ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2019; 24(5): 856-865
doi: 10.25083/rbl/24.5/856.865

Received for publication, July, 4, 2017
Accepted, March, 18, 2018

Original paper

Decolourization of four different azo dyes and their mixtures in anaerobic batch reactors

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Abstract Biological treatment of wastewaters containing textile dyes, particularly azo dyes, is very difficult under aerobic conditions. However, better decolourization of textile dyes can be obtained under anaerobic conditions. The azo bond (-N=N-), which is the chromophore group in the chemical structure of azo dyes, can be much more easily broken down under anaerobic conditions. This study aims at evaluating the anaerobic treatment potential of azo dye bearing textile wastewaters in terms oforganic matter removal, decolourization, and biogas generation. For this purpose, several 200 mL batch bioreactors containing anaerobic sludge, synthetic textile wastewater and azo dyes at various concentrations were incubated at 35ºC. The azo dyes used were Remazol Brilliant Violet 5R, Acid Orange 8, Naphtalene Blue Black and Remazol Black B. Significant biogas production was obtained in the presence of 10-500 mg/L of azo dyes. Hence, inhibitory effect of azo dyes on glucose biodegradation was limited even at high concentrations. Besides, decolourization efficiency reached almost 98% depending on the molecular structure of azo dyes. However, COD removal remained usually below 50% due to inhibition caused by aromatic amines, which are produced as a result of anaerobic degradation of azo dyes.

Keywords Anaerobic treatment, azo dyes, biogas, decolourization.

To cite this article: AKTAŞ O, NARIN YT, SAHINKAYA E. Decolourization of four different azo dyes and their mixtures in anaerobic batch reactors. *Rom Biotechnol Lett*. 2019; 24(5): 856-865. DOI: 10.25083/rbl/24.5/856.865

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Introduction

Most textile wastewaters are highly coloured, because they may contain dye at concentrations between 5 and 200 mg/L and even 1 mg/L of dyes can make the water coloured. Therefore dye-containing wastewaters may create serious esthetical problems in the receiving water bodies even at very low concentrations. Their colour, nonbiodegradability and toxicity also create problems in biological treatment systems. Particularly azo dyes, which constitute 60-70% of dyes used in textile finishing industry, are of major concern because of their toxicity (CERVANTES & al [1]; HAI & al [2]).

Dye-containing textile wastewaters can be treated by several physicochemical methods. However, these methods are usually very costly. Besides, chemical precipitation methods produce high amounts of sludge which results in further disposal problems. Therefore, biological systems are required for an efficient treatment of high amounts of dye-containing wastewaters. However, COD/BOD_5 ratios ranging between 3 and 4 in textile industry wastewaters show that these wastewaters may be hardly biodegradable. Particularly synthetic dyes such as azo dyes are refractory to microbial degradation and usually cannot be treated by conventional aerobic biological systems. The structural classification of dyes can be made according to the molecular structure and chromophore group of the dye molecule. Chromophore group is the light absorbing part of the dye molecule which also provides fixation of dye on the fabric. Azo dye is a general name for dyes characterized by having at least one azo bond (-N=N-). Azo dyes cause colour, turbidity and toxicity in receiving waters and may bioaccumulate in human tissue through food chain (ÇINAR & al [3]).

Literature studies have shown that it is possible to biologically degrade and decolourize azo dyes by anaerobic processes, where their reduction is mainly performed by fermentative bacteria (CERVANTES & al [1]). A study which involved biodegradation of an azo dye containing wastewater in a two-stage anaerobic system showed that colour removal mainly occurred in the acidogenic reactor rather than the methanogenic one (FIRMINO & al [4]).

Azo dye reduction is an electrochemical reaction in which azo dyes are used as final electron acceptors by microorganisms. During this reaction, azo bonds are cleaved by azo reductase enzyme. Therefore, the first step in decolourization of azo dye containing wastewaters is the formation of anaerobic condition, which providescleavage of azo bond by reduction. A carbon and energy source is always required for reduction of azo dyes by bacteria under anaerobic conditions. Only very few aerobic bacterial species can utilize azo dyes as a growth substrate (PANDEY & al [5]). On the other hand, some Fungi are capable of decolourizing some azo dyes (e.g. *Curvularia clavata* for Reactive Black 5) by utilizing them as the sole source of carbon and nitrogen (NEOH & al [6]). Glucose, ethanol, acetate, starch and organic substrates with more complex structures were used in previous literature studies (ÇINAR & al [3]). The released electrons from organic oxidation are used for azo dye reduction. Thereby, the azo bond, which is responsible for colour, is broken and decolourization is achieved. Since this reaction is inhibited by oxygen, it should occur under anaerobic conditions. Only very few special aerobic species (e.g. *Acinetobacter baumannii* YNWH 226) have the ability to decolourize azo dyes through specific azoreductase enzymes (LI & al [7]). It was shown in literature studies that anaerobic degradation of azo dyes are provided by some specific bacterial species through the activity of nonspecific azoreductase enzymes and these species dominated the microflora by a rapid growth in the presence of azo dyes (YOU & al [8]).

However, under anaerobic conditions, azo dyes are transformed into aromatic amines which are known to have toxic effects in biological wastewater treatment and also in receiving water bodies. On the other hand, aromatic compounds can be degraded through exclusion of the hydroxyl group (OH^-) and the succeeding ring cleavage at aerobic conditions. Aromatic amines can thus be mineralized under aerobic conditions after ring cleavage and hydroxylation. Therefore, after an anaerobic treatment of azo dye-containing wastewaters, usually an aerobic treatment step is required such that aerobic microorganisms can utilize aromatic amines as carbon source as well as other organic matter which may remain nonbiodegraded in a preceding anaerobic reactor. Although aromatic amines, the anaerobic degradation products of azo dyes, are known to be carcinogenic and more toxic than azo dyes, azodyes may exert more toxicity than their degradation products in some cases (SINGH & al [9]).

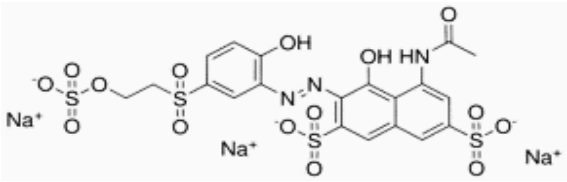
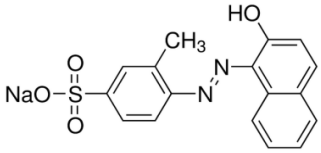
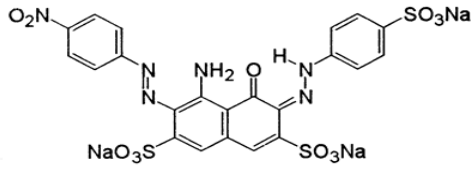
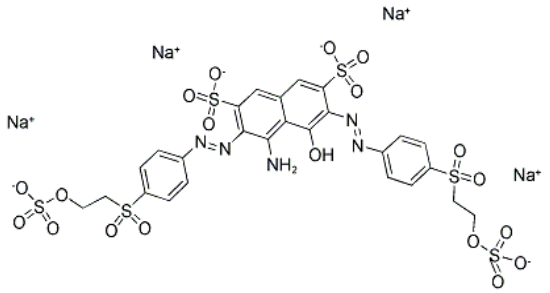
The present study aimed at investigating the degradation and decolourization of four azo dyes with different chemical structures and their mixtures at varying concentrations in batch vessels under anaerobic conditions in the presence of a growth substrate and nutrients. In addition, biogas formation and organic matter removal were also monitored to investigate the inhibitory effects of azo dyes or their degradation products on anaerobic microorganisms. The novelty of the paper is testing decolourization of mixtures of azo dyes using RES (color number) parameters together with biogas formation potentials.

Materials and Methods

1. Azo dyes used in the experiments

Water-soluble azo dyes with different chemical structures and chromophore characteristics were used in the study, namely Remazol Brilliant Violet 5R (RBV-5R), Acid Orange 8 (AO8), Naphthalene Blue Black (NBB), Remazol Black B (RBB) and their mixtures. The main characteristics of azo dyes used in the experiments are given in Table 1.

Table 1. Azo dyes used in the experiments

Dye	Chemical Formula	Molecular weight (g/mol)	Maximum absorbance wavelength (λ_{max})	Molecular structure
RBV-5R	$C_{20}H_{16}N_3Na_3O_{15}S_4$	735.587	560 nm	
AO8	$C_{17}H_{13}N_2NaO_4S$	364.35	488 nm	
NBB	$C_{22}H_{13}N_6Na_3O_{12}S_3$	718.54	610 nm	
RBB	$C_{26}H_{21}N_5Na_4O_{19}S_6$	991.82	595 nm	

2. Batch experiments

Anaerobic sludge, dye solution and synthetic nutrient mixture involving glucose with a total volume of 100 mL were added to 200 mL bottles. Bottles were screw-capped with aluminum cap and rubber septum in order to prevent air intake. Biogas accumulating in the head-space of bottles was regularly measured by water-displacement technique. In six different runs, organic matter and colour removal and biogas formation performances were tested for varying concentrations (10, 50, 100, 200 and 500 mg/L) of four azo dye types. Specific methanogenic activity tests were limited to measurement of overall biogas formation. These tests were performed to have an idea on anaerobic degradation and inhibitory characteristics of each azo dye used within the scope of the study. The concentrations of each dye used in the batch runs are shown in Table 2. Before starting the batch runs, each batch was fed with a synthetic nutrient solution involving sodium acetate for two times with 15 days of duration for each feeding. In total, three control reactors were incubated under the same conditions with the other batches. Two of the control reactors (C1 and C2) did not receive dye and synthetic solution. The other control reactor (C3) receivedsynthetic nutrient solution including glucose, but did not receive dye. Anaerobic granular sludge was obtained from a lab-scale UASB (up-flow anaerobic sludge bed) reactor and it was added to batches numbered 1-8 and control batches C1 and C2. The sludge concentrations in each batch bottle were 142 mg MLSS/L (106 mg MLVSS/L). On the other hand, batches numbered 9-10 and control batch C3 were seeded with a sludge taken from the anoxic-aerobic tank of a textile wastewater treatment plant. The same sludge was used for each batch in succeeding runs after the supernatant was discarded. The pH ranged between 7.3-7.8 in the batches. The batches were incubated at 35°C in a temperature controlled room. The synthetic nutrient solution used in the experiments consisted of 2000 mg/L glucose, 200 mg/L NH_4CL, 70 mg/L KH_2PO_4, 300 mg/L $NaHCO_3$, 25 mg/L $CaCl_2$, 30 mg/L $MgCl_2$, 40 mg/L $FeSO_4.7H_2O$, 5 mg/L $MnSO_4$,5 mg/L $CoCl_2.6H_2O$, 5 mg/L $CuSO_4.5H_2O$, 5 mg/L $ZnSO_4.7H_2O$, 2 mg/L $NiSO_4.6H_2O$. COD of the synthetic solution was about 2000 mg/L. Dyes were added from stock solutions of 10 g/L.

Table 2. Dye concentrations in batches (mg/L)-abbreviations of dyes

Batch No	Sludge	RUN 1	RUN 2	RUN 3	RUN 4	RUN 5	RUN 6
1	Anaerobic granule	10-RBV5R	10-RBV5R	100-RBV5R	100-RBV5R	50-mix	500-RBV5R
2	Anaerobic granule	100-RBV5R	100-RBV5R	200-RBV5R	200-RBV5R	500-RBV5R	500-AO8
3	Anaerobic granule	10-AO8	10-AO8	100-AO8	100-AO8	50-mix	500-NBB
4	Anaerobic granule	100-AO8	100-AO8	200-AO8	200-AO8	500-AO8	500-RBB
5	Anaerobic granule	10-NBB	10-NBB	100-NBB	100-NBB	100-mix	-
6	Anaerobic granule	100-NBB	100-NBB	200-NBB	200-NBB	500-NBB	-
7	Anaerobic granule	10-RBB	10-RBB	100-RBB	100-RBB	100-mix	-
8	Anaerobic granule	100-RBB	100-RBB	200-RBB	200-RBB	500-RBB	-
9	Anoxic-Aerobic	10-RBV5R	10-RBV5R	100-RBV5R	100-RBV5R	0	-
10	Anoxic-Aerobic	100-RBV5R	100-RBV5R	200-RBV5R	200-RBV5R	0	-

3. Analyses

COD, MLSS and MLVSS measurements were performed according to the *Standard Methods* (APHA-AWWA-WFC [10]). The bottles were sampled regularly by a syringe through the rubber septum for COD and colour analysis. Before colour and COD analyses, samples were centrifuged and then filtered through a 0.45 µm pore sized membrane. Colour was measured spectrophotometrically at the maximum absorbance wavelengths of each dye. Absorbance was also measured at three wavelengths: 436 nm (yellow), 525 nm (red) and 620 nm (blue) according to the new Turkish standards based on EN ISO 7887 and these were called as RES (colour number) parameters throughout the text.

Results and Discussion

COD, colour and biogas production were measured with respect to time in batch reactors. The results obtained for 6 runs are provided. In the first run, 10 and 100 mg/L of each dye were tested for a total duration of 19 days. COD removal ratios varied between about 20-70%. Similarly, biogas formation also ranged drastically and was between 22-97 mL. The variations in COD removal and biogas formation were related to differences in the anaerobic conditions of each bottle because of some air intake during samplings performed with a syringe. Methanogens are particularly inhibited by increased oxidation-reduction potential (ORP). Biogas formation was very low (2-5 mL) in control batches C1 and C2 which did not receive dye and synthetic solution as expected. On the other hand, biogas formation in the other control batch C3, which did not receive dye but received glucose-containing synthetic solution, was comparable (24 mL) with batches 9 and 10 (22 and 26 mL) which were seeded with the same anoxic-aerobic sludge. This showed that the RBV-5R dye was not inhibitory even to methanogens at a concentration up to 100 mg/L. Colour removal was also tested at maximum absorbance wavelength and at wavelengths corresponding to RES parameters (Figure 1). Colour was mostly removed during the first five days of RUN 1. Slight increases were observed in colour afterwards, and this was attributed to autooxidation of a portion of the aromatic amines back to dyes due to intake of some air during samplings.

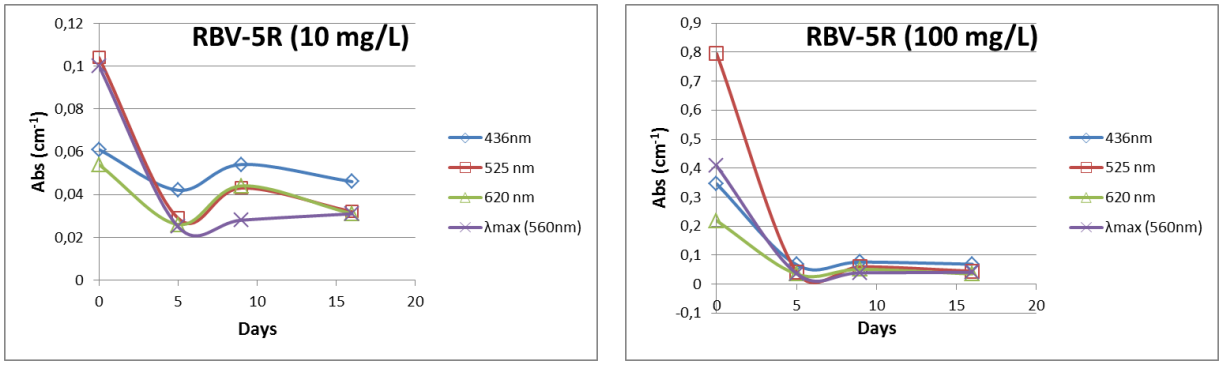


Figure 1. Colour removal in RUN 1 for RBV-5R (Batches 9 and 10) seeded with textile industry sludge.

In RUN 2, COD removal efficiencies were about 50±10%. In none of the batches, organic matter was completely converted to biogas. Biogas formation rate was about 0.30 L/gCOD$_{removed}$ on average. In anaerobic reactors, typical values for biogas formation are reported in the literature to be in the range of 0.10-0.30 L/gCOD$_{removed}$ (AYAZ & al [11]). The biogas formation was satisfactory in most of the batches except Batch 3 which was probably subject to oxygen intake (Figure 2). Biogas formation was much higher in batches 9 and 10 which were seeded with sludge taken from a textile wastewater treatment plant. Even though this seed sludge was not obtained fromstrictly anaerobic conditions, it was acclimated to dye-bearing textile wastewater. Colour removal occurred particularly in the first 2 or 4 days of the batches (Figure 3). Colour removal efficiencies obtained by measurement of absorbance at λ_{max} were 86%, 98%, 80% and 96% respectively for azo dyes RBV-5R, AO8, NBB and RBB at an initial dye concentration of 100 mg/L. Reliable colour removal ratios could not be obtained at dye concentrations of 10 mg/L because of biosorption and desorption of dyes, continuously occurring during the batches. Investigation of colour removal in terms of removal of RES parameters revealed that discharge limits of 0.09, 0.07 and 0.05 cm^{-1}as given in the regulations, respectively for absorbances at 436 nm, 525 nm and 620 nm, could not be achieved in most of the batches. However, the results showed that anaerobic treatment could achieve effluent colour very close to these limits. Final absorbances were 0.111, 0.101, 0.208 and 0.303 cm^{-1} at 436 nm, 0.036, 0.026, 0.099 and 0.173 cm^{-1} at 525 nm, and 0.026, 0.017, 0.137 and 0.174 cm^{-1} at 620 nm, respectively for azo dyes RBV-5R, AO8, NBB and RBB.

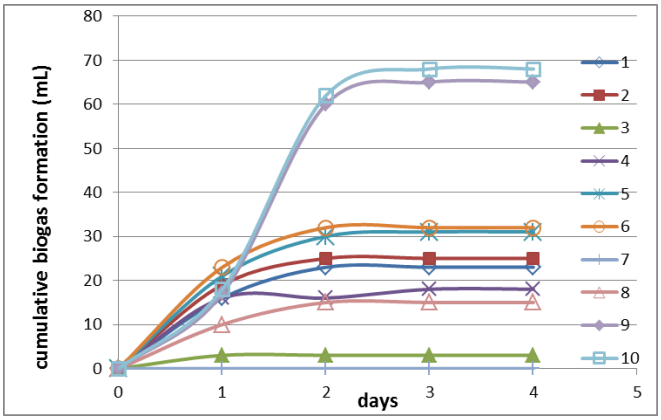


Figure 2. Biogas formation in RUN 2 (Batches 1-10).

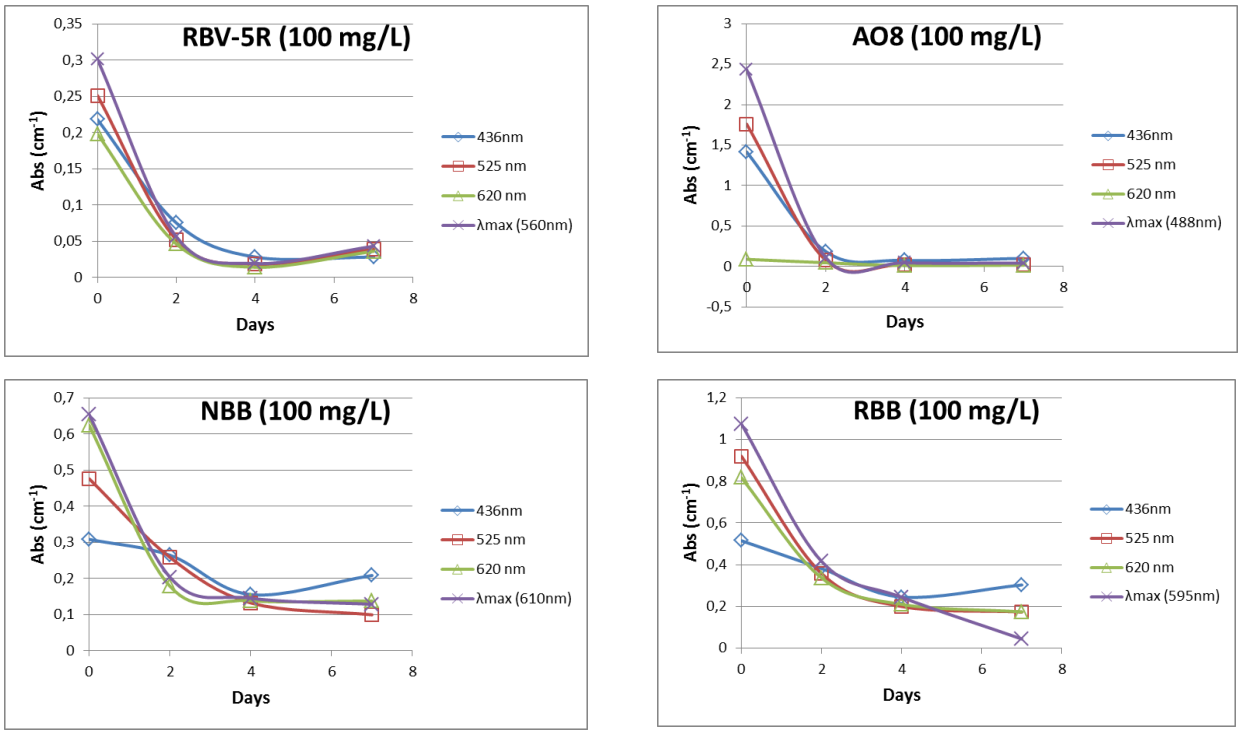


Figure 3. Colour removal in RUN 2.

Initial dye concentrations were increased up to 200 mg/L in RUN 3. Similar to previous runs, COD removal partially occurred. However biogas formation was completed within 2 days (Figure 4). More rapid biogas formation compared to previous runs can be explained by acclimation of methanogens since the same sludge had been used in preceding runs. Biogas formation ranged drastically from zero up to 62 mL depending on the formation of anaerobic conditions. Differences were not significant between the batches with initial dye concentrations of 100 and 200 mg/L. Colour removal was also observed within the first two days of the run for RBV-5R and AO 8 dyes. However NBB dye continued to be degraded until the fifth day although most degradation occurred during the first two days. Degradation of RBB was much slower. Atinitial RBB concentration of 200 mg/L, absorbance at λ_{max} decreased to 1 cm^{-1} from an initial value of 3 cm^{-1} with a removal efficiency of approximately 67% at the end of two days, and further decreased to 0.5 cm^{-1} at the end of the 5^{th} day and 0.4 cm^{-1} at the end of the 12^{th} day. Also in the case of 100 mg/L initial RBB concentration, the final absorbance was as high as 0.3 cm^{-1}, which was much higher than the other dyes. This showed that RBB was less biodegradable compared to the other three azo dyes. The final absorbances were in the increasing order of AO8, RBV-5R, NBB and RBB. Colour removal ratios were 98% for AO8, 90% for RBV-5R and NBB, and 87% for RBB.

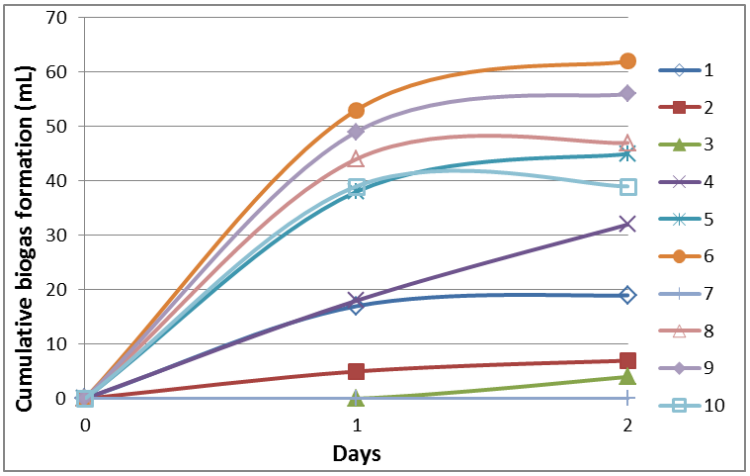


Figure 4. Biogas formation in RUN 3 (Batches 1-10).

RUN 4 was a repetition of RUN 3 with the same initial dye concentrations. Therefore the results obtained were very similar to RUN 3. However, colour removal efficiencies were higher in this run with 97% for RBV-5R, 98% for AO8, 96% for NBB and 93% for RBBat the end of 11 days. Final absorbance values at λ_{max} were 0.024 cm^{-1} and 0.042 cm^{-1} for RBV-5R, 0.077 cm^{-1} and 0.096 cm^{-1} for AO8, 0.042 cm^{-1} and 0.099 cm^{-1} for NBB, 0.165 cm^{-1} and 0.384 cm^{-1} for RBB, respectively at initial dye concentrations of 100 and 200 mg/L. Similar to RUN 3, highest colour remained at the end of the batches with RBB.

Single dye solutions for each dye at 500 mg/L and dye mixtures containing 50 and 100 mg/L of each dye were tested in RUN 5 (see Table 2). Biogas formation occurred within the first two days even at the highest dye concentrations of 500 mg/L (Figure 5). However the highest biogas formation occurred in batches 9 and 10 which were only fed with glucose-containing synthetic solution and did not receive any dye. Lower biogas formations in the dye-added batches can be attributed to inhibition caused by the aromatic amines formed as a result of anaerobic degradation of dyes rather than the azo dye itself, because otherwise dye-mediated inhibition would prevent biogas formation even at the start of the run. Lowest biogas formation was in batch 6 which received 500 mg/L NBB. Removal of colour and RES parameters occurred particularly in the first two days in parallel to biogas formation and no colour removal occurred after the 7^{th} day. Colour removal had the highest efficiency and rate for RBV-5R. For AO8, removal efficiency was similarly high, but the rate was lower. Decolourization was faster for NBB and RBB compared to AO8, but the remaining colour was much higher for these dyes (Abs at λ_{max}: 2.5 cm^{-1} for NBB and 0.59 cm^{-1} for RBB). Removal of RES parameters also occurred within the first 2 days in the batches with dye mixtures of 50 and 100 mg/L of each dye (Figure 6).

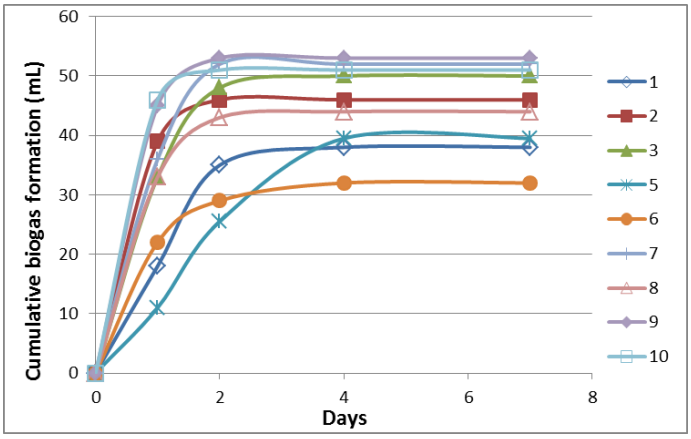


Figure 5. Biogas formation in RUN 5 (Batches 1-10).

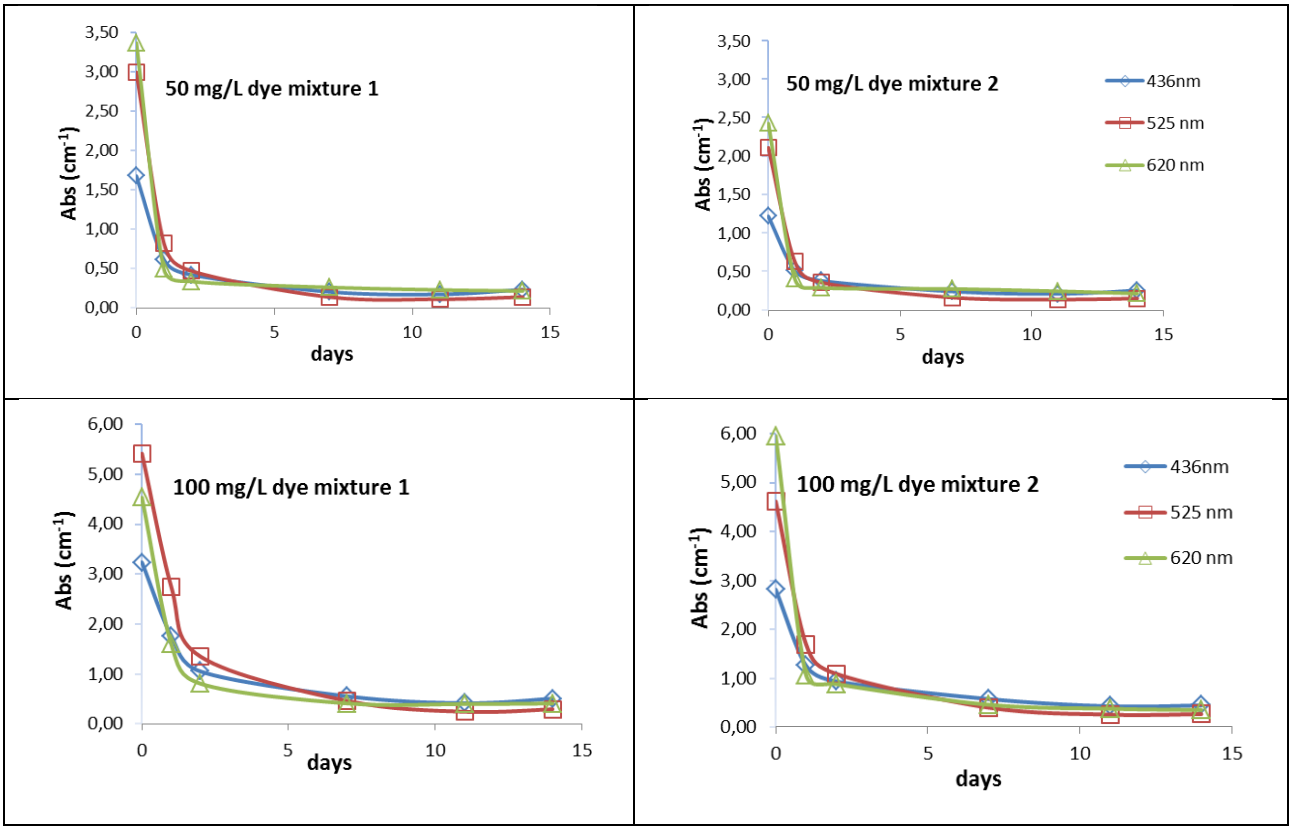


Figure 6. Removal of RES parameters from dye mixtures in RUN 5.

In RUN 6, batches with 500 mg/L of each dye were repeated (see Table 2). The difference of this run from the previous ones was that biogas formation was very low in the first two days, and mainly occurred during the following two days. The reason for this was that, bacteria required an adaptation period since one month had passed after the last feeding of the bottles, which had been done in RUN 5. Also, inhibition caused by high dye concentrations probably delayed the activity of microorganisms. Cumulative biogas formations were 60, 44, 36 and 58 mL for RBV-5R, AO8, NBB and RBB dyes, respectively. In this run, the least biogas production occurred in the batch involving 500 mg/L NBB indicating that the highest inhibition was caused by this dye or its biodegradation products. The higher inhibition caused by NBB can be attributed to the presence of a nitro (-NO_2) group in its structure, and azo dyes with a nitro group are known to be much more toxic than the aromatic amines generated in their reduction (MENDEZ-PAZ & al [12]). On the other hand, sulfonic groups, which were present in the four dyes of our study, are also considered as a rate limiting factor in azo dye reduction and also known to pose a more recalcitrant character for degradation of aromatic amines at anaerobic conditions (MENDEZ-PAZ & al [12]). Also, sulfonated aromatic amines are more resistant to mineralization in aerobic conditions (PANDEY & al [5]). Colour

removal also occurred mostly between days 2 and 4 similar to biogas formation. The results obtained in this and previous runs showed that colour removal and biogas formation occurred in parallel. That means, reduction of dyes occurred simultaneously with biodegradation of glucose. Table 3 shows colour removal at λ_{max} and in terms of RES parameters. Efficiencies were between 89 and 99% even at such a high dye concentration of 500 mg/L. The smallest removal efficiencies and highest final concentrations were obtained with NBB. Figure 7 shows the colour removal profiles for each dye. Lowest absorbances were obtained for RBV-5R and AO8 at the end of the batches.

Table 3. Decolourization in RUN 6 at initial dye concentrations of 500 mg/L

Dye	λmax (nm)	Initial $Abs_{\lambda max}$ (cm^{-1})	Final $Abs_{\lambda max}$ (cm^{-1})	Removal ratio (%)
RBV-5R	560	1.585	0.087	94.5
AO8	488	8.684	0.080	99
NBB	610	17.072	1.928	88.7
RBB	595	8.976	0.225	97.5

The results showed that decolourization of azo dyes were dependent on their molecular structures. The azo dyes RBV-5R and AO8, which involved three aromatic rings in their molecular structures, were found to be more biodegradable than NBB and RBB, which involved four aromatic rings. The results obtained in our study were in accordance with literature studies, which also showed that different azo dyes had different biodegradabilities. For example in one study, 150-2400 mg/L of Reactive Black 5 and Reactive Red 24 azo dyes with concentrations of 150-2400 mg/L were degraded with efficiencies of 93.4-99.8% within 24 hours in batch anaerobic reactors, whereas another azo dye Reactive Blue 49 could only be treated with efficiencies of 16.5-22.9% within 72 hours (KARATAŞ [13]). Another study for decolourization of an azo dye in the presence of a synthetic solution in an anaerobic membrane bioreactor showed that COD removal remained at about 55-60% similar to our study (SPAGNI & al [14]). In the same study, methane production was inhibited at a level of 80-85% at a much higher concentration (3200 mg/L) of an anaerobic dye Reactive Orange 16 (SPAGNI & al [14]). However, inhibition of biogas production was much lower at dye concentrations of less than 500 mg/L in our study.

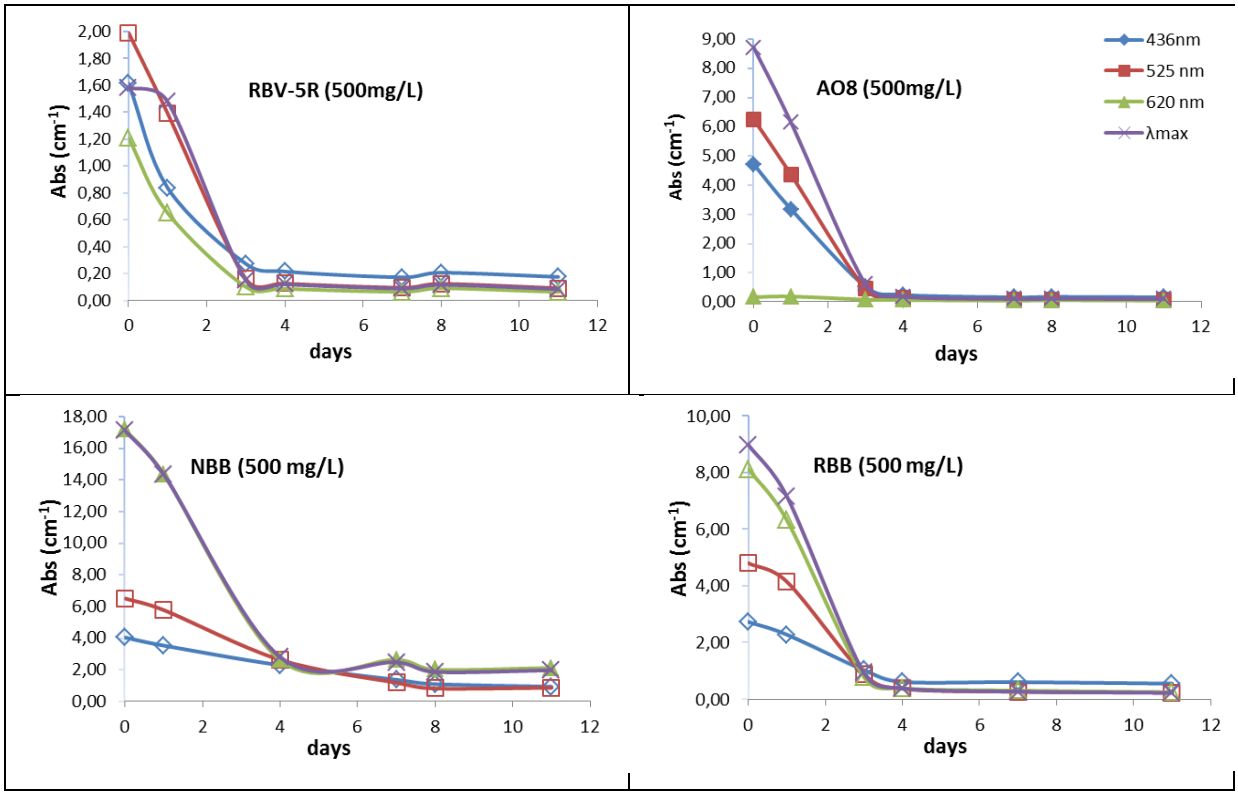


Figure 7. Colour removal (at λ_{max} and RES parameters) in RUN 6 at initial dye concentrations of 500 mg/L.

In a continuous bioreactor, higher organic matter removal efficiencies and lower effluent dye concentrations can be expected. In our previous study with a synthetic textile wastewater involving only RBV-5R dye, almost complete colour removal and more than 95% COD removal was observed in a continuous-flow anaerobic membrane bioreactor (MBR), whereas colour removal was only 30-50% in the parallel aerobic MBR (YURTSEVER & al [15]). Therefore, the inhibition of methanogenic activity in the present study should be predominantly attributed to the accumulation of aromatic amines at batch conditions rather than the azo dye itself. Fast biogas formation in the first day, even at the highest dye concentration of 500 mg/L, supports this hypothesis. In accordance with our results, MENDEZ-PAZ & al [12] reported that azo dye Acid Orange 7 had no toxic effects on the anaerobic microorganisms although they are known to be toxic to every living cell. This contradictory situation can be explained by diffusion limitation. Azo dye toxicity is supposed to be caused by intervention of these compounds between DNA base pairs. However, permeation of azo dye through the cell membrane is the rate limiting step for intracellular degradation process (CHANG & al [16]). Therefore, a non-specific and presumably extracellular mechanism was proposed by several researchers (MENDEZ-PAZ & al [12]; GONÇALVES & al [17]). Hence, cell extracts are expected to more efficiently reduce azo dyes compared with the entire cells, particularly in the case of sulfonated azo dyes (MENDEZ-PAZ & al [12]) as in the case of our study testing four different sulfonated azo dyes. On the other hand, aromatic amines can much more easily permeate through the cell membrane and cause toxicity (MENDEZ-PAZ & al [12]). A more recent study showed that azo dyes with concentration of 600 mg/L could causesignificant inhibition on overall (decolorizing and methanogenic) performance of anaerobic methanogenic wastewater treatment due to enrichment effect of azo dyes in tightly bound extracellular polymeric substances (DAI & al [18]). Another recent study showed the importance of acclimation in anaerobic biodegradation of dyes including azo dyes. It was shown that species belonging to Acidobacteria, Firmicutes, Bacteroidetes, Chloroflexi and Proteobacteria dominated the microflora in the acclimated sludge (CUI & al [19]).

Conclusion

Anaerobic batch studies showed that four different azo dyes (Remazol Brilliant Violet 5R, Acid Orange 8, Napthalene Blue Black, Remazol Black B) were successfully decolourized at concentrations between 10 and 500 mg/L in the presence of 2000 mg/L COD-equivalent glucose and nutrients. Colour removal efficiency was as high as about 90-99% even at the highest initial dye concentration of 500 mg/L. The colour removal efficiencies and the remaining dye concentrations differed greatly with respect to the azo dye. Higher dye biodegradation was obtained with RBV-5R and AO8 dyes, and biodegradability was lower for NBB and RBB dyes. Inhibition of methanogens, as monitored by biogas formation, was attributed to the aromatic amines formed during the anaerobic process rather than the azo dye itself. Therefore, considering the toxicity and carcinogenicity of the formed aromatic amines, further aerobic treatment is recommended in order to treat the aromatic amines produced during the anaerobic process.

Acknowledgements

This work was supported by Istanbul Medeniyet University Scientific Research Project Funding (IMU BAP) under Grant number FBA-2013-354.

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