic 4-ABS, 1-AN-4-S, 1-AN-5-S, and 8-ANOH-3,6-DS; aerobic 2-ABS, 3-ABS, 4-ABS, 2,4-DABS, 1-AN-4-S, and 3-AN-2,7-DS). Even at concentrations up to 1000 mg l^{-1}, no inhibition was observed. These concentrations are in excess of those expected in wastewater discharges. Therefore, the sulfonated aromatic amines would not be expected to have an inhibitory effect on sequential anaerobic/aerobic treatment system used to treat sulfonated azo dye containing wastewaters.

5.3.3 Bioreactor

The aerobic bioreactor in this study was originally used as the aerobic post treatment in a sequential anaerobic/aerobic bioreactor that treated the sulfonated azo dye MY10. This reactor was exposed to 4-ABS, one of the degradation products of MY10. However, 4-ABS was only degraded after bioaugmentation with a 4-ABS degrading enrichment culture derived from Rhine sediment (Chapter 4). Afterwards, the anaerobic stage was disconnected and the aerobic stage was used for the continuous degradation of sulfonated aromatic amines. The main results of the aerobic bioreactor are shown in Figure 5.2. This figure is divided into four graphs, which will be discussed separately.

In Figure 5.2A, the load of the ABS isomers and the HRT are shown. During period I, 4-ABS was fed to the reactor. In period II, 2-ABS was added together with 4-ABS. During period III, 3-ABS was fed to the reactor together with in the other ABS isomers (Table 5.1). Finally, in the last two periods, 2- and 4-ABS were fed at increasing loading rates to determine the maximum loading rate.

Figure 5.2B indicates the removal efficiencies of the reactor. Since the reactor was originally bioaugmented with a 4-ABS-degrading enrichment culture, degradation of this compound was expected and observed in period I. Due to problems with sludge settling at the end of period I, lower removal efficiencies were observed. Therefore, the settler configuration was adjusted by using higher flow rates and adjusting the tubing, and this resulted in a better removal of 4-ABS in period II and III. In period II, 2-ABS was also added to the influent. Thirty days after starting this addition, degradation of 2-ABS was clearly shown. These results were confirmed during batch experiments with sludge from the bioreactor and with the original enrichment culture used to bioaugment the reactor (results not shown). Surprisingly, the original bioaugmented 4-ABS-degrading enrichment culture was still able to degrade 4-ABS and could also degrade 2-ABS after 2 years of storage in the refrigerator (4 °C).

Introduction of 3-ABS in period III did not result in degradation of 3-ABS. Also this result was confirmed in batch experiments with bioreactor sludge and the original enrichment culture. Bioreactor sludge and the original bioaugmented 4-ABS-degrading enrichment

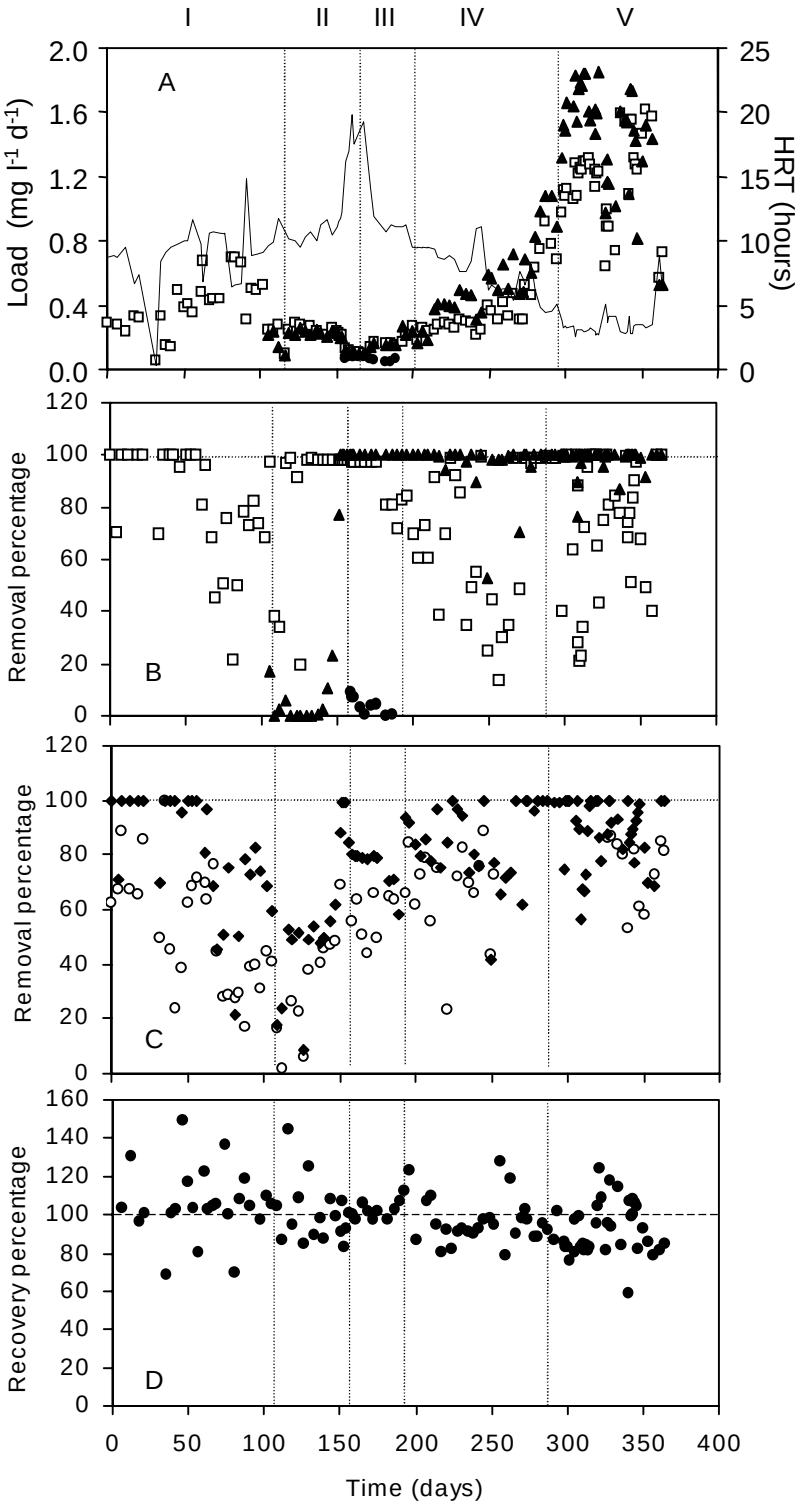

Figure 5.2A-D *A) Loading rate of 2-ABS (▲), 3-ABS (●), and 4-ABS (□) and hydraulic retention time (HRT, —); B) Percentage removal of 2-ABS (▲), 3-ABS (●), and 4-ABS (□); C) COD removal percentage measured (◆) and calculated (○); D) Percentage of sulfate recovery (●) and average (---).*

culture were not able to degrade 3-ABS (results not shown). In period IV and V only 2- and 4-ABS were added in the reactor. During the period IV, the HRT was decreased and during period V the influent concentrations of these compounds were gradually increased from 80 to

200 mg l^{-1}. High removal efficiencies of 2- and 4-ABS were still observed. The maximum degradation rates achieved were around 1.6-1.8 g ABS l^{-1} d^{-1} (Figure 5.2A) for both compounds. Sometimes the lower removal efficiencies of 4-ABS were observed during period IV and V. These lower efficiencies were again due to problems occurring in the settler, which were most probably aggravated by the decrease of the HRT. The 4-ABS degradation was more affected by settler problems than 2-ABS degradation.

Figure 5.2C gives the COD removal of the reactor. The measured and calculated COD removal during the five periods is depicted in this figure and shows the same trend. The calculated COD removal was determined by the difference in the theoretical influent and effluent COD concentrations of the ABS isomers. The measured COD sometimes was lower, indicating occasional accumulation and discharge of degradation intermediates. The batch degradation experiments of 2- and 4-ABS (Table 5.2) indicated a complete mineralization of the isomers. The desulfonation of 2- and 4-ABS in the reactor was also observed and shown in Figure 5.2D. The average sulfate recovery, a mineralization product 2- and 4-ABS, was high (99 ± 19%) during all 5 periods.

The bioreactor showed high removal efficiencies even at a total ABS concentration of 364 mg l^{-1} and at an HRT of 2.8-3.3 hours generally complete removal could be achieved. The maximum degradation rate achieved during the experiments was 1.6-1.8 g l^{-1} d^{-1} for 2-ABS and 1.3-1.5 g l^{-1} d^{-1} for 4-ABS at the similar low HRTs.

5.3.4 Kinetic parameters

The OUR, 2-ABS and sulfate analyses during the kinetic assay are shown in Figure 5.3, together with the model fit according to a (growth) Monod model. Figure 5.3 clearly showed the mineralization of 2-ABS by formation of the mineralization product sulfate and the increase in OUR. Although the model seems to fit the measurements reasonably well, the initial linear increase of the OUR cannot be explained by growth alone as this would have resulted in an exponential increase of the OUR. An explanation for this phenomenon could lay in a shortage of a trace metal or a nutrient for the microorganisms or mass transfer limitation of oxygen to the bacteria. Therefore, the parameter values which were estimated (Y=0.17 mg VSS mg^{-1} COD, K_s=28 mg COD l^{-1} and μ_{max}= 0.077 h^{-1}) cannot be considered reliable.

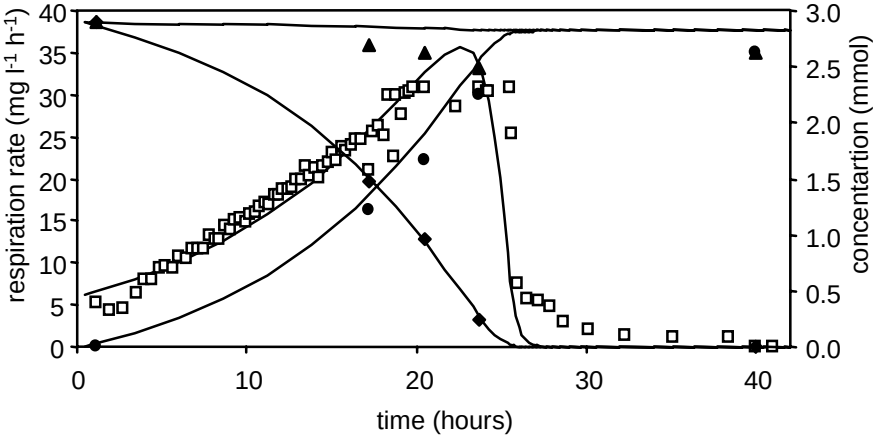

Figure 5.3 *Respiration rate (□) of 2-ABS degrading culture together with the removal of 2-ABS (◆) and sulfate production (●) and mass balance (▲) based on 2-ABS and sulfate concentration (lines represents the model fit).*

5.4 Discussion

The aim of this study was to investigate which sulfonated aromatic amines are readily degraded by natural mixed cultures. The results from this study showed that natural bacterial populations generally are not able to readily degrade many sulfonated aromatic amines. Therefore, many of these compounds will accumulate in the environment and it is not surprising that these compounds can be found in surface waters where they are a substantial polluting factor (Lange et al. 1995; Malle 1978; Zerbinati et al. 1994; Zerbinati et al. 1997).

None of the sulfonated aromatic amines tested were degraded anaerobically. This is in agreement with the limited experimental data available in the literature (Kuhn and Suflita 1989, Razo-Flores et al., 1996). However, there are a few reports of other sulfonated aromatic compounds being degraded under anaerobic conditions. For example 4-sulfobenzoate was eliminated under sulfate reducing conditions (Kuhn and Suflita 1989). Furthermore, there is evidence that linear alkylbenzenesulfonates and other sulfonated aromatic are utilized and degraded as a sulfur source under sulfur limiting anaerobic conditions (Cook 1998; Denger et al. 1996; Jensen 1999; Prats et al. 1999).

Aerobic degradation of 2- and 4-ABS isomers was clearly demonstrated in batch experiments as well in bioreactor experiment. However, aerobic degradation of 3-ABS could not be consistently observed during these experiments. Aerobic degradation of these compounds and 3-ABS has been described in literature before most probably due to usage of other inoculum sources (Feigel and Knackmuss 1988; Thurnheer et al. 1986). The degradation of 4-ABS was possible by a co-culture of two stains, *Hydrogenophaga palleronii* and *Agrobacterium radiobacter*, that collaborated to mineralize this compound (Feigel and Knackmuss 1988; Feigel and Knackmuss 1993). A similar co-culture was obtained during our enrichment

experiments (unpublished). The fact that two strains are involved in the degradation of 4-ABS could indicate that it is not easy to mineralize this compound.

Degradation of the sulfonated aromatic amines, in this study, was only observed with inoculum sources that were historically polluted with sulfonated aromatic amines. Therefore, the conclusion can be made that inoculation source, which have been exposed to these compounds, such as the Rhine (Malle 1978) and activated sludge from Aarle-Rixtel (treating textile wastewater), are a good source for specialized xenobiotic degradation microbial activity. Similar results were obtained for a bioreactor treating 3-nitrobenzenesulfonic acid and 3-ABS, in which degradation occurred only with activated sludge originating from a plants treating industrial wastewater (Kolbener et al. 1994).

Biodegradation of aminonaphthalenesulfonic acids was not found in this study. From previous studies the only aminonaphthalenesulfonic acids reported to be aerobically degraded so far are amine and sulfonic acid substituents located next to each other or both on different rings (Tan and Field 2000). The two aminonaphthalenesulfonic acids tested in this study did meet these criteria but also had an extra sulfonated group as substituent, which may have been the reason for their recalcitrance.

Both anaerobic and aerobic toxicity experiments were conducted at fairly high concentrations of sulfonated aromatic amine (1.0 g l^{-1}). Due to the presence of a sulfonic acid group the sulfonated aromatic amines are highly soluble and therefore, these compounds are not easily taken up and therefore do not show any toxic effect. For the same reason sulfonated aromatic amines are less genotoxic and carcinogenic than their unsulfonated analogues (Jung et al. 1992). The results obtained indicate that sulfonated aromatic amines do not harm the microorganisms involved in the anaerobic and aerobic treatment systems, which are required for the biodegradation of sulfonated azo dyes and other wastewater components. However, biodegradation is also not commonly present.

The continuous aerobic bioreactor used in this study was able to successfully degrade 2- and 4-ABS. However, this system was not able to degrade 3-ABS. The ability to degrade these two ABS isomers remained in the reactor for a long period (380 days). To our knowledge this is the first bioreactor with natural bioaugmented enrichment culture that is able to degrade these compounds (Tan and Field 2000). Only Thurnheer et al. (1988), who operated a chemostat at high HRT inoculated with five isolated cultures, could degrade seven benzenesulfonic acids, including the three ABS isomers. However, this chemostat is not comparable with the activated sludge system tested during these experiments, which is more realistic to wastewater treatment systems.

The fact that the original bioaugmented enrichment culture was also able to degrade 2- and 4-ABS after two years of storage in a refrigerator was surprising. This culture was originally

enriched on 4-ABS. Thus storage for two years did not affect the ability of this culture to degrade 4-ABS. The ability of this enrichment culture to degrade of 2-ABS was not tested before storage and showed to be new.

The kinetic assay that was used, and based on substrate, product and OUR measurements, could not be used to estimate kinetic parameters, probably due to a shortage of a trace metal or a nutrient or mass transfer limitation of oxygen. It may be better to obtain these parameters from classical experiments in which the sludge retention time of a continuous bioreactors is varied and effluent substrate concentrations are measured (Tchobanoglus and Burton 1991). However, these experiments clearly showed an increase in OUR coupled with the removal of 2-ABS and production of mineralization product sulfate.

Ruff et al. (1999) showed that 14 sulfonated aromatic compounds, five of which were sulfonated aromatic amines, were degraded by a mixed culture under sulfur limited conditions. Therefore, they presumed that the capacity to degrade these compounds extensively is widespread in the environment. However, this paper showed contradictory results. The reason for this may be a lack of sulfur limitations in the in sediments or wastewater treatment environments.

This research clearly showed that 2- and 4-ABS were degraded aerobic conditions. However, biodegradation of other sulfonated aromatic amines was not possible under aerobic and anaerobic conditions. These compounds showed no acute toxic effect for activated sludge and were not toxic for anaerobic granular sludge. Therefore, it is expected that many of these compounds have no negative influence and remain untreated in treatment systems. Since many different types of sulfonated azo dyes are currently be utilized, a wide variety of sulfonated aromatic amines will be formed under anaerobic conditions that will not easily be biodegraded and will constitute an important part of the untreated COD fraction in sulfonated azo dye containing wastewater after treatment.

6

Summary and discussion

6.1 Summary

Azo dyes constitute a major class of environmental pollutants accounting for 60 to 70% of all dyes and pigments used (Carliell et al. 1998; Zollinger 1987). These compounds are characterized by aromatic moieties linked together with an azo group (-N=N-). The release of azo dyes into the environment is a concern due to coloration of natural waters and due to the toxicity, mutagenicity and carcinogenicity of the dyes and their biotransformation products (Chung and Cerniglia 1992; Chung et al. 1981; Reid et al. 1984; Rosenkranz and Klopman 1989; Rosenkranz and Klopman 1990).

Therefore, considerable attention has been given to evaluating the fate of azo dyes during wastewater treatment and in the natural environment. The review of the literature (Chapter 1) revealed that azo dyes are generally persistent to aerobic degradation. On the other hand, the azo bond is readily reduced under anaerobic conditions by microorganisms provided with an electron donating co-substrate, yielding aromatic amines. Since the aromatic amines are colorless, the reduction process is associated with the decolorization of the dye.

The aromatic amines generally resist any further biodegradation in anaerobic environments. However, most types of aromatic amines are susceptible to mineralization by aerobic bacteria. Based on these findings, the complete biological mineralization of azo dyes will generally require anaerobic and aerobic phases of degradation (Figure 6.1)(Field et al. 1995).

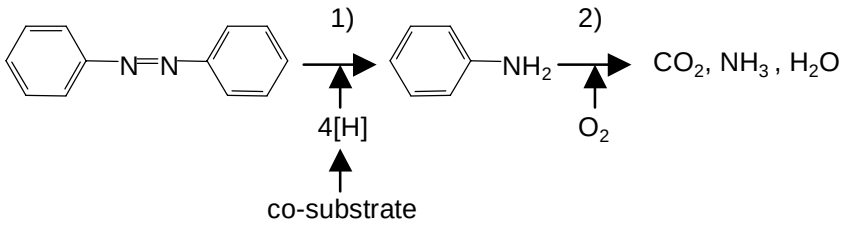

Figure 6.1 *Mineralization of an azo dye with the anaerobic azo dye reduction (1) and the aerobic oxidation of the aromatic amine (2) to their mineralization products.*

The aim of this dissertation was to explore methods of obtaining complete biodegradation of azo dyes in bioreactor systems that combine anaerobic and aerobic phases. In this research, two approaches were considered. The first approach, indicated as integrated anaerobic/aerobic treatment, evaluated the potential of combining the anaerobic and aerobic phases in one bioreactor concurrently. This integration of the two different redox conditions was achieved by addition of oxygen to anaerobic granular sludge, enabling an aerobic zone in the bulk liquid phase and outer periphery of the granules and an anaerobic zone inside the granules. The second approach, indicated as sequential anaerobic/aerobic treatment, was achieved by

first treating azo dyes in an upflow anaerobic sludge blanket (UASB) reactor, followed by treatment of the biotransformation products in an aerobic activated sludge reactor.

For these studies, several model azo dye compounds were use. Their chemical structures are shown in Figure 6.2 (Mordant Orange 1 (MO1), Mordant Yellow 10 (MY10) and 4-phenylazophenol (4-PAP)). The expected biotransformation products from these dyes are several aromatic amines. The chemical structure of these compounds are shown in Figure 6.2 (5-aminosalicylic acid (5-ASA), 1,4-phenylenediamine (1,4-PDA), 4-aminobenzenesulfonic acid (4-ABS), 4-aminophenol (4-AP) and aniline).

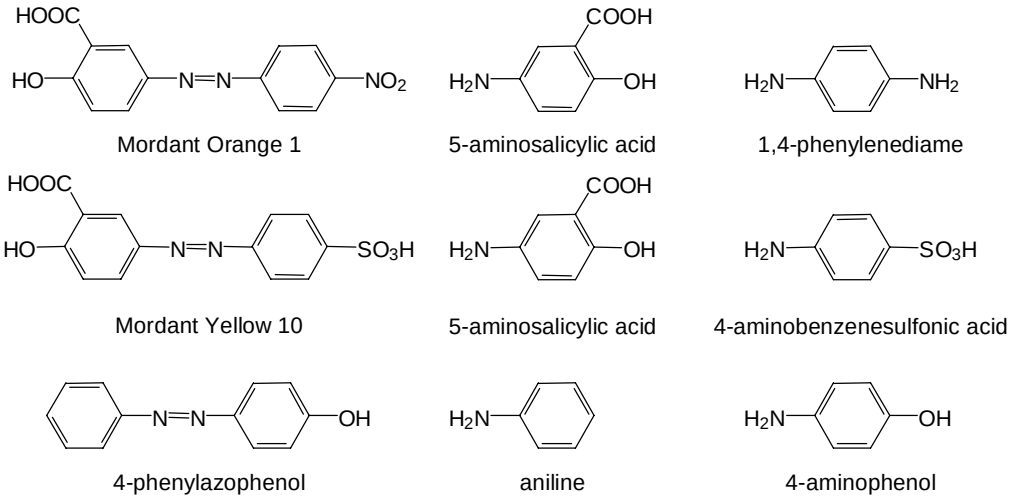

Figure 6.2 *Azo dyes and aromatic amines tested during this research.*

6.1.1 Azo reduction in integrated anaerobic/aerobic systems

In Chapter 2, the potential of azo dye reduction in integrated anaerobic/aerobic bioreactor systems was evaluated by measuring the impact of oxygen exposure to granular sludge on the rate of the azo dye MO1 reduction. The effect of different co-substrates on the oxygen tolerance and azo dye-reduction rates was evaluated.

Without the addition of oxygen under anaerobic conditions, co-substrate acts as an electron donor and the azo dye as an electron acceptor (Figure 6.1). Azo dye-reduction rates can be highly influenced by the co-substrate used (Nigam et al. 1996; Razo-Flores et al. 1997; Van der Zee et al. 2000a). Under these conditions, the results clearly show that ethanol accelerated the azo dye-reduction rate significantly compared to acetate due to production of interspecies hydrogen that was used for the reduction of the azo dye (Razo-Flores et al. 1997). The addition of acetate as co-substrate had the same effect as no addition, indicating that electrons were donated by endogenous substrate and not by acetate.

In the presence of oxygen and without co-substrate, azo dye reduction will not occur due to the absence of electron donor and oxidation of the endogenous substrate. If oxygen is present, a co-substrate is required for azo dye reduction. The co-substrate acts as electron donor for

azo dye reduction and it furthermore creates anaerobic microniches in which the azo dye reduction can take place. Due to the low diffusion rate of oxygen into the biofilm compared to that of the co-substrate, oxidation of co-substrate by facultative bacteria will proceed in the periphery of the biofilm and anaerobic microniches are created inside the biofilm. In this way, both anaerobic and aerobic processes take place next to each other (Bishop and Yu 1999; Nielsen et al. 1990) (Figure 6.3). The azo dye-reduction rate of MO1 remained high but decreased by additions of increasing amounts of oxygen when ethanol was used as co-substrate. The other co-substrate, acetate, only facilitates the formation of anaerobic microniches in the presence oxygen. However, the azo dye-reduction rate maintained at the same low level due to the presence of electron donating endogenous substrate if increasing amounts of oxygen were added. The presence of anaerobic microniches was confirmed by the presence of the anaerobic process of methane production during exposure to oxygen. Since ethanol gave high azo dye-reduction rates, both in the presence of oxygen and under anaerobic conditions, this co-substrate was used for further investigations during this Ph.D. study.

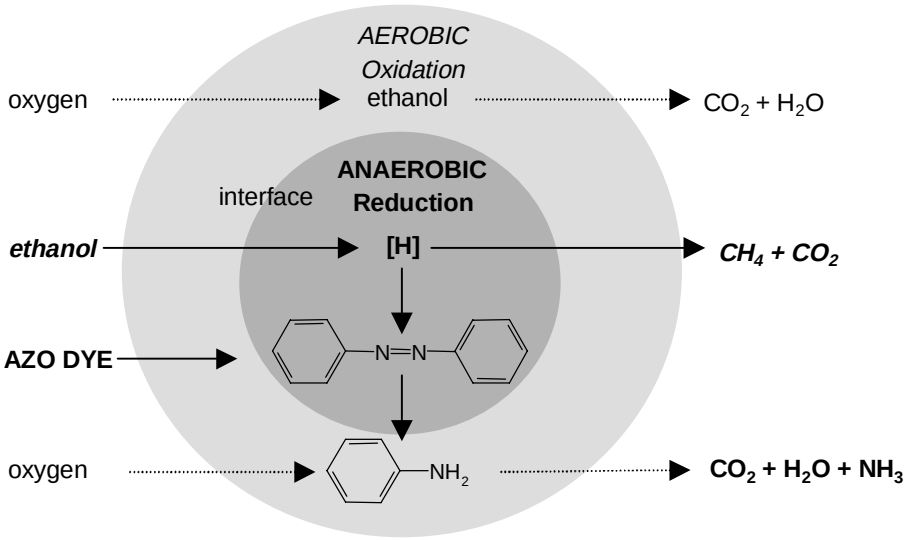

Figure 6.3 *Theoretical representation of the mineralization of an azo dye under integrated anaerobic/aerobic conditions, with inside anaerobic and outside aerobic conditions.*

6.1.2 Mineralization of azo dyes in integrated anaerobic/aerobic systems

In Chapter 3, the integrated anaerobic/aerobic treatment concept for the mineralization of the azo dyes MY10 and 4-PAP at different initial oxygen concentrations was evaluated in batch systems using granular anaerobic sludge as biomass and ethanol as co-substrate. The concept of the integrated anaerobic/aerobic degradation of azo dyes is depicted in Figure 6.3. Since the influence of oxygen on the azo dye reduction was tested in Chapter 2, special attention

was paid to the aerobic biodegradation of the formed aromatic amines 4-AP, aniline, 5-ASA and 4-ABS.

Both azo dyes, MY10 and 4-PAP, were readily reduced under the integrated anaerobic/aerobic conditions applied. Under these conditions, the azo dye reduction products of 4-PAP, aniline and 4-AP, were readily removed if sufficient oxygen was available. However, since 4-AP is eliminated under sterile conditions when exposed to air, this compound was most probably removed via autoxidation (Haller 1978). Aniline was mineralized under aerobic conditions because in aerobic bioassays with granular sludge and aniline as sole substrate, stoichiometric amounts of oxygen were used.

The reduced products of MY10 azo dye reduction, 4-ABS and 5-ASA, behaved differently. During the integrated anaerobic/aerobic biodegradation experiments of MY10, insufficient amounts of oxygen were available. The amount of co-substrate compared to the amount of oxygen was not in balance. An excess addition of co-substrate oxidized all the available oxygen. Subsequently, insufficient oxygen was available for the degradation of the aromatic amines. After readdition of oxygen, fast aerobic degradation of 5-ASA was observed. As extra oxygen was added during these experiments, one could argue if real integrated anaerobic/aerobic conditions were present.

In contrast to 5-ASA, the sulfonated aromatic amine 4-ABS accumulated indicating that this compound was not initially degraded. Consequently, various inoculum sources were screened for 4-ABS degradation, resulting in the cultivation of an aerobic 4-ABS-degrading enrichment culture derived from Rhine sediments. Addition of the 4-ABS-degrading enrichment culture to the granular sludge, a procedure known as bioaugmentation, resulted in the complete mineralization of MY10 in batch and bioreactor experiments. However, for successful mineralization of the accumulated 4-ABS intermediate, high amounts of oxygen were required: 4-ABS was only degraded at the highest oxygen levels if additional oxygen was applied. By comparison, lower oxygen levels supported the degradation of 5-ASA. Autoxidation of 5-ASA was also observed since 5-ASA was slowly removed in sterile control batches exposed to air (Jensen et al. 1993; Jensen et al. 1992). However, active batches showed a faster removal, which indicates that 5-ASA removal was primarily via biodegradation.

In the course of this Ph.D. study, several integrated anaerobic/aerobic bioreactor experiments were performed with MY10 and 4-PAP by supplying oxygen to the recirculation line of an expanded granular sludge bed (EGSB) reactor fed with ethanol as co-substrate. The results obtained were similar to those of the batch experiments in Chapters 2 and 3. The azo dyes were successfully reduced in the aerated EGSB reactors. Both aromatic amines released from the reduction of the azo dye 4-PAP were recovered if no oxygen was added to the EGSB

reactor. The aromatic amines were degraded (aniline) or removed by autoxidation (4-AP) if sufficient oxygen was available (Tan et al. 1999). During integrated anaerobic/aerobic EGSB reactor experiments with MY10, aerobic degradation of the reduced intermediate, 5-ASA, was observed. However, the other reduced intermediate, 4-ABS was degraded only after bioaugmentation of the 4-ABS-degrading enrichment culture. Since the 4-ABS-degrading enrichment culture required high amounts of oxygen, the oxygen supply had to be increased to obtain complete 4-ABS degradation. The increased oxygen supply, however, was unfavorable for the granular sludge structure. Filamentous biomass developed and the granular structure was no longer evident after continued operation. This resulted in excessive biomass washout and deterioration of the bioreactor performance (unpublished).

6.1.3 Mineralization of azo dyes in sequential anaerobic/aerobic systems

In Chapter 4, the sequential anaerobic/aerobic treatment concept for the mineralization of the azo dye MY10 was evaluated in a continuous bioreactor. The sequential anaerobic/aerobic conditions were easily applied by using in sequence an anaerobic bioreactor followed by a aerobic bioreactor. The anaerobic reactor was an EGSB type anaerobic reactor filled with granular sludge and the aerobic reactor consisted of an activated sludge reactor connected to a settler. Under the anaerobic conditions, the azo dye MY10 was reduced and all the co-substrate ethanol was anaerobically degraded. High recovery of both aromatic amines, 4-ABS and 5-ASA, in the effluent of the anaerobic bioreactor confirmed azo dye reduction. During the course of the experiments, the recovery of 5-ASA in the anaerobic effluent decreased. Anaerobic biodegradation experiments indicated that 5-ASA was anaerobically degraded (Razo-Flores et al. 1997). However, the 5-ASA-degradation rate was insufficient for complete 5-ASA mineralization in the anaerobic reactor. Therefore, aerobic post treatment for the removal of 5-ASA was required.

In this way, the substrate subsequently supplied to the aerobic activated sludge reactor mainly consisted of the reduced aromatic amines 5-ASA and 4-ABS. Bacterial activity for the mineralization of 5-ASA was readily developed. Directly after start of the operation, 5-ASA removal was nearly complete. However, the other aromatic amine 4-ABS was not degraded at all, even after an extended period of operation of 245 days. Therefore, the 4-ABS-degrading enrichment culture derived from Rhine River sediment was bioaugmented into the aerobic bioreactor. After bioaugmentation, 4-ABS degradation occurred immediately. The enrichment culture retained the ability to degrade 4-ABS for the entire duration of the extended operation of the aerobic bioreactor (1.5 years), which indicates successful incorporation of the 4-ABS-degrading enrichment culture into the activated sludge. Evidence that 4-ABS was mineralized was based on the high recovery of sulfate in the effluent of the aerobic reactor and in batch experiments with bioreactor sludge.

6.1.4 Biodegradability of sulfonated aromatic amines during wastewater treatment

A wide variety of sulfonated azo dyes used in the textile industry will release many different sulfonated aromatic amines. The biodegradation of sulfonated aromatic compounds is not commonly present in the environment (Chapter 1). In the previous chapters, it was observed that the degradation of the sulfonated aromatic amine 4-ABS only proceeded after bioaugmentation of a specialized enrichment culture (Chapter 3 and 4). Therefore, the ultimate biodegradability of the sulfonated aromatic amines is questionable. To investigate this matter, the fate of sulfonated aromatic amines with natural mixed cultures was evaluated in aerobic and anaerobic batch assays as well as in a continuous aerobic bioreactor (Chapter 5). The sulfonated aromatic amines tested were 2-ABS, 3-ABS, 4-ABS, 2,4-diaminobenzenesulfonic acid (2,4-DABS), 3-amino-4-hydroxybenzenesulfonic acid (3-A-4-HBS), 1-aminonaphthalene-4-sulfonic acid (1-AN-4-S), 1-aminonaphthalene-5-sulfonic acid (1-AN-5-S), 2-aminonaphthalene-1,5-disulfonic acid (2-AN-1,5-DS), 3-aminonaphthalene-2,7-disulfonic acid (3-AN-2,7-DS), and 8-aminonaphthol-3,6-disulfonic acid (8-ANOH-3,6-DS). Furthermore, the biodegradation of the three ABS isomers was tested in the aerobic activated sludge reactor used in the sequential anaerobic/aerobic conditions for MY10 (Chapter 4).

None of the tested sulfonated compounds was degraded under the applied anaerobic conditions. Furthermore, of the tested sulfonated aromatic amines only 2- and 4-ABS were aerobically mineralized. High sulfate recovery, oxygen uptake and carbon dioxide production clearly confirmed these findings. The other sulfonated aromatic amines tested with more complicated structures were not degraded by any of the inoculum sources used.

The 4-ABS-degrading enrichment culture, which was originally isolated from aerobic Rhine River sediments, was also responsible for 2-ABS biodegradation. Both 2- and 4-ABS were also degraded using activated sludge from a wastewater treatment plant treating domestic and textile wastewater. The Rhine and this wastewater treatment plant are historically exposed to sulfonated aromatic compounds. Biodegradation of 4-ABS was not observed with various other inoculum sources from unpolluted environments. Therefore, the conclusion can be made that biomass sources that are historically exposed to sulfonated aromatic compounds are a good source for sulfonated aromatic amines degrading microorganisms.

Only a few biotechnological approaches for the removal of sulfonated aromatic amines were found in literature (Chapter 1). Therefore, the behavior of these compounds was evaluated in an activated sludge reactor. This reactor was capable of degrading 4-ABS due to the originally bioaugmented 4-ABS-degrading enrichment culture (Chapter 4). Biodegradation of 2-ABS was observed a month after its addition, whereas 3-ABS was not degraded in this bioreactor. High sulfate recovery and good COD removal indicated mineralization of both ABS isomers.

Batch respiration experiments with bioreactor sludge and 2-ABS clearly showed an increasing oxygen uptake rate, which was coupled with 2-ABS degradation. Furthermore, sulfate production confirmed the mineralization of 2-ABS. In the bioreactor, the maximum degradation rates of 2- and 4-ABS were around 1.6 g l^{-1} d^{-1} at a HRT of 2.8-3.3 hours. As already was mentioned in Chapter 5, this high rate at low HRT implies that the 2- and 4-ABS degrading biomass was successfully incorporated in the activated sludge.

Furthermore, isolation and identification experiments on the 4-ABS-degrading enrichment culture were conducted (unpublished). The DNA of the enrichment culture was isolated and PCR was performed, amplifying the V6V8 region of the 16S rDNA. Amplicons were analyzed by denaturing gradient gel electrophoresis (DGGE is a DNA fingerprinting technique that is often used in the microbial ecology to show the composition of mixed bacterial cultures). DGGE analysis revealed that the 4-ABS-degrading enrichment culture consisted of two strains. Furthermore, after cloning and sequencing, a part of the 16S rRNA gene was matched to sequences of the NCBI database on internet (http://www.ncbi.nlm.nih.gov/blast/ blast.cgi?Jform=0). The co-culture consisted most probably of the strains *Hydrogenophaga* sp. and *Ochrobactrum* sp.. Surprisingly, the *Hydrogenophaga* sp. strain in our co-culture had a 98% similarity (rRNA basis) with the strains *Hydrogenophaga* sp. DSM 5680 from the 4-ABS degrading co-culture isolated in Germany (Feigel and Knackmuss 1993). This strain is responsible for the initial biodegradation of 4-ABS and forms 5-suflocatechol via a dioxygenase. The other strain of this co-culture (*Agrobacterium* sp.) uses this compound for growth (Dangmann et al. 1996). Most probably, the *Hydrogenophaga* sp. in our co-culture is also responsible for the initial degradation of 4-ABS. A literature search revealed that the other strain in our co-culture, *Ochrobactrum* sp., could also degrade other aromatic xenobiotic compounds (Lechner et al. 1995; Song et al. 2000). Unfortunately, no single strain was isolated out of the co-culture and therefore no further experiments were conducted with this 4-ABS-degrading enrichment culture.

The aerobic degradation of sulfonated aromatic amines was only limited to two of the three ABS isomers, 2- and 4-ABS. Degradation of these compounds was accomplished in batch experiments and in the activated sludge bioreactor. Other substituted ABS compounds and aminonaphthalenesulfonic acids (ANS) were not susceptible for aerobic biodegradation (Table 6.1). However, there are some specialized isolated cultures that are able to mineralize ANS compounds (Chapter 1). Future research should focus on finding suitable inoculum sources for the biodegradation of the substituted ABS and ANS compounds and showing successful bioaugmentation of the isolated ANS-degrading cultures in wastewater treatment plants.

As many different azo dyes contain the sulfonic acid group and many different sulfonated aromatic amines are formed after the anaerobic treatment of these dyes, a part of the remaining COD fraction of treated wastewater with sulfonated azo dyes will consist of the recalcitrant substituted ABS and ANS compounds. Fortunately, these compounds did not show any toxicity towards anaerobic granular sludge and aerobic activated sludge (Chapter 5) and are not as harmful as the unsulfonated aromatic amines (Chung and Cerniglia 1992; Jung et al. 1992; Levine 1991; Rosenkranz and Klopman 1990).

6.2 Discussion

6.2.1 Sequential versus integrated anaerobic/aerobic conditions

The aim of this dissertation was to explore methods to obtain the complete biodegradation of azo dyes in batch and bioreactor systems that combine anaerobic and aerobic phases. The integrated and sequential anaerobic/aerobic conditions were evaluated. Under both conditions, mineralization of azo dyes was achieved via anaerobic azo dye reduction followed by aerobic degradation of aromatic amines. Since in this thesis, both integrated and sequential anaerobic/aerobic conditions were applied with anaerobic granular sludge systems as biomass, a comparison will be made between both conditions.

The first step in the biodegradation of azo dyes is the anaerobic azo dye reduction. Under both integrated and sequential anaerobic/aerobic conditions, the azo dye reduction occurred. However, faster azo dye-reduction rates were observed under anaerobic conditions (Chapter 2). Under anaerobic conditions, co-substrate only donates electrons for the azo dye reduction whereas under integrated anaerobic/aerobic conditions, co-substrate also acts to create anaerobic microniches. The formation of anaerobic microniches occurs due to facultative biofilm bacteria that oxidize co-substrate. Facultative bacteria in the outer layers of the granular biofilm rapidly oxidize the co-substrate. As a result, the penetration of oxygen into the granular biofilm is limited and anaerobic conditions are maintained in the inside of the granule (Field et al. 1995). In actively respiring biofilms, the depth of oxygen penetration is lowered by increasing oxygen consumption rates (Kudlich et al. 1996; Kurosawa and Tanaka 1990) and the depth of oxygen penetration is limited to a few hundred μm (Costerton et al. 1994; De Beer et al. 1993; Hooijmans et al. 1990; Muller et al. 1994; Nielsen et al. 1990) and depends on the oxygen concentration in the bulk liquid phase.

Aerobic degradation of co-substrate under integrated anaerobic/aerobic conditions will usually give rise to deficiency of either oxygen or co-substrate. This negative effect will not occur under the sequential anaerobic/aerobic conditions. A deficiency of oxygen or co-substrate under integrated anaerobic/aerobic conditions is unfavorable for the mineralization of the azo dye. In Chapter 2 and 3, it was described that an excess of co-substrate caused a

shortage of oxygen resulting in the absence of aerobic degradation of the aromatic amines. Readdition of oxygen resulted in aerobic degradation of the aromatic amines (Chapter 3). Similar results were observed in an integrated anaerobic/aerobic granular sludge bioreactor in which strict aerobic methanotrophic bacteria were outcompeted for the oxygen by facultative anaerobic bacteria (Shen et al. 1996). An excess of oxygen may also result in shortage of electron donor for azo dye reduction. This was observed in the integrated anaerobic/aerobic EGSB bioreactor treating MY10 (described above). Too much oxygen resulted in a severe decline of the azo dye-reduction efficiency in the bioreactor. In view of these problems concerning co-substrate and oxygen in the integrated anaerobic/aerobic bioreactor, the conclusion can be made that separation of anaerobic and aerobic systems as in the sequential anaerobic/aerobic conditions is much easier to control.

There are some reports in which addition of oxygen to granular sludge under integrated anaerobic/aerobic conditions was shown possible and beneficial for biomass and COD removal. Under these conditions, an excess of substrate compared to oxygen was added. The substrate was degraded in a combination of anaerobic and aerobic processes. However, the oxygen was not required for a certain specific biodegradation such as aerobic degradation of the aromatic amines during integrated anaerobic/aerobic mineralization of azo dyes (Kato et al. 1993a; Kato et al. 1993b; Shen and Guiot 1995; Zitomer 1998; Zitomer and Shrout 1998).

In view of the advantages of sequential above integrated anaerobic/aerobic conditions, i.e. faster anaerobic azo dye reduction, better usage of co-substrate and oxygen, successful bioaugmentation and easier control of bioreactors, sequential anaerobic/aerobic conditions are recommended for the mineralization of azo dyes.

6.2.2 Fate of sulfonated and unsulfonated aromatic amines

In practice, many different azo dyes are used and therefore many different aromatic amines will be formed. The fate of the tested aromatic amines is shown in Table 6.1. Azo dye reductions generally occurs under anaerobic conditions and aromatic amines are not readily degraded under these conditions. Only some aromatic amines substituted with hydroxyl and carboxyl groups, like 5-ASA, are degraded under anaerobic conditions (Kalyuzhnyi et al. 2000; Kuhn and Suflita 1989; Razo-Flores et al. 1996; Razo-Flores et al. 1999; Razo-Flores et al. 1997). Also in this research (Chapter 4), anaerobic degradation of 5-ASA was observed. All the other tested sulfonated and unsulfonated aromatic amines were not degraded under the anaerobic conditions (Table 6.1). This clearly implies the requirement of an aerobic post treatment for anaerobically treated azo dyes containing wastewater.

Under aerobic conditions, two different removal processes can take place with aromatic amines. The first one is the biological degradation and the second one is a non-biological

removal via autoxidation. Both removal processes were observed during this research (Table 6.1).

Table 6.1 Removal process of different sulfonated and unsulfonated aromatic amines tested and described in this thesis.

Compound	**Removal process**		
	Anaerobic degradation	Aerobic degradation	Autoxidation
5-aminosalicylic acid	slow degradation	fast degradation	slow autoxidation
4-aminophenol	no degradation	no degradation	fast autoxidation
aniline	no degradation	fast degradation	no autoxidation
2-aminobenzenesulfonic acid	no degradation	specialized enrichment	no autoxidation
3-aminobenzenesulfonic acid	no degradation	in one occasion	no autoxidation
4-aminobenzenesulfonic acid	no degradation	specialized enrichment	no autoxidation
2,4-diaminobezenesulfonic acid	no degradation	no degradation	no autoxidation
1-aminonaphthalene-4-sulfonic acid	no degradation	no degradation	no autoxidation
3-aminonaphthalene-2,7-disulfonic acid	not tested	no degradation	no autoxidation
2-aminonaphthalene-1,5-disulfonic acid	not tested	no degradation	no autoxidation
1-aminonaphthalene-5-sulfonic acid	no degradation	not tested	not tested
3-amino-4-hydroxybenzenesulfonic acid	no degradation	not tested	not tested

The unsulfonated compounds, aniline and 5-ASA, were readily degraded by the biomass present in batch and bioreactor systems. Sulfonated aromatic amines were not readily aerobically degraded and require specialized enrichment cultures for their biodegradation. During this research, only degradation of 2- and 4-ABS was achieved by these specialized cultures. The other substituted ABS and ANS compounds were not aerobically degraded (Table 6.1). There is evidence that under sulfur-limited conditions many sulfonated aromatic compounds are biodegraded and used as sulfur source (Ruff et al. 1999). However, sulfur-limited conditions are unrealistic for wastewater treatment and will therefore not occur. A conclusion of this research is that substituted ABS and ANS compounds are not easily degraded under natural conditions and remain recalcitrant in wastewater treatment plants.

The other removal process of aromatic amines is via autoxidation. Autoxidation of two aromatic amines, 4-AP and 5-ASA, was observed during this research. Autoxidation is a process in which oxygen reacts via free radical mechanisms with the aromatic compound to form polymeric compounds. The aromatic amines are probably more susceptible to these kind of reactions than any other aromatic compounds (Field et al. 1995). Fast autoxidation of 4-AP and 2-AP was observed in aerobic biodegradation experiments of mono-substituted phenols

(Haller 1978). Due to the 4-AP structure in 5-ASA, 5-ASA will also undergo autoxidation reactions (Jensen et al. 1993; Jensen et al. 1992). Especially compounds with ortho and para substituted hydroxyl groups in aromatic amines are susceptible for these autoxidation reactions. Examples of these compounds are 1,2-aminonaphthol (observed during aerobic biodegradation experiments of this compound in our laboratory), 4-chloro2-aminophenol (Beunink and Rehm 1990) and even some sulfonated o-aminohydroxynaphthalenes (Kudlich et al. 1999). The formed polymeric autoxidation products are dark-colored and mainly non-biodegradable (Beunink and Rehm 1990; Haller 1978) and the autoxidation products of 5-ASA and some sulfonated o-aminohydroxynaphthalenes were characterized (Jensen et al. 1993; Jensen et al. 1992; Kudlich et al. 1999). Insoluble and non-biodegradable polymeric autoxidation products can easily be removed from the wastewater. However, there is still a remaining and unwanted recalcitrant COD fraction which has to be treated.

6.3 Concluding remarks and implications

Under integrated and sequential anaerobic/aerobic conditions, all the azo dyes (MO1, MY10 and 4-PAP) were decolorized. Furthermore, decolorization of azo dyes is possible under integrated anaerobic/aerobic conditions if an electron donating and anaerobic microniches creating co-substrate is available. For the removal of aromatic amines, different processes occurred such as anaerobic biodegradation (5-ASA),aerobic biodegradation (aniline, 5-ASA, 2- and 4-ABS) and autoxidation (4-AP and 5-ASA).

A sequential anaerobic/aerobic bioreactor is recommended for the biodegradation of azo dyes. The co-substrate is readily degraded and fastest azo dye reduction will occur under the anaerobic conditions. Under aerobic conditions, the aromatic amines will generally be biodegraded or removed via autoxidation processes. Also bioaugmentation of a specialized aerobic degrading culture can easily be achieved in an aerobic bioreactor. Successful bioaugmentation of the 4-ABS-degrading enrichment culture was achieved in the aerobic stage of the sequential anaerobic/aerobic bioreactor.

Only two sulfonated aromatic amines, 2- and 4-ABS, were aerobically degraded. The substituted ABS and ANS compounds tested were not aerobically degraded and none of the sulfonated aromatic amines were anaerobically degraded. Therefore, many sulfonated aromatic amines formed during reduction of sulfonated azo dyes are not susceptible to anaerobic or aerobic biodegradation. The effluent of treated wastewater with sulfonated azo dyes will still contain these recalcitrant sulfonated aromatic amines and further research is required on the biodegradation and removal of these recalcitrant sulfonated aromatic amines.

6.4 Samenvatting

Azokleurstoffen vormen een belangrijke groep van milieuverontreinigende verbindingen. 60 tot 70% van alle kleurstoffen en pigmenten die worden gebruikt bestaan uit deze azokleurstoffen (Carliell et al. 1998; Zollinger 1987). Deze verbindingen zijn aromatische ringstructuren die onderling verbonden zijn door zogenaamde azogroep (-N=N-). De emissie van deze azokleurstoffen is onwenselijk omdat deze stoffen de oppervlaktewateren kleuren en sommige van deze verbindingen en hun biodegradatieproducten toxisch, mutageen en carcinogeen zijn (Chung and Cerniglia 1992; Chung et al. 1981; Reid et al. 1984; Rosenkranz and Klopman 1989; Rosenkranz and Klopman 1990).

Veel aandacht is besteed aan de het lot van deze verbindingen tijdens afvalwaterzuivering en onder natuurlijke omstandigheden. De literatuurstudie, beschreven in Hoofdstuk 1, laat duidelijk zien dat azokleurstoffen meestal niet worden afgebroken onder aërobe omstandigheden. Daarentegen wordt de azogroep van deze kleurstoffen eenvoudig gereduceerd door anaërobe micro-organismen als er cosubstraat aanwezigheid is. Tijdens deze reductie worden aromatische aminen gevormd en omdat deze aromatische aminen kleurloos zijn, is azoreductie een ontkleuringsproces.

Aromatische aminen worden in het algemeen niet afgebroken onder anaërobe condities, maar de meeste aromatische aminen worden wel afgebroken door aërobe bacteriën. Daarom is er voor de volledige microbiologische afbraak van azokleurstoffen zowel een anaërobe als een aërobe degradatiefase nodig (Figuur 6.1)(Field et al. 1995).

Het doel van dit onderzoek was methoden te vinden voor de volledige biodegradatie van azokleurstoffen in verschillende bioreactorsystemen waarbij anaërobe en aërobe fases worden gecombineerd. Tijdens dit onderzoek zijn twee verschillende systemen onderzocht.

Ten eerste is de zogenaamde geïntegreerde anaërobe/aërobe afbraak van azokleurstoffen onderzocht. Hierbij vindt de afbraak van de azokleurstof in één bioreactor plaats waar zowel anaërobe als aërobe condities aanwezig zijn. De integratie van twee verschillende redoxcondities werd tijdens dit onderzoek tot stand gebracht door het toevoegen van zuurstof aan anaëroob korrelslib. Hierdoor ontstaan aërobe condities in de vloeistoffase en in de buitenste laag van de korrel. Binnenin de korrel blijft het anaëroob.

Ten tweede is de sequentiële anaërobe/aërobe afbraak van azokleurstoffen onderzocht. Dit werd bewerkstelligd door azokleurstoffen te behandelen in een reactorsysteem dat bestond uit een anaërobe bioreactor gevolgd door een aërobe actiefslib-bioreactor.

Voor dit onderzoek zijn verschillende model-azokleurstoffen onderzocht. De chemische structuurformules van deze verbindingen zijn weergegeven in Figuur 6.2 (Mordant Orange 1 (MO1), Mordant Yellow 10 (MY10) en 4-fenylazofenol (4-PAP)). De verwachte anaërobe omzettingsproducten van deze azokleurstoffen zijn de aromatische aminen die ook in Figuur

6.2 zijn weergegeven (5-aminosalicylzuur (5-ASA), 1,4-fenylenediamine (1,4-PDA), 4-aminobenzeensulfonzuur (4-ABS), 4-aminofenol (4-AP) en aniline).

6.4.1 Azoreductie in geïntegreerde anaërobe/aërobe systemen

In Hoofdstuk 2 is de mogelijkheid van de geïntegreerde anaërobe/aërobe bioreactor onderzocht door de invloed van zuurstof op de azoreductiesnelheid van MO1 door korrelslib te bepalen. In dit hoofdstuk is het effect van verschillende hoeveelheden zuurstof op de azoreductiesnelheid met verschillende cosubstraten onderzocht.

Zonder toevoeging van zuurstof, onder anaërobe condities, wordt het cosubstraat gebruikt als elektronendonor en treedt de azokleurstof op als elektronenacceptor (Figuur 6.1). De azoreductiesnelheid wordt in grote mate beïnvloed door het type cosubstraat dat wordt gebruikt (Nigam et al. 1996; Razo-Flores et al. 1997; Van der Zee et al. 2000a). De resultaten van de experimenten laten duidelijk zien dat ethanol in vergelijking tot acetaat een positief effect had op de azoreductiesnelheid. De reden hiervoor is dat bij de afbraak van ethanol “interspecies” waterstof wordt gevormd dat de azokleurstof reduceert. Dit is niet het geval bij acetaat (Razo-Flores et al. 1997). Toevoegen van acetaat geeft dezelfde reductiesnelheden als in het geval dat er geen cosubstraat wordt toegevoegd. Dit duidt erop dat de elektronen voor de reductie worden gedoneerd door het endogene substraat en niet door acetaat.

Zonder cosubstraat en in aanwezigheid van zuurstof treedt azoreductie niet op doordat er geen elektronendonor aanwezig is voor de azoreductie. Het aanwezige endogene substraat wordt door zuurstof geoxideerd. In aanwezigheid van zuurstof is er daarom een cosubstraat nodig om azoreductie te laten plaatsvinden. Het cosubstraat, de elektronendonor voor azoreductie, creëert tevens de zogenaamde anaërobe microniches waar de azoreductie plaatsvindt. Door de lage diffusiesnelheid van zuurstof in de biofilm in vergelijking met die van het cosubstraat, wordt het cosubstraat geoxideerd in de buitenste lagen van de biofilm door facultatieve bacteriën. Binnenin de biofilm blijft het hierdoor anaëroob en worden anaërobe microniches gevormd. Op deze manier kunnen anaërobe en aërobe processen zich naast elkaar afspelen in één en dezelfde biofilm (Bishop and Yu 1999; Nielsen et al. 1990) (Figuur 6.3). De azoreductiesnelheid van MO1 was hoog maar nam af met hogere concentraties aan zuurstof en met ethanol als cosubstraat. Acetaat, het andere geteste cosubstraat, stimuleert alleen de vorming van anaërobe microniches. In aanwezigheid van zuurstof en met acetaat als cosubstraat bleef de azoreductiesnelheid op hetzelfde niveau als met endogeen substraat. De aanwezigheid van anaërobe microniches werd bevestigd door de vorming van methaan in aanwezigheid van zuurstof. Omdat het cosubstraat ethanol hoge azoreductiesnelheden gaf, zowel in aanwezigheid van zuurstof als onder anaërobe condities, is dit cosubstraat verder gebruikt tijdens het promotieonderzoek.

6.4.2 Mineralisatie van azokleurstoffen in geïntegreerde anaërobe/aërobe systemen

In Hoofdstuk 3 is het geïntegreerde anaërobe/aërobe biodegradatieconcept voor de mineralisatie van de azokleurstoffen onderzocht. Biodegradatie van de azokleurstoffen MY10 en 4-PAP werd met behulp van anaëroob korrelslib en met verschillende initiële hoeveelheden zuurstof en ethanol als cosubstraat getest. Het concept van de geïntegreerde anaërobe/aërobe biodegradatie van azokleurstoffen is weergeven in Figuur 6.3. In Hoofdstuk 2 is de aandacht uitgegaan naar de invloed van zuurstof op de azoreductie en in Hoofdstuk 3 is de aandacht gevestigd op mineralisatie van de gevormde aromatische amines, 4-AP, aniline, 5-ASA en 4-ABS.

Beide azokleurstoffen, MY10 en 4-PAP, werden snel gereduceerd onder de toegepaste geïntegreerde anaërobe/aërobe condities. De azoreductieproducten van 4-PAP, aniline en 4-AP, werden tevens verwijderd onder deze condities als er voldoende zuurstof aanwezig was. De verwijdering van 4-AP is echter waarschijnlijk het gevolg van auto-oxidatiereacties, want ook onder steriele condities in aanwezigheid van zuursof verdween 4-AP uit de oplossing (Haller 1978). De andere aromatische amine, aniline, werd gemineraliseerd omdat tijdens biodegradatie-experimenten stoichiometrische hoeveelheden zuurstof werden opgenomen.

De reductieproducten van MY10, 4-ABS en 5-ASA, lieten een ander beeld zien. Tijdens de geïntegreerde anaërobe/aërobe afbraakexperimenten van MY10 was te weinig zuurstof aanwezig om verdere afbraak van aromatische aminen te bewerkstelligen. De hoeveelheid zuurstof ten opzichte van de hoeveelheid cosubstraat was tijdens deze experimenten niet in balans. Door een overmaat aan cosubstraat werd alle zuurstof verbruikt via preferente oxidatie van het cosubstraat. Hierdoor was er te weinig zuurstof beschikbaar voor de biodegradatie van de aromatische aminen. Na toevoeging van een extra hoeveelheid zuurstof werd 5-ASA snel afgebroken, maar het is de vraag of er in dit geval daadwerkelijk sprake is geweest van geïntegreerde anaërobe/aërobe condities.

Het andere afbraakproduct van MY10, de gesulfoneerde aromatische amine 4-ABS, accumuleerde en dit duidt erop dat deze verbinding niet makkelijk wordt afgebroken. Om de afbraak van 4-ABS te verbeteren werden verschillende inoculumbronnen gescreend op de afbraak van 4-ABS, wat resulteerde in de cultivatie van een 4-ABS-afbrekende ophopingscultuur die werd verkregen uit Rijnsediment. Toevoeging van deze cultuur aan korrelslib, een handelwijze die bekend staat als “bioaugmentation”, resulteerde in de volledige mineralisatie van MY10. Voor een goede afbraak van 4-ABS waren hoge zuurstofconcentraties nodig, terwijl 5-ASA wél bij lagere zuurstofconcentraties werd afgebroken. Auto-oxidatie was deels verantwoordelijk voor de verwijdering van 5-ASA want 5-ASA werd langzaam verwijderd in steriele controle-batches in aanwezigheid van zuurstof

(Jensen et al. 1993; Jensen et al. 1992). In actieve batches verliep de verwijdering echter veel sneller, hetgeen aangeeft dat 5-ASA voornamelijk werd verwijderd via biodegradatie.
In de loop van dit promotieonderzoek zijn meerdere geïntegreerde anaërobe/aërobe bioreactorexperimenten uitgevoerd met als azokleurstoffen MY10 en 4-PAP. De geïntegreerde anaërobe/aërobe condities werden bewerkstelligd door zuurstof toe te voegen aan de recyclestroom van een “expanded granular sludge bed” (EGSB) bioreactor. Tijdens deze experimenten werd ethanol als cosubstraat gebruikt. De resultaten die werden verkregen zijn vergelijkbaar met die van de batchexperimenten in Hoofdstuk 2 en 3. De azokleurstoffen werden eenvoudig gereduceerd in een beluchte EGSB-bioreactor. Beide aromatische aminen die gevormd waren bij de azoreductie van 4-PAP werden teruggevonden als er geen zuurstof werd toegevoegd aan de EGSB-reactor. De aromatische aminen werden afgebroken (aniline) of verwijderd via auto-oxidatie (4-AP) als er voldoende zuurstof aanwezig was (Tan et al. 1999). Tijdens het geïntegreerde anaërobe/aërobe EGSB-bioreactorexperiment met MY10, werd aërobe biodegradatie van het azoreductieproduct 5-ASA waargenomen. Het andere azoreductieproduct, 4-ABS, werd alleen afgebroken als aan de reactor een 4-ABS-afbrekende ophopingscultuur werd toegevoegd. Echter, omdat de 4-ABS-afbrekende ophopingscultuur hoge zuurstofconcentraties nodig had, werd de hoeveelheid zuurstof verhoogd. Dit had negatieve effecten op de structuur van het korrelslib. Doordat filamenteuze biomassa werd gevormd, was de korrelslibstructuur niet meer aanwezig. Hierdoor trad uitspoeling van de biomassa op, waardoor de verwijderingscapaciteit van de bioreactor ernstig verslechterde (niet gepubliceerd).

6.4.3 Mineralisatie van azokleurstoffen in sequentieel anaërobe/aërobe systemen

In Hoofdstuk 4 is het sequentiële anaërobe/aërobe behandelingsconcept voor de afbraak van azokleurstoffen in een bioreactor getest. De sequentiële anaërobe/aërobe condities zijn eenvoudig te creëren door eerst een anaërobe en vervolgens een aërobe reactor toe te passen. Als anaërobe reactor werd een met korrelsib gevulde EGSB-bioreactor gebruikt en de aërobe reactor bestond uit een aërobe actiefslib-reactor met een bezinker. De azokleurstof, MY10, werd eenvoudig gereduceerd en het grootste deel van het cosubstraat werd afgebroken in de anaërobe EGSB-bioreactor. Beide aromatische aminen, 4-ABS en 5-ASA, werden in hoge percentages teruggevonden in het effluent van de EGSB-bioreactor. Dit feit bevestigde de volledige azoreductie van MY10. Gedurende dit bioreactorexperiment werd het percentage 5-ASA dat werd teruggevonden lager. Door anaërobe afbraak van experimenten werd aangetoond dat 5-ASA anaëroob werd afgebroken (Razo-Flores et al. 1997). Helaas was de anaërobe 5-ASA-biodegradatiesnelheid te laag voor een volledige anaërobe afbraak. Daarom is aërobe nabehandeling van 5-ASA nog altijd noodzakelijk.

Door sequentiële anaërobe/aërobe behandeling van de azokleurstoffen bestaat het substraat voor de aërobe stap voornamelijk uit de azoreductieproducten, de aromatische aminen 4-ABS en 5-ASA. De bacteriële activiteit voor de mineralisatie van 5-ASA ontwikkelde zich snel in de aërobe actiefslib-reactor. Meteen na de start van het experiment werd 5-ASA bijna volledig verwijderd. De andere aromatische amine, 4-ABS, werd echter helemaal niet verwijderd, zelfs niet na een periode van 245 dagen. Daarom is de 4-ABS-afbrekende ophopingscultuur, die verkregen is uit het Rijnsediment, toegevoegd aan de aërobe bioreactor. Na de toevoeging van deze ophopingscultuur werd 4-ABS onmiddellijk afgebroken. De afbraak van 4-ABS bleef hierna tot aan het eind van het experiment aanwezig (1.5 jaar). Dit geeft duidelijk aan dat de 4-ABS-afbrekende ophopingscultuur in de slibvlokken is gaan zitten. Ondersteunend bewijs voor de mineralisatie van 4-ABS was dat sulfaat, een van de mineralisatieproducten van 4-ABS, in stoichiometrische hoeveelheden werd aangetroffen in het effluent van de bioreactor en in de vloeistoffase van batches van 4-ABS biodegradatie-experimenten.

6.4.4 Biodegradeerbaarheid van gesulfoneerde aromatisch amines gedurende afvalwaterzuivering

Veel verschillende gesulfoneerde azokleurstoffen worden gebruikt in de textiel industrie. Hierdoor ontstaan na anaërobe behandeling tevens ook veel verschillende gesulfoneerde aromatische aminen. De biodegradatie van deze gesulfoneerde aromatische amines is meestal niet eenvoudig onder natuurlijke omstandigheden (Hoofdstuk 1). In Hoofdstuk 3 en 4 werd aangetoond dat de afbraak van 4-ABS alleen optrad nadat een speciale ophopingscultuur aan de systemen was toegevoegd. Daarom is de het de vraag of deze gesulfoneerde aromatische aminen worden afgebroken. Om het lot van dit soort verbindingen met natuurlijk gemengde bacteriepopulaties te onderzoeken zijn experimenten uitgevoerd onder aërobe en anaërobe batch-condities en in een aërobe continue bioreactor (Hoofdstuk 5). De gesulfoneerde aromatische aminen die zijn getest zijn 2-ABS, 3-ABS, 4-ABS, 2,4-diaminobenzenesulfonzuur (2,4-DABS), 3-amino-4-hydroxybenzenesulfonzuur (3-A-4-HBS), 1-aminonaftaleen-4-sulfonzuur (1-AN-4-S), 1-aminonaftaleen-5-sulfonzuur (1-AN-5-S), 2-aminonaftaleen-1,5-disulfonzuur (2-AN-1,5-DS) 3-aminonaftaleen-2,7-disulfonzuur (3-AN-2,7-DS) en 8-aminonaftol-3,6-disulfonzuur (8-ANOH-3,6-DS). Tevens zijn de drie ABS-isomeren getest in de aërobe actiefslib-reactor die ook is gebruikt voor de sequentiële anaërobe/aërobe biodegradatie van MY10 (Hoofdstuk 4).

Geen van de geteste gesulfoneerde verbindingen werd anaëroob afgebroken. Tevens werden, van de geteste gesulfoneerde aromatische amines, alleen 2- en 4-ABS onder aërobe condities afgebroken. Stoichiometrische sulfaatproductie, zuurstofopname en koolstofdioxideproductie bevestigde biodegradatie van deze bevindingen. De andere gesulfoneerde verbindingen,

verbindingen met meer gecompliceerde structuren, werden niet afgebroken door de verschillende inoculumbronnen die werden getest.

De 4-ABS-afbrekende ophopingscultuur, die origineel uit de Rijn werd geïsoleerd, was ook in staat om 2-ABS af te breken. Beide verbindingen werden ook afgebroken door actief slib afkomstig van een afvalwaterzuiveringsinstallatie die tevens textielafvalwater behandelt. De Rijn en deze afvalwaterzuiveringsinstallatie zijn historisch gezien blootgesteld aan gesulfoneerde aromatische verbindingen. Daarom kan de conclusie worden getrokken dat dit soort inoculumbronnen een goede bron zijn voor micro-organismen om deze verbindingen af te kunnen breken.

Slechts een paar biotechnologische oplossingen voor de verwijdering van gesulfoneerde aromatische aminen zijn in de literatuur beschreven (Hoofdstuk 1). Daarom werd het gedrag van deze verbindingen geëvalueerd in een actiefslib-reactor. Deze reactor was in staat om 4-ABS af te breken omdat aan deze reactor een 4-ABS-afbrekende ophopingscultuur was toegevoegd (Hoofdstuk 4). Ook werd biodegradatie van 2-ABS waargenomen, een maand nadat deze component werd toegevoegd aan de reactor. Biodegradatie van 3-ABS werd niet waargenomen gedurende dit reactorexperiment.

De mineralisatie van 2- en 4-ABS werd bevestigd door hoge sulfaatproductie en een goede chemisch zuurstofverbruik-(CZV)-verwijdering. Batch respiratie-experimenten met bioreactorslib en 2-ABS lieten duidelijk zien dat de toename in zuurstofverbruiksnelheid was gekoppeld aan de afbraak van 2-ABS en een toename in sulfaatproductie bevestigde de mineralisatie van 2-ABS. De maximale afbraaksnelheid die tijdens de bioreactorexperimenten werd bereikt lag voor 2-ABS en 4-ABS rond de 1.6 g l^{-1} d^{-1} bij een hydraulische verblijftijd van 2.8-3.3 uren. Zoals al werd opgemerkt in Hoofdstuk 4 laat deze hoge afbraaksnelheid bij lage verblijftijden duidelijk zien dat de 2- en 4-ABS-afbrekende biomassa in het actief slib geïncorporeerd is.

Verdere isolatie- en identificatie-experimenten werden uitgevoerd met de 4-ABS-afbrekende ophopingscultuur (niet gepubliceerd). Het DNA van de ophopingscultuur werd geïsoleerd. Het V6V8 gedeelte van het 16S rDNA werd vermenigvuldigd (geamplificeerd) via een PCR reactie. Deze stukken DNA werden geanalyseerd met behulp van denaturerende gradiënt gel-elektroforese (DGGE, dit is een moleculaire techniek die vaak wordt gebruikt in de microbiële ecologie om de samenstelling van gemengde bacterieculturen aan te tonen). De DGGE-analyse liet zien dat de 4-ABS-afbekende ophopingscultuur uit twee bacterieculturen bestond. Na kloneren en sequensen van een gedeelte van het 16S rRNA, werd dit vergeleken met de NCBI-database op het internet (http://www.ncbi.nlm.nih.gov/blast/blast.cgi?Jform=0). De resultaten lieten zien dat de cocultuur waarschijnlijk bestaat uit de volgende bacteriestammen: *Hydrogenophaga* sp. en *Ochrobactrum* sp.. Heel verrassend bleek

dat de *Hydrogenophaga* sp. in onze cocultuur voor 98% (rRNA basis) hetzelfde is als de *Hydrogenophaga* sp. DSM 5680 uit de 4-ABS-afbrekende cocultuur uit Duitsland (Feigel and Knackmuss 1993). Deze stam is verantwoordelijk voor de initiële afbraak van 4-ABS en vormt 5-suflocatechol via een dioxygenase. De andere bacteriestam (*Agrobacterium* sp.) uit deze cocultuur gebruikt deze verbinding om op te groeien (Dangmann et al. 1996). Zeer waarschijnlijk is de *Hydrogenophaga* sp. in onze cocultuur ook verantwoordelijk voor de initiële biodegradatie van 4-ABS. Een literatuuronderzoek liet zien dat de andere stam in onze cocultuur, *Ochrobactrum* sp., in staat is om aromatische verbindingen af te breken (Lechner et al. 1995; Song et al. 2000). Helaas is geen van de bacteriën uit de cocultuur geïsoleerd en daarom is geen verder onderzoek aan deze 4-ABS-afbrekende ophopingscultuur verricht.

De aërobe afbraak van de gesulfoneerde aromatische amines was beperkt tot twee van de drie ABS-isomeren, 2- en 4-ABS. Biodegradatie van deze verbindingen werd waargenomen tijdens batch- en bioreactorexperimenten. De andere gesubstitueerde ABS- en aminonaftaleensulfonzuren-(ANS)-verbindingen werden niet aëroob afgebroken (Tabel 6.1). Sommige gespecialiseerde en geïsoleerde bacterieculturen zijn in staat dit soort verbindingen af te breken (Hoofdstuk 1). Toekomstig onderzoek moet zich daarom richten op het verrijken van goede inoculumbronnen voor de afbraak van gesubstitueerde ABS- en ANS-verbindingen. Deze en de geïsoleerde culturen dienen vervolgens te worden getest in aërobe afvalwaterzuiveringen.

Veel verschillende azokleurstoffen bevatten de sulfonzuurgroep ($-SO_3H$). Daarom zullen veel verschillende gesulfoneerde aromatische amines worden gevormd na de anaërobe behandeling van deze azokleurstoffen. Omdat de meeste van deze verbindingen niet makkelijk worden afgebroken zal een deel van de CZV-fractie na behandeling van afvalwater met gesulfoneerde azokleurstoffen bestaan uit gesubstitueerde ABS- en ANS-verbindingen. Uit onderzoek bleek dat gesulfoneerde aromatische aminen gelukkig niet toxisch waren voor anaëroob korrelslib en aëroob actiefslib (Hoofdstuk 5) en minder schadelijk voor mensen dan de niet-gesulfoneerde aromatische aminen (Chung en Cerniglia 1992; Jung et al. 1992; Levine 1991; Rosenkranz and Klopman 1990).

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ABS	aminobenzenesulfonic acid
AHBS	aminohydroxybenzenesulfonic acid
AHNDS	aminohydroxynaphthalenedisulfonic acid
AHNS	aminohydroxynaphthalenesulfonic acid
ANDS	aminonaphthalenedisulfonic acid
ANOHDS	aminonaphtholdisulfonic acid
ANS	aminonaphthalenesulfonic acid
AP	aminophenol
ASA	aminosalicylic acid
BOS	benzosulfonic acid
BS	benzenesulfonic acid
CBS	chlorobenzenesulfonic acid
COD	chemical oxygen demand
DABS	diaminobenzenesulfonic acid
DGGE	denaturing gradient gel electrophoresis
DO	dissolved oxygen
EGSB	expanded granular sludge blanket
EtOH	ethanol
GC	gaschromatography
HBS	hydroxybenzenesulfonic acid
HPLC	high pressure liquid chromatography
HRT	hydraulic retention time
IC_{50}	concentration which leads to 50% inhibition of the activity
IHOP	initial headspace oxygen percentage
K_S	substrate affinity
MO1	Mordant Orange 1
MY10	Mordant Yellow 10
NBS	nitrobenzenesulfonic acid
NDS	naphthalenedisulfonic acid
NS	naphthalenesulfonic acid
OUR	oxygen uptake rate
PAP	phenylazophenol
PDA	phenylenediamine
TS	toluenesulfonic acid
UASB	upflow anaerobic sludge blanket
μ_{max}	maximal growth rate
UV	ultra violet
VSS	volatile suspended solids
Y	biomass yield

Mijn proefschrift is af! Na bijna vijf jaar werk, onderzoek en schrijven is het einde van mijn promotieonderzoek in zicht. Hiervoor ben ik natuurlijk een heleboel mensen dank verschuldigd. Als eerste bedank ik Jim voor zijn enthousiaste inzet en subtiele manier van begeleiden als copromotor. Jim, van jouw wetenschappelijke inzichten en heldere structurering heb ik veel geleerd. Ik had me geen beter copromotor kunnen wensen. Verder bedank ik Gatze voor zijn rol als promotor tijdens dit promotieonderzoek. Als een constante factor was jij altijd op de achtergrond aanwezig en greep je in op de juiste momenten. Natuurlijk wil ik Elías bedanken voor zijn hulp, advies, luisterend oor en vriendschap tijdens de eerste twee jaar van mijn promotieonderzoek. Elías, toen jij terug was in Mexico heb ik gemerkt wat je voor mijn promotieonderzoek betekende. Por supuesto, quiero agradecer a Elías por su ayuda, amistad y consejos durante los primeros dos años de mi investigación de doctorado. Elías, cuando regresaste a México, me di cuenta de lo mucho que significaste para mi investigación.

Verder is dit werk, beschreven in dit proefschrift, mede tot stand gekomen door de hulp van een groot aantal studenten die onderzoek hebben uitgevoerd onder mijn begeleiding. Francesc, Joris, Richard, Arjan, Anna, Peter, Annemarie en Ellen, allemaal bedankt voor jullie inzet. Daarnaast heb ik nog twee gastmedewerkers mogen begeleiden. Laura en Elena, helaas viel het onderwerp van jullie onderzoeken net buiten de scope van dit proefschrift dus er is niets van terug te vinden. Maar evengoed, bedankt voor de goede samenwerking! Además, supervisé dos colaboradores visitantes. Laura y Elena, desgraciadamente el tema de su investigación quedo fuera el enfoque de esta tesis, por lo tanto no lo incluí. Pero de todos modos, gracias por la buena cooperación.

Natuurlijk heb ik heel vaak gebruikt gemaakt van de hulp van allerlei collegae en andere mensen. Zo bedank ik Johannes, Ilse, Geert, Sjoerd en Jo voor de hulp bij allerlei analyses en Heleen en Liesbeth voor de hulp bij administratieve aangelegenheden. Ik bedank Vinnie in het bijzonder voor al zijn tijd, hulp en technische kennis van allerlei apparatuur. Hierdoor hebben mijn opstellingen altijd goed kunnen draaien. Elías, Shyung, “Paco” en Frank, bedankt voor de goede samenwerking op het Lab728. Tevens wil ik de heren van de respiratie, John, Hardy, Jaap en Henri, bedanken voor de hulp en kennis bij allerlei moeilijke respiratievraagstukken en -modellen. Ook wil ik Antoon en Kees van het laboratorium voor Microbiologie bedanken voor de hulp bij het werk met verschillende DNA-technieken op het gebied van de microbiële ecologie en ik wil Mirjam, Frank, Edwin (muchas gracias) en Kees bedanken voor het nakijken en corrigeren van delen van teksten van dit proefschrift. Luc en Joke bedankt voor het maken van de mooie omslag van het proefschrift.

Ik heb de AIO-kamer mogen delen met een groot aantal collegae en gastmedewerkers. Sybren, José-Luis, Anneke, Rene, Huib, Chiel, Frank, Cristina, Michiel, Jaap en Dale, ik hoop dat jullie het net zo leuk vonden om met mij een kamer te delen als ik dat met jullie. Gelukkig dat er naast de serieuze zaken ook nog tijd was voor een goed gesprek, spel en ontspanning.
Naast het onderzoek heb ik tijdens mijn promotieonderzoek nevenactiviteiten ontplooid waar ik met plezier aan terugdenk, zoals allerlei gevolgde cursussen, de WIMEK-AIO-raad en -bestuur, afvalwateroverleg (Adriaan bedankt voor de goede samenwerking), congresbezoek (San Diego, Mérida en Eilat waren toch wel hoogtepunten) en niet te vergeten de lunches met de "jonge" AIO's. Daarnaast waren de vrijdagmiddagborrels, volleybal, zaalvoetbal, wielrennen en voetbal van essentieel belang voor mij om het promotieonderzoek even te vergeten. Ik wil iedereen bedanken die hier op enige manier bij betrokken is geweest.
Pa en Ma bedankt voor jullie onvoorwaardelijke steun mijn hele leven lang, zonder deze steun had ik nooit bereikt wat ik nu heb bereikt! Pa, tevens bedankt voor het halen van actiefslib uit de afvalwaterzuivering van Aarle-Rixtel.
Als laatste wil ik mijn vriendin (en vrouw) Jolanda heel erg bedanken voor alles wat ze voor mij deze promotietijd heeft betekend en al veel langer betekent. Lieve Jolanda, door jouw steun, relativeringsvermogen, geduld en liefde zijn de afgelopen vijf jaar een stuk makkelijker verlopen. Na deze laatste drukke periode van schrijven en afronden van het proefschrift gaan we samen genieten van alles wat ons pad kruist. Onlangs hebben we het mooiste wat ons tot nu toe overkomen is mogen beleven, de geboorte van onze zoon Rik. Lieve Rik, al zul je nog niet begrijpen wat er staat, maar pappa heeft na zijn promotie meer tijd om samen met jou te spelen, lachen, stoeien en plezier te maken.

Nico

Nico(demus) Christiaan Gerardus Tan werd geboren op 27 november 1970 te Eibergen. In 1989 behaalde hij zijn VWO-diploma aan het Carolus Boromeus College te Helmond en ging hij moleculaire wetenschappen studeren aan de Landbouwuniversiteit Wageningen.

Tijdens zijn studie deed hij twee afstudeervakken, de eerste bij de vakgroep Organische Chemie gericht op de organische synthese uitgaande van s-carvon en de tweede bij de vakgroep Microbiologie met als onderwerp de anaërobe afbraak van tolueen onder mangaanreducerende condities. Vervolgens deed hij twee stages op het gebied van de milieubiotechnologie: bij Bioclear in Groningen onderzocht hij een biologisch membraanluchtfilter en bij TNO-Milieubiotechnologie in Delft deed hij onderzoek aan de biologische afbraak van 1,2-dichloroethaan in een membraanbioreactor. In 1995 studeerde hij af als moleculair wetenschapper met als specialisatie chemie en milieuchemie.

Na zijn studie heeft hij drie maanden gewerkt als practicumbegeleider bij de vakgroep Fysische- en Kolloïdchemie van de Landbouwuniversiteit. Vervolgens heeft hij drie maanden onderzoek gedaan naar het kweken van magnetotactische bacteriën als junioronderzoeker bij de vakgroep Fysische en Colloïdchemie aan de Universiteit Utrecht.

Van mei 1996 tot mei 2000 heeft hij gewerkt aan zijn promotieonderzoek bij de vakgroep Milieutechnologie van de Landbouwuniversiteit in Wageningen. Dit onderzoek heeft geresulteerd in dit proefschrift. Sinds mei 2000 is hij werkzaam als wetenschappelijk onderzoeker en projectleider bij het ATO, een instituut van Wageningen Universiteit en Researchcentrum.

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