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Effect of Anaerobic Granular Sludge in Degradation of Two Azo dyes

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ABSTRACT: The study 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. Subsequently, aniline was degraded further in the presence of oxygen by the facultative aerobic bacteria present in the anaerobic granular sludge. Acid Orange and Reactive Black were also degraded, if inoculum from aerobic enrichment cultures were added to the batch experiments. Due to rapid autoxidation of Acid Orange, 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.

Key words: Acid Orange, Reactive Black, Azo dyes, Enrichment

INTRODUCTION

The growth of the world population, the development of various industries, and the use of fertilizers and pesticides in modern agriculture have overloaded not only the water resources but also the atmosphere and the soil with pollutants (Shah *et al.,* 2013). In the last few decades the handling of wastewater appeared to be one of the most important. Textile industry which is one of the largest water consumers in the world produces the wastewater comprising of various recalcitrant agents such as dye, sizing agents and dying aid. Therefore it has to be really concerned in releasing these types of wastewater to the environment. In the disposal of textile wastewater, color is of very important due to the aesthetic deterioration as well as the obstruction of penetration of dissolved oxygen and sun light into natural water bodies (Shah *et al.,* 2013). Most of the researches have investigated decoloriztion of azo dyes under batch anaerobic condition. Iik and and Sponza (2003) studied decolorization of Congo Red and Direct Black 38 Escherichia coli and Pseudomonas sp. cultures in anaerobic, aerobic, and microaerophilic condition. They reported that anaerobic conditions were more favourable than other conditions for decolorization. Setiadi and Van Loosdrecht (1997) demonstrated about.70% color removal efficiency, in an anaerobic reactor treating reactive azo dye. Some previous batch studies, following decolorization efficiencies were obtained for reactive Black-5; 75 and 95% color removal in 10 and 30 h, respectively (Iýk and Sponza, 2004), 95% in 48 h (Oxspring *et al.,* 1996) and 79% in 10 h (Beydilli *et al.,* 1998). Zero order reaction kinetic with respect to dye concentration has also been reported by several researchers (Brown, 1981), whereas some others der Zee *et al.,* 2001; Iýk and Sponza, 2004). Azo dye reduction leads to the formation of aromatic amines. Aromatic amines are generally not degraded and accumulate under anaerobic conditions (Brown & Hamburger, 1987., Field *et al.,* 1997) with the exception of a few aromatic amines characterized by the presence of hydroxyl and/or carboxyl groups (Heider & Fuchs 1997. Kuhn & 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 (Razo-Flores *et al.,* 1996, Brown & Laboureur, 1983a, Brown & Laboureur, 1983b). However, it should be noted that some aromatic amines are readily autoxidized in the presence of oxygen to humic like oligomeric and polymeric structures (Manalne, 1960). 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

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activity of granular sludge could be maintained after prolonged exposure to oxygen (Parris, 1980). 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.

MATERIALS & METHODS

Sample of Methanogenic granular sludge from an upflow anaerobic sludge blanket (UASB) reactor treating wet oxidized industrial effluent and from an UASB reactor treating effluent of an alcohol distillery 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. The basal medium used in all batch experiments contained (mg/L): $NaHCO_3$ (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 mg/L). The biogases in the headspace of the batches and ethanol in the liquid phase were analyzed via gas chromatographic methods. The volatile suspended solids (VSS) were measured according to Standard Methods for Examination of Water and Wastewater (Kato *et al.,* 1993a). 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 ethanol. The aromatic amines used in the aerobic biodegradation assays were analyzed spectrophotometrically 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 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 ml samples were injected with a Marathon autosampler. The azo dyes and their corresponding aromatic amines were detected spectrophotometrically. 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 at a flow rate of 300 ml min-1 first through a Separations GT-103 degaser and afterwards through two reverse phase C18 columns. 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. The assay serum flasks were flushed with N2 / CO2 (70% / 30%) gas for 5 minutes. 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 inoculums 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, 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 [23]. Integrated anaerobic/aerobic 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), active aerobic aromatic amines degrading enrichments (5% v/v), ethanol (2.5 g COD/L) and 180 mg Acid Orange and closed with a butyl rubber septum and a crimp-seal aluminum cap.

Integrated anaerobic/aerobic 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 from an integrated anaerobic/aerobic reactor treating Reactive Black-containing-artificial wastewater), ethanol (2.0 g COD/L) and 60 mg Reactive Black and closed with a butyl rubber septum and a crimp-seal aluminum cap. Afterwards, all assay serum flasks were flushed with N2 / CO2 (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.

RESULTS & DISCUSSION

Four different aromatic amines are potentially formed by the anaerobic reduction of Acid Orange and Reactive Black. The aromatic amines are 4-ABS and 5-ASA from Acid Orange; 4-AP and aniline from Reactive

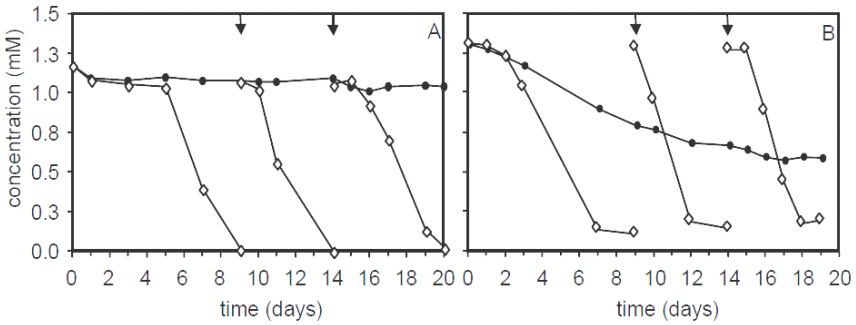

Fig. 1. Time courses of the aerobic biodegradation of 4-ABS by aerobic (A) and 5-ASA by a 4-ABS enrichment culture (B); legend: compound + inoculum (△), sterile control (•); arrows indicate new additions of compound in the inoculated batches.

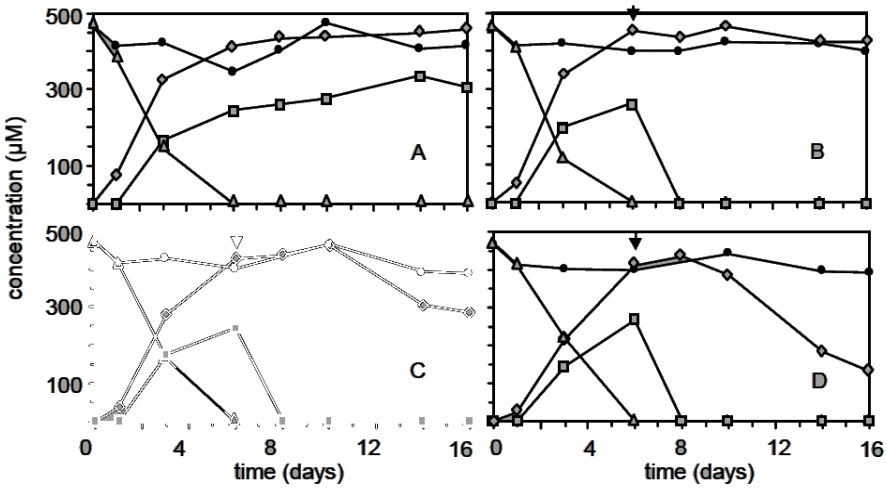

Fig. 2. A-D Concentration of AO (△), 5-ASA (□) and 4-ABS (◊) during the integrated anaerobic/aerobic AO degradation experiment at different IHOPs: 0 (A0, 5 (B), 10 (C), and 20 (D). Concentration of AO in the sterile control (•); arrows indicate a second addition of oxygen corresponding to the IHOP

Black 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 (Fig. 1A). Several other inoculum sources were tested (activated sludge, forest humus soil and anaerobic granular sludge) but none of the others supported degradation of 4-ABS. The enrichment culture developed after three feedings of 4-ABS was also able to degrade 5-ASA (Fig. 1B). The sterilized control and control lacking inoculums showed some decreases in the 5-ASA concentration due to slow autoxidation of 5-ASA. Two different inoculum sources, the aerobic and the 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 Acid Orange indicate that this compound was rapidly autoxidised. 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. Integrated anaerobic/aerobic conditions were created after addition of oxygen to co-cultures of anaerobic granular sludge with aerobic aromatic amine degrading enrichment cultures. Fig. 2 shows the results of the integrated anaerobic/aerobic Acid orange

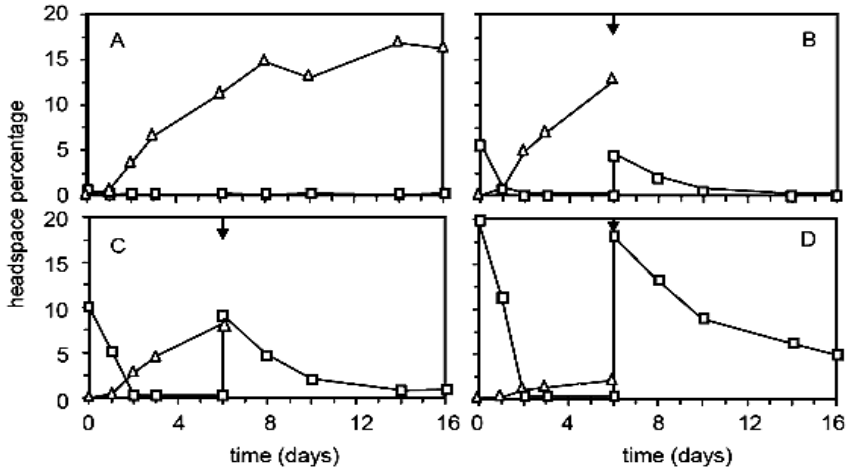

Fig. 3. A-D Percentage of the methane (Δ) and oxygen (□) in the headspace of the assays during the integrated anaerobic/aerobic AO degradation experiment at different IHOPS: 0 (A), 5 (B), 10 (C), and 20 (D); arrows indicated a second addition of oxygen corresponding to the IHOP after flushing the batches with N_2/CO_2 (70 / 30%)

experiment. The reduction of Acid orange 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 Acid orange occurred. Both aromatic amines, 4-ABS and 5-ASA, accumulated in the integrated anaerobic/aerobic Acid orange biodegradation experiment (Fig. 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 (Fig. 2A). Due to fast respiration of the added ethanol at higher IHOPs (Fig. 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 (Fig. 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 (Fig. 2C-D). The formation of methane even in the presence of oxygen was also observed (Fig. 3). This observation confirms the presence of anaerobic micro niches in which methanogenic conversions and azo dye reduction can occur. Fig. 3B-D also shows that the oxygen added on day 6 was taken up for the degradation of the aromatic amines. Fig. 4 shows the results for the integrated anaerobic/aerobic Reactive Black biodegradation experiment at different IHOPs applied. The Fig. clearly shows a rapid reduction of Reactive Black at all IHOPs applied. As was observed with Acid orange, the reduction of Reactive Black even occurred in presence of oxygen due to the addition of ethanol. Again it was observed that in sterile controls no degradation of Reactive Black occurred. The formation of both aromatic amines, 4-AP and aniline, is also clearly shown in Fig. 4. As expected, the anaerobic degradation of both of these compounds did not occur (Fig. 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 (Fig. 4B) due to limited amounts of oxygen remaining (Fig. 5B). The main degradation mechanism for 4-AP was probably autoxidation, as was indicated from the aromatic amine degradation studies. 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 (Kato *et al.*, 1993a). However, microorganisms capable of biodegradation of 5-ASA are less numerous (APHA, 1985). 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 (Gheewala & Annachhatre, 1997). In this study, only bacteria from aerobic were able to degrade 4-ABS, although other sources were tested. Furthermore, the developed enrichment was also able to degrade 5-ASA. 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 (Stolz *et al.*, 1992). Aromatic amines with one or more hydroxyl group tend to autoxidize in presence of oxygen. This autoxidation process was observed for Acid orange and as well for 5-ASA (Feigel & Knackmuss, 1993). Autoxidation rates for Acid orange were orders of magnitude faster compared to 5-ASA. Due to its slower autoxidation rates, biodegradation of 5-ASA was possible whereas

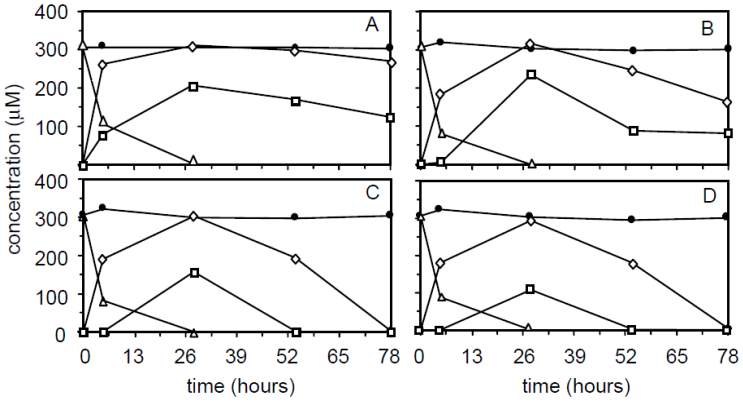

Fig. 4. A-D Concentrations of RB (Δ), 4-AP (□) and aniline (◊) during athe integrated anaerobic/aerobic RB degradation experiment at different IHOPs: 0 (A), 10 (B), 20 (C), and 30 (D). Concentration of 4-PAP in the sterile control (•) at 10 IHOP.

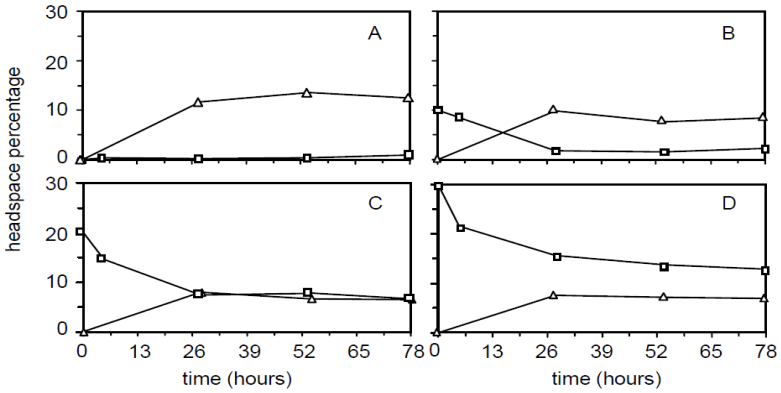

Fig. 5. A-D Percentage of the methane (Δ) and Oxygen (□) in the headspace of the batches during the integrated anaerobic/aerobic RB degradation experiment at different IHOPs: 0 (A), 10 (B), 20 (C), and 30 (D)

autoxidation rates appeared to outcompete biodegradation rates in the case of Acid orange. The autoxidation products such as oligomeric and polymeric humic substances are not very susceptible for biodegradation. Azo dyes generally need an anaerobic and an aerobic step to become completely mineralized (Kato *et al.*, 1933b). A sequential anaerobic/aerobic degradation was described for azo dyes Acid Orange 10, Acid Red 14 and Acid Red 18 (Jensen *et al.*, 1992). Alternatively, an integrated anaerobic/aerobic defined bacterial co-culture was shown to successfully biodegrade the azo dye Mordant Yellow 3 (Field *et al.*, 1995). 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 Reactive Black and Acid orange 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 micro niches in which azo dye reduction can occur and second, to donate reducing equivalencies for the azo dye reduction. 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 Reactive Black 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 Reactive Black 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

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