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Azo Dye Reduction by Methanogenic Granular Sludge Exposed to Oxygen

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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.

Keywords: *Methanogenic, Azo dye, oxygen, Mordant Orange*

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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 1983 b), 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 1983 a; 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. 1993 b; 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. Materials and Methods

2.1. Biomass

Methanogenic granular sludge from a full-scale upflow anaerobic sludge blanket (UASB) reactor treating wet oxidized industrial effluent and from a full-scale UASB reactor treating effluent of an alcohol distillery at Ankleshwar, Gujarat, India 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. Basal Medium

The basal medium used in all batch experiments contained (mg l^{-1}): $NaHCO_3$ (5000), NH_4 Cl (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_4 $SeO_3 \cdot 5H_2O$ (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}).

2.3. 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 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 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 5 A (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. 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 UV-1800 spectrophotometer (Shimadzu, Japan) 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 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.

2.4. 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 N2/CO2 (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 N2/CO2 (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 N2/CO2 (70%/30%) gas for 5 minutes. The N2/CO2 (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}. Methane, oxygen and co-substrates were measured gas

chromatographically and MO1 was measured spectrophotometrically.

2.5. Chemicals

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

3. Results

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 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). Granular sludge-I was more tolerant to oxygen and this sludge had a higher acetoclastic activity than granular sludge-II.

Table 1. The acetoclastic methanogenic activity and oxygen activity of Granular sludge–I & II

Biomass source	Acetoclastic activity of control (Gcod-$CH_4 g^{-1}$ VSS d^{-1})	IC_{50}
Granular sludge-I	0.53	17.7
Granular sludge-II	1.10	25.8

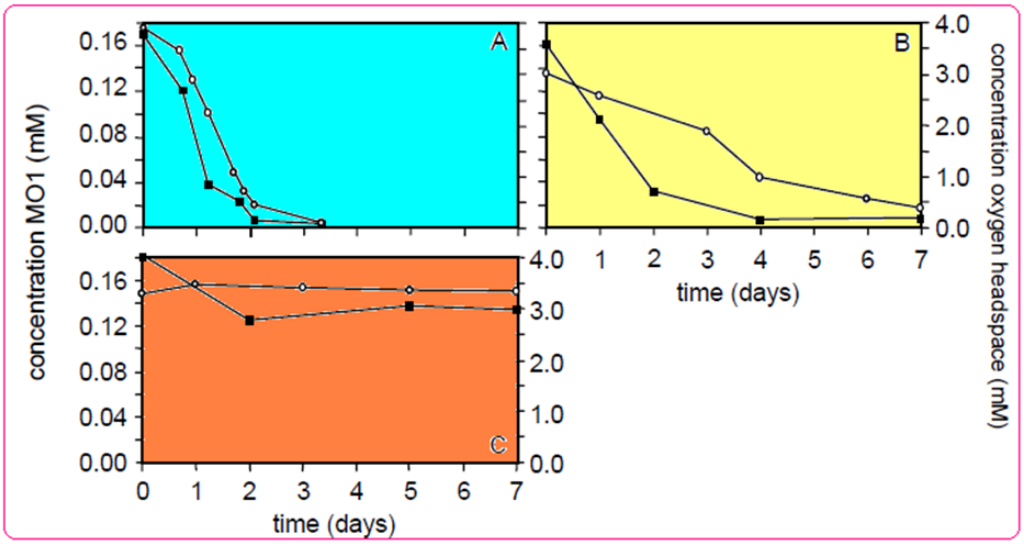

Figure 1. Time courses of MO1 (O) and oxygen (■) at 10 IHOP with ethanol (A), or acetate (B) as co-substrate, and no co-substrate (C) and granular sludge-I biomass source

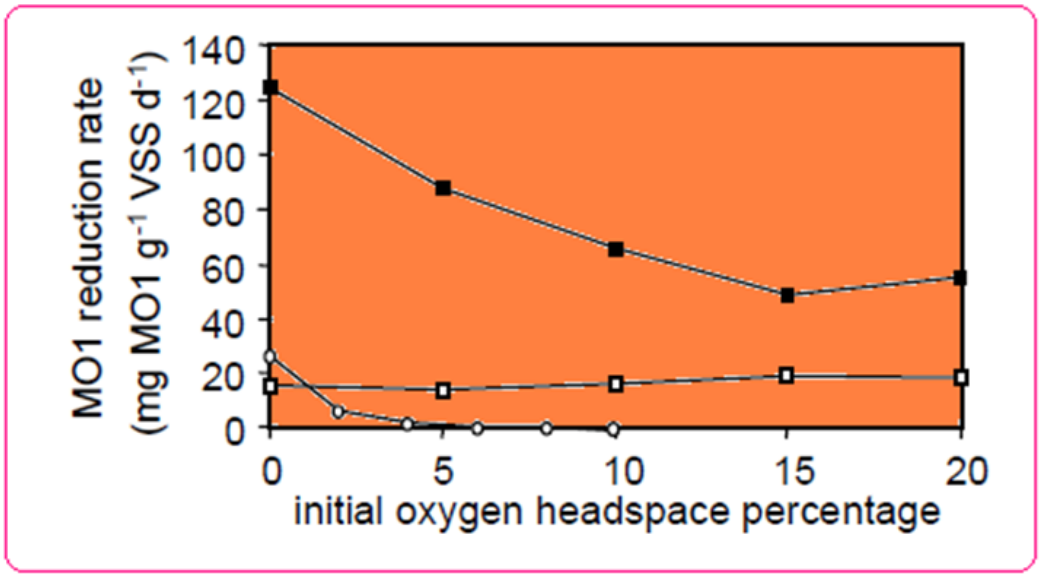

Figure 2. Reduction rates of MO1 in the presence of oxygen with granular sludge-I as biomass source with acetate (□) or ethanol (■) as co-substrate, or no co-substrate (○)

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 1 shows typical time-courses of MO1 exposed to 10 IHOP with granular sludge-I 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 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 granular sludge-II (data not shown).

Figure 2 shows the azo dye-reduction rate as a function of the IHOP for granular sludge-I. 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.

In the absence of oxygen, acetate and no co-substrate supported much lower azo dyereduction 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 granular sludge-II (Figure 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 granular sludge-I. Exposure to oxygen up to 20 IHOP had no effect on the rate. When co-substrate was lacking, granular sludge-II had reducing activity only in the complete absence of oxygen.

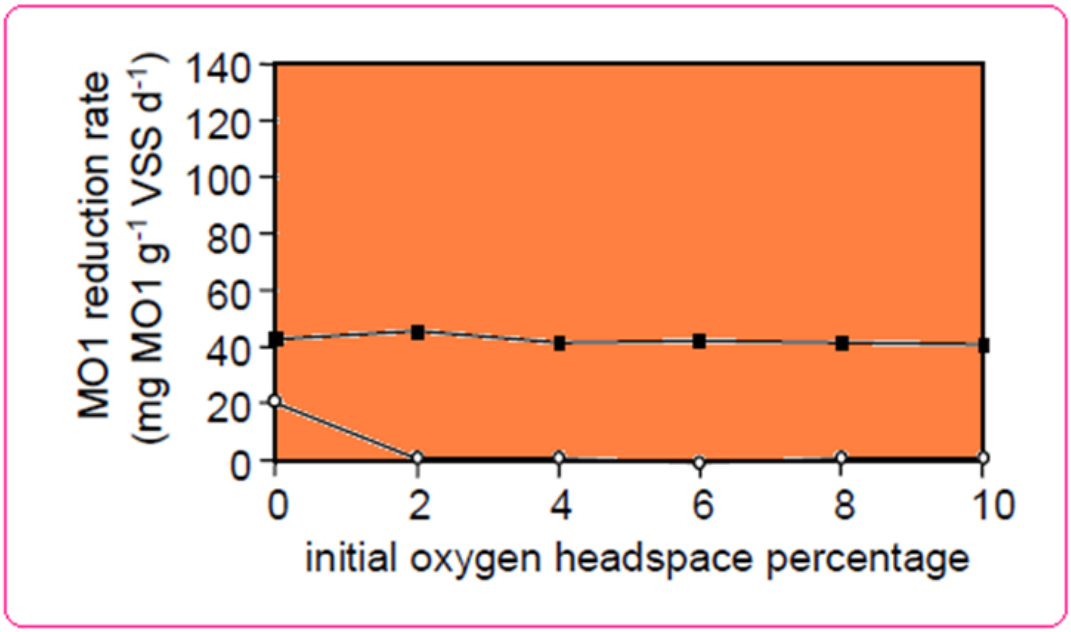

Figure 3. Reduction rates of MO1 in the presence of oxygen with granular sludge-II as biomass source with ethanol (■) as co-substrate, or no co-substrate (○)

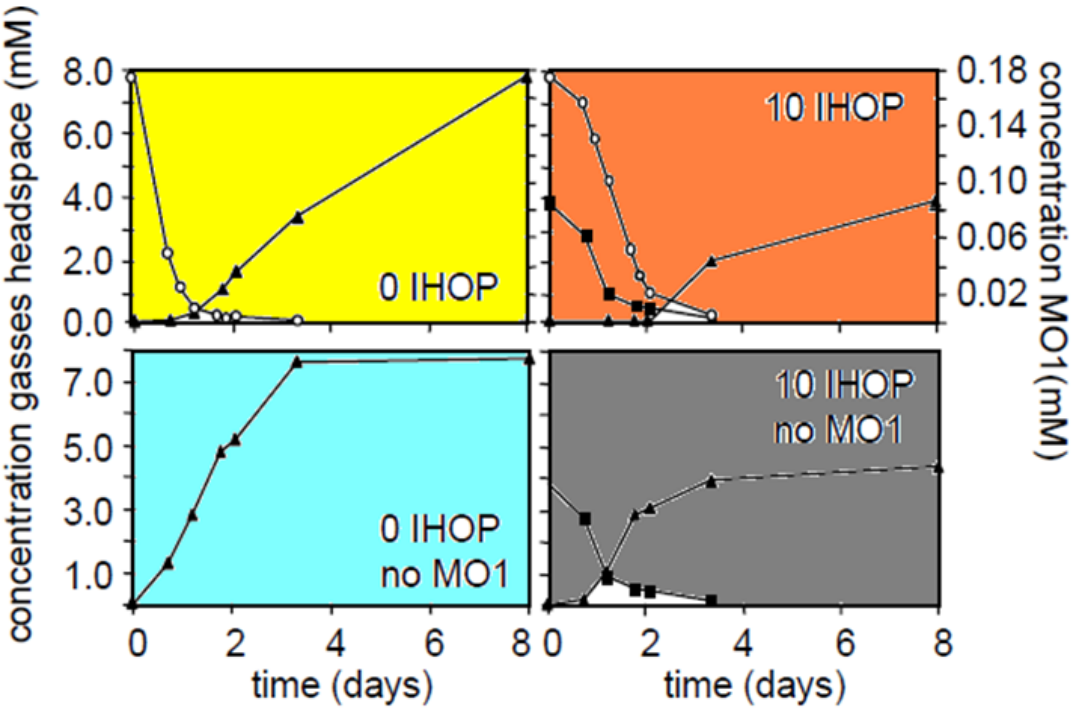

Figure 4. Methane (▲) production and oxygen (■) consumption in the presence of 0 and 10 IHOP, and with or without MO1(○), with granular sludge-I plus ethanol as co-substrate (IHOP = initial headspace oxygen percentage)

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 granular sludge-I with 0 and 10 IHOP and ethanol as co-substrate (Figure 4). If no azo dye was added, methane production occurred when oxygen was present.

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 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.

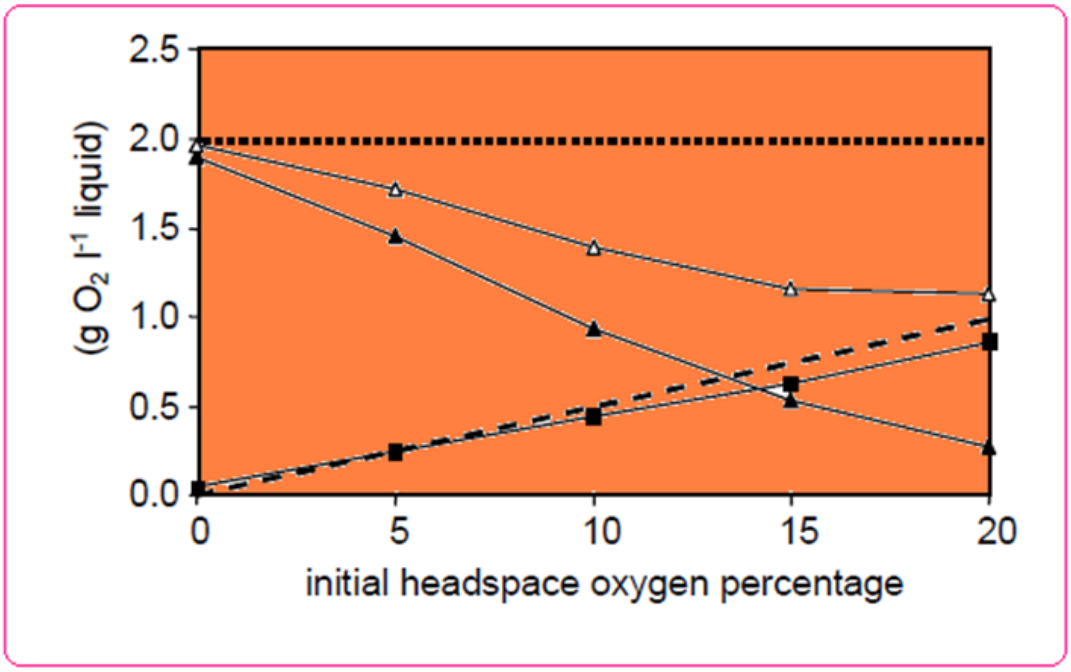

Figure 5. COD mass balance as a function of the IHOP for granular sludge-I plus ethanol as co-substrate after two days (COD expressed as g O_2 l^{-1} liquid; oxygen uptake (■), methane production (▲), sum methane production + oxygen uptake (△), ethanol supplied (▪▪▪) and oxygen supplied (----), methane values used were calculated from measurements after eight days)

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). Table 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. Percentage recovery of aromatic amines

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

4. Discussion

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 result in consumption of oxygen. Ethanol was more rapidly respired aerobically than acetate in granular sludge. Also, granular sludge had a higher oxygen consuming activity than other granular sludge, indicating that the former granular sludge contained a higher concentration of facultative anaerobes, as had been observed previously (Kato et al. 1993 a). 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 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 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. 1993 b) and the unusually high tolerance of methanogens in granular sludges to oxygen toxicity (Kato et al. 1993 a). 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.

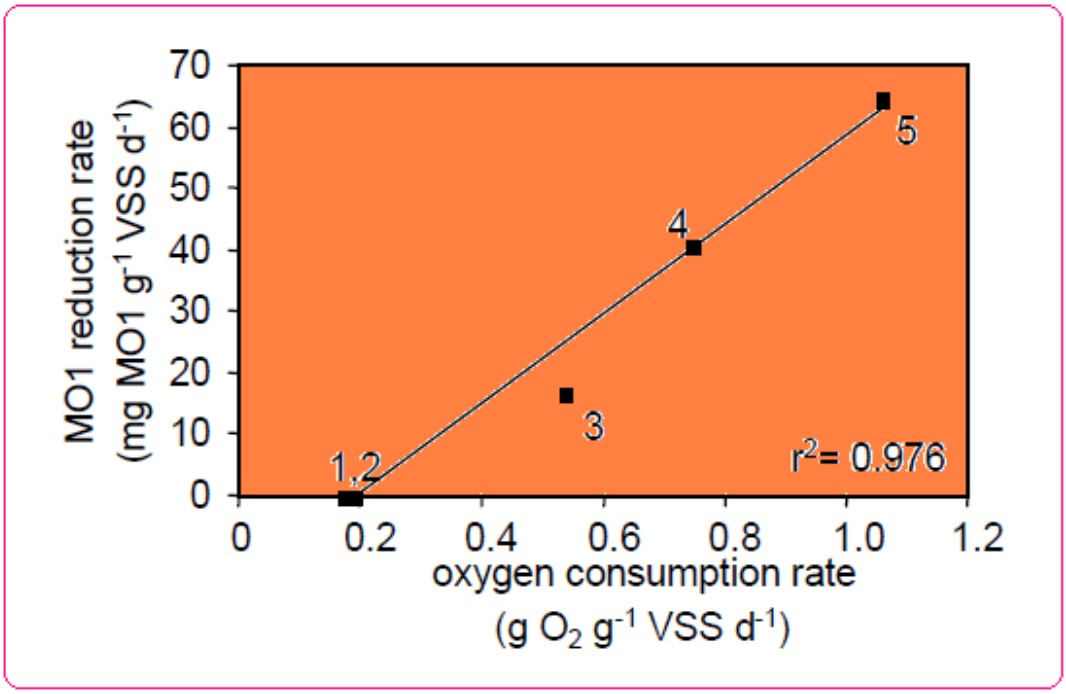

Figure 6. Correlation between oxygen consumption rate and azo dye-reduction rate at 10 IHOP (1 = Granular sludge-I with no co-substrate; 4 = Granular sludge-II with ethanol as co-substrate; 5 = Nedalco granular sludge with ethanol as co-substrate)

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. 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 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.

References

[1] An H., Yi Q., Xiasheng G. and Walter Z.T. (1996). Biological treatment of dye wastewaters using an anaerobic oxic system. Chemosphere 33 (12): 2533-2542.

[2] Anliker R. (1979). Ecotoxicology of Dyestuffs-A joint effort by industry. Ecotoxicology and Environmental Safety 3: 59-74.

[3] APHA (1985). Standard methods for the examination of water and wastewater, 16th edition. American Public Health Association, Washington DC.

[4] Brown D. and Hamburger B. (1987). The degradation of dyestuffs: Part III-Investigations of their ultimate degradability. Chemosphere 16 (7): 1539-1553.

[5] Brown D. and Laboureur P. (1983 a). The aerobic biodegradability of primary aromatic amines. Chemosphere 12 (3): 405-414.

[6] Brown D. and Laboureur P. (1983 b). The degradation of dyestuffs: Part I–Primary biodegradation under anaerobic conditions. Chemosphere 12 (3): 397-404.

[7] Carliell C.M., Barclay S.J., Naidoo N., Buckley C.A., Mulholland D.A. and Senior E. (1995). Microbial decolourisation of a reactive azo dye under anaerobic conditions. Water SA 21 (1): 61-69.

[8] Carliell C.M., Godefroy S.J., Naidoo N., Buckley C.A., Senior E., Mulholland D. and Martineigh B.S. (1994). Anaerobic decolourisation of azo dyes, Seventh International Symposium on Anaerobic Digestion, RSA Litho (Pty) Ltd, Goodwood, South Africa, Cape Town, South Africa, pp. 303-306.

[9] Chung K.T. and Cerniglia C.E. (1992). Mutagenicity of azo dyes: Structure-activity relationships. Mutation Research 277: 201-220.

[10] Costerton J.W., Lewandowski Z., De Beer D., Caldwell D., Korber D. and James G. (1994). Biofilms, the customized microniche. Journal of Bacteriology 176 (8): 2137-2142.

[11] De Beer D., Van Den Heuvel J.C. and Ottengraf S.P.P. (1993). Microelectrode measurements of the activity distribution in nitrifying bacterial aggregates. Applied and Environmental Microbiology 59 (2): 573-579.

[12] Donlon B.A., Razo-Flores E., Lettinga G. and Field J.A. (1996). Continuous detoxification, transformation and degradation of nitrophenols in upflow anaerobic sludge blanket (UASB) reactors. Biotechnology and Bioengineering 51: 439-449.

[13] Donlon B.A., Razo-Flores E., Luijten M., Swarts H., Lettinga G. and Field J.A. (1997). Detoxification and partial mineralization of the azo dye mordant orange 1 in a continuous upflow anaerobic sludge-blanket reactor. Applied Microbiology and Biotechnology 47 (1): 83-90.

[14] Field J.A., Stams A.J.M., Kato M. and Schraa G. (1995). Enhanced biodegradation of aromatic pollutant in coculture of anaerobic and aerobic bacterial consortia. Antonie van Leeuwenhoek 67: 47-77.

[15] FitzGerald S.W. and Bishop P.L. (1995). Two stage anaerobic/aerobic treatment of sulfonated azo dyes. Journal of Environmental Science and Health A 30 (6): 1251-1276.

[16] Gingell R. and Walker R. (1971). Mechanisms of azo reduction by *Streptococcus faecalis* II. The role of soluble flavins. Xenobiotica 1 (3): 231-239.

[17] Hooijmans C.M., Briasco C.A., Huang J., Geraats B.G.M., Barbotin J.N., Thomas D. and Luyben K.C.A.M. (1990). Measurement of oxygen concentration gradients in gelimmobilized recombinant *Escherichia coli*. Applied Microbiology and Biotechnology 33 (6): 611-618.

[18] Kato M.T., Field J.A. and Lettinga G. (1993 a). High tolerance of methanogens in granular sludge to oxygen. Biotechnology and Bioengineering 42 (11): 1360-1366.

[19] Kato M.T., Field J.A. and Lettinga G. (1993 b). Methanogenesis in granular sludge exposed to oxygen. FEMS Microbiology Letters 114 (3): 317-323.

[20] Keck A., Klein J., Kudlich M., Stolz A., Knackmuss H.J. and Mattes R. (1997). Reduction of azo dyes by redox mediators originating in the naphthalenesulfonic acid degradation pathway of Sphingomonas sp. Strain BN6. Applied and Environmental Microbiology 63 (9): 3684-3690.

[21] Konopka A. (1993). Isolation and characterization of a subsurface bacterium that degrades aniline and methylanilines. FEMS Microbiology Letters 111 (1): 93-99.

[22] Kudlich M., Bishop P.L., Knackmuss H.J. and Stolz A. (1996). Simultaneous anaerobic and aerobic degradation of the sulfonated azo dye Mordant Yellow 3 by immobilized cells from a naphthalenesulfonate-degrading mixed culture. Applied Microbiology and Biotechnology 46 (5-6): 597-603.

[23] Kudlich M., Keck A., Klein J. and Stolz A. (1997). Localization of the enzyme system involved in anaerobic reduction of azo dyes by *Sphingomonas* sp. Strain BN6 and effect of artificial redox mediators on the rate of azo dye reduction. Applied and Environmental Microbiology 63 (9): 3691-3694.

[24] Kurosawa H. and Tanaka H. (1990). Advances in immobilized cell culture: development of co-immobilised mixed culture system of aerobic and anaerobic micro-organisms. Process Biochemistry 25 (6): 189-196.

[25] Loidl M., Hinteregger C., Ditzelmuller G., Ferschl A. and Streichsbier F. (1990). Degradation of aniline and monochlorinated anilines by soil-born *Pseudomonas acidovorans* strains. Archives of Microbiology 155 (1): 56-61.

[26] Muller W., Winnefeld A., Kohls O., Scheper T., Zimelka W. and Baumgart H. (1994). Real and pseudo oxygen gradients in Ca-alginate beads monitored during polarographic PO-2-measurements using Pt-needle microelectrodes. Biotechnology and Bioengineering 44 (5): 617-625.

[27] Nielsen L.P., Christensen P.B., Revsbech N.P. and Sorensen J. (1990). Denitrification and oxygen respiration in biofilms studied with a microsensor for nitrous oxide and oxygen. Microbial Ecology 19 (1): 63-72.

[28] Oren A., Gurevich P. and Henis Y. (1991). Reduction of nitrosubstituted aromatic compounds by the halophilic anaerobic eubacteria *Haloanaerobium pravealens* and *Sporabacter marismortui*. Applied and Environmental Microbiology 57: 3367-3370.

[29] Pagga U. and Brown D. (1986). The degradation of dyestuffs: Part II. Behaviour of dyestuffs in aerobic biodegradation tests. Chemosphere 15 (4): 479-491.

[30] Razo-Flores E., Donlon B.A., Field J.A. and Lettinga G. (1996). Biodegradability of Nsubstituted aromatics and alkylphenols under methanogenic conditions using granular sludge. Water Science and Technology 33 (3): 47-57.

[31] Razo-Flores E., Luijten M., Donlon B.A., Lettinga G. and Field J.A. (1997). Complete biodegradation of the azo dye azodisalicylate under anaerobic conditions. Environmental Science and Technology 31 (7): 2098-2103.

[32] Seshadri S., Bishop P.L. and Agha A.M. (1994). Anaerobic/aerobic treatment of selected azo dyes in wastewater. Waste Management 14 (2): 127-137.

[33] Shaul G.M., Holdsworth T.J., Demmpsey C.R. and Dostal K.A. (1991). Fate of water soluble azo dyes in the activated sludge process. Chemosphere 22: 107-119.

[34] Shen C.F. and Guiot S.R. (1995). Long term impact of dissolved O2 on the activity of anaerobic granules. Biotechnology and Bioengineering 49: 611-620.

[35] Shen C.F., Miguez C.B., Borque D., Groleau D. and Guiot S.R. (1996). Methanotroph and methanogen coupling in granular biofilm under O2-limited conditions. Biotechnology Letters 18 (5): 495-500.

[36] Vaidya A.A. and Datye K.V. (1982). Environmental pollution during chemical processing of synthetic fibers. Colourage 14: 3-10.

[37] Weber E.J. (1991). Studies of benzidine-based dyes in sediment-water systems. Environmental Toxicology and Chemistry 10 (5): 609-618.

[38] Zaoyan Y., Ke S., Guangliang S., Fan Y., Jinshan D. and Huanian M. (1992). Anaerobicaerobic treatment of dye waste-water by combination with activated sludge. Water Science and Technology 26: 2093-2096.

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