

The effect of biological sulfate reduction on anaerobic color removal in anaerobic–aerobic sequencing batch reactors

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Abstract Combination of anaerobic–aerobic sequencing processes result in both anaerobic color removal and aerobic aromatic amine removal during the treatment of dye-containing wastewaters. The aim of the present study was to gain more insight into the competitive biochemical reactions between sulfate and azo dye in the presence of glucose as electron donor source. For this aim, anaerobic–aerobic sequencing batch reactor fed with a simulated textile effluent including Remazol Brilliant Violet 5R (RBV 5R) azo dye was operated with a total cycle time of 12 h including anaerobic (6 h) and aerobic cycles (6 h). Microorganism grown under anaerobic phase of the reactor was exposed to different amounts of competitive electron acceptor (sulfate). Performance of the anaerobic phase was determined by monitoring color removal efficiency, oxidation reduction potential, color removal rate, chemical oxygen demand (COD), color, specific anaerobic enzyme (azo reductase) and aerobic enzyme (catechol 1,2-dioxygenase), and formation of aromatic amines. The presence of sulfate was not found to significantly affect dye

decolorization. Sulfate and azo dye reductions took place simultaneously in all operational conditions and increase in the sulfate concentration generally stimulated the reduction of RBV 5R. However, sulfate accumulation under anaerobic conditions was observed proportional to increasing sulfate concentration.

Keywords Anaerobic color removal · Sulfate · Remazol Brilliant Violet-5R · Sequencing batch reactor

Introduction

The control of water pollution has become of increasing importance in recent years due to the increase in population, development, and assortment of industries. In particular, increasing demand for textile products makes textile industry one of the main sources of water pollution problems. In fact, the main problem comes from dyes which are mainly used to color synthetic and natural fabrics. Azo dyes are the largest class of dyes due to more economical synthesis. Because the release of dyes together with their breakdown products into the environment constitutes serious problems, the release of colored effluents into the environment is undesirable [1]. Most dyes are known toxic; however, their components and breakdown products can be more toxic. To overcome this problem, much attention has been focused on the effective treatment of dyes discharged from the dyeing and textile industries. Biological methods are commonly considered to be the most effective treatment applications since they present lower operating costs and improved applicability [2, 3]. Biological processes applied for decolorization of textile effluents are based on anaerobic and aerobic treatment. Though anaerobic treatment removes the color of the dye, aromatic amines

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resulting from decolorization process are not mineralized under anaerobic conditions and tend to accumulate to toxic levels [4, 5]. This has led to the development of two-stage sequencing anaerobic–aerobic biological treatments, in which the reduced breakdown products are oxidized in the second aerobic stage. Hence, combination of anaerobic aerobic sequencing processes result in both anaerobic color removal and aerobic aromatic amine removal during the treatment of dye containing wastewaters [6–10].

So far, so many reactor types have been used to provide effective treatment for textile wastewaters such as fluidized bed, upflow anaerobic sludge blanket reactors, and packed bed reactors [11, 12]. Recent studies have indicated the success of sequential biological systems in achieving the complete biodegradation of azo dyes [3, 13]. Sequencing batch reactor (SBR) systems, applied to anaerobic color removal and aerobic aromatic amine biodegradation offer various advantages including minimal space requirements, ease of management, and possibility of modifications during trial phases through on-line control of the treatment strategy. The main steps in a SBR system includes fill, react, settle, decant, and idle. That fill, react, settle and draw operations can be provided in a single reactor make the SBR operation flexible. In SBR operations, a cycle is repeated continuously and all the operations can be achieved in a single reactor. SBR has the ability for achieving the complete biodegradation of azo dyes by providing decolorization of textile wastewaters with the added possibility of metabolite mineralization in the aerobic period [2, 14, 15]. SBR can tolerate mechanism of microorganism. Since the reaction time can be adjusted in SBRs, anaerobic performance of a biological reactor with often-variable organic loads can be easily controlled.

Classically, anaerobic color removal is based on oxidation–reduction reactions [12, 16–18]. The failure of many anaerobic bioreactors to operate reliably and with constant performance has underlined the need for more basic information on the biological color removal, parameters affecting color removal should be determined. One of the most important points to be emphasized is availability of the electron acceptors in the anaerobic phase. As anaerobic color removal occurs by the way of reduction of the azo dye which acts as final electron acceptor in the microbial electron transport chain, the presence of competitive electron acceptors in anaerobic zone can be a rate-controlling factor for the dye removal [19]. Competitive electron acceptors such as oxygen, nitrate, sulfate, and ferric ion may compete with the azo dye for reducing equivalents and result in insufficient color removals under anaerobic conditions. Sulfate is normally found in textile processing wastewaters and generally coming from the salts added to the dye baths for ionic strength adjustment [20]. The effect of sulfate on anaerobic color removal efficiency differs greatly in literature. It has been reported

in so many reports that sulfate has different effects on azo dye biodegradation. Color removal may be either stimulated or competitively suppressed [14, 20–22]. When sulfate is reduced under anaerobic conditions by sulfate-reducing bacteria (SRB), sulfide, which is known as bulk reductant, is generated and can in turn serve as an electron donor. Sulfide generation is found to also contribute to the reduction of azo dyes [20, 23, 24].

It is unclear sulfate has adverse effect or has an inconclusive effect. In this research, the relative contribution of azo reduction mechanisms was investigated with respect to the role of sulfate. The purpose of the study was to assess whether there is deterioration of azo degrading capacity of microorganism over time after the sulfate reducing conditions in anaerobic–aerobic sequencing batch reactor.

Materials and methods

Simulated textile wastewater

The synthetic textile effluent fed to the reactor was composed of nutrient elements required for microbiological growth. All inorganic nutrients were provided in excess, making textile dye the limiting substance. The following nutrients in mg were used to prepare 1 L of SBR feed: NH_4Cl (80), K_2HPO_4 (35), KH_2PO_4 (30), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (500), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (367), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (5), H_3BO_3 (0.04), ZnCl_2 (0.05), $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.038), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.5), $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (0.05), $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ (0.09), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (1), $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (0.092), NaCl (127) and EDTA (5.4).

Electron sources

Glucose (contributed to COD of 1,085 mg/L) was used to provide readily biodegradable carbon source, and at the same time to provide the electrons for the reductive cleavage of the azo dye. Azo dye with a concentration of 100 mg/L was used to ensure the colored wastewater in each cycle. Electron acceptors used for the experiments were azo dye and sulfate. The studied azo dye was the monoazo reactive dye Remazol Brilliant Violet 5R (C.I Reactive Violet 5), (Sigma-Aldrich, Germany). For the experiments for sulfate, the chemical sodium sulfate was used with a concentration up to 300 mg/L (see Table 1). It should be noted that the measured sulfate concentration at the beginning of the anaerobic cycles were different from the additional amount of sulfate to the reactor.

Reactor setup and experimental design

Sludge from a textile wastewater treatment plant was used to start a SBR; however, during the study two identical

Table 1 Electron sources and concentrations in the reactors

Name of compounds	Role of compounds	Concentration of compounds
Glucose	Electron donor	1,120 mg/L
Azo dye	Electron acceptor	100 mg/L
Sulfate	Electron acceptor	Up to 300 mg/L SO ₄ ²⁻

SBRs were used. SBR I was continuously operated under control conditions. It was the reactor for the sludge enrichment under control conditions in which the electron acceptor was only azo dye. After the sludge was enriched the SBR II was started. Data were always taken from the SBR II. SBR II was operated under control and sulfate-reducing conditions to observe effect of sulfate on azo dye reduction. Each SBR consisted of a 6.5-L vessel (Bioflo 110, New Brunswick Scientific Co., Edison, NJ, USA) with an effective working volume of 5 L. The contents of the SBRs were mixed by a single-shaft impeller system at a speed of 450 rpm. An internal pH controller (Bioflo 110 system) was used to control the pH between 7.2 and 7.3 by adding 0.2 M HCl and 0.2 M NaOH. The temperature was maintained at (25 ± 0.5) °C. Each SBR system was operated with a solid retention time (SRT) of 15 days. Care was taken to totally exclude atmospheric oxygen from the SBRs by continually sparging with nitrogen gas during anaerobic reaction phases. Oxygen was provided in excess to the aerobic phase of the SBRs by air pumps to maintain the aerobic cycles of study runs. Oxidation–reduction potential (ORP) in the reactors was measured continuously using a ORP Meter (M 300, Mettler Toledo, Greifensee, Switzerland) equipped with a Redox Electrode. Operation of the SBRs was based on 12 h total cycles consisting of a start phase (10 min), an influent phase (3 min) in which 2.5 L fresh medium was supplied, an anaerobic reaction phase (6 h), an aerobic reaction phase (6 h), a biomass withdrawal phase (5 min) in which 300 mL (15 days SRT, considering also biomass removed with the effluent) mixed reactor liquor was withdrawn, a settling phase (30 min), and an effluent withdrawal phase (5 min). The effluent was

withdrawn in the middle of the reactor, leaving a working volume of 2.5 L at the beginning of the next cycle. Summary of operational conditions tested for color removal was shown in Table 2.

Experiments were initiated with sludge inoculation to the SBR I. Sludge was collected from the wastewater treatment plant (Kahramanmaraş, Turkey) that receives colored textile effluents. The sludge mixed with simulated wastewater was transferred to the SBR I. The reactor was operated initially for 90 days after being started with the sludge from textile wastewater treatment plant. Further, the enriched biomass from the SBR I was equally divided into two SBRs to start the experiments. SBR I was continuously operated under control conditions in which electron acceptor was only azo dye. SBR II was operated through control and run 7 under control and sulfate-reducing conditions to investigate the effect of sulfate on anaerobic azo dye reduction. The reactors were operated initially for 90 days after being started with the sludge from textile wastewater treatment plant. Anaerobic cycles were monitored after the steady state in the SBR was reached. The SBR was considered to be running stable when for at least 3 days, COD removal, sulfate removal, color removal, and ORP profiles within a cycle were constant. All assays were run in triplicate, and data given in the figures were averages of the replicates.

Analytical procedures

Color was measured at maximum wavelengths of 560 nm in spectrophotometer (Chebios Optimum-One UV–VIS Spectrometer, Rome, Italy). The samples were centrifuged (Eppendorf Centrifuge 5415R, Hamburg, Germany) at 10,000 rpm for 5 min and the absorbance values of supernatants were recorded for color measurements. Biomass concentration was measured as absorbance at 600 nm followed by reference to an experimentally derived standard curve ($ABS_{600\text{ nm}} = 0.9529\text{ MLSS} + 320.74$; $R^2 = 0.9799$). The soluble COD was measured calorimetrically. First the samples were centrifuged for 5 min at 10,000 rpm.

Table 2 Summary of operational conditions tested for color removal

	Electron donors and COD equivalents	Electron acceptors and COD equivalents ^a
Control	Glucose 1,085 COD	Only Azo dye
RUN 1 (R1)	Glucose 1,085 COD	Azo dye and 70 mg/L SO ₄ ²⁻ –47 COD
RUN 2 (R2)	Glucose 1,085 COD	Azo dye and 120 mg/L SO ₄ ²⁻ –80 COD
RUN 3 (R3)	Glucose 1,085 COD	Azo dye and 180 mg/L SO ₄ ²⁻ –120 COD
RUN 4 (R4)	Glucose 1,085 COD	Azo dye and 190 mg/L SO ₄ ²⁻ –127 COD
RUN 5 (R5)	Glucose 1,085 COD	Azo dye and 360 mg/L SO ₄ ²⁻ –241 COD
RUN 6 (R6)	Glucose 1,085 COD	Azo dye and 480 mg/L SO ₄ ²⁻ –320 COD

^a Only COD equivalents of sulfate were calculated (–0.667 g COD/g SO₄²⁻); sulfate concentrations measured in the reactor (beginning of the anaerobic cycle) were considered in the table

Sulfide was measured using commercially prepared HACH testing kits (HACH DR/5000). Methods were the current US EPA approved (Standard Method COD, 5220D; Standard Method Sulfide (S^{-2} , 4500D) and procedures are equivalent to Standard Methods [25]. The total dissolved organic carbon concentration was determined by a TOC analyzer (Teledyne Tekmar, Torch, USA) equipped with an auto sampler.

An ion chromatography (Dionex ICS-3000) was used to measure the concentrations of sulfate and sulfite (Dionex, Sunnyvale, CA, USA) with Ion Pac AG19 guard and AS19 analytical columns. Eluent containing 8 mM sodium carbonate $Na_2(CO_3)$ and 1.5 mM sodium hydroxide (NaOH) was prepared and used for the sulfate and sulfite ion analyses with a flow rate of 1 ml/min.

The formation of intermediates from the degradation of RBV-5R was analyzed by use of an HPLC system equipped with SPD-M20A diode array detector (DAD) (Shimadzu Co. Kyoto, Japan). Column used in HPLC was of Inertsil ODS-3 V (250 mm \times 4.6 mm ID 5 μ m). The mobile phase used for separation of aromatic amines was a mixture of acetonitrile and water in a 3:7 ratio. The flow rate was 1 ml/min. The UV-visible detector was set at 254 nm [17]. Aromatic amine standards were generated by the use of sodium dithionite ($Na_2S_2O_4$) for the cleavage of the $-N=N-$ linkage followed by HPLC analysis of the reduction products of RBV-5R. Chemical reduction was performed under 65 °C with a dit/col (w:w) ratio of 10. Chemical reaction time was adjusted to 5 min.

Enzyme assays

Azo reductase enzyme which is released extracellularly, known to be responsible for the reduction of azo dyes. The key enzyme used as indicator of aerobic aromatic amine biodegradation was catechol 1,2-dioxygenase (C12DO). Cell-free extract (CFE) was prepared for quantifying the activities of the key enzymes involved. For the enzyme assays, approximately 25 mL of bacterial suspension was taken from the experimental reactor per hour and centrifuged at 6,000 rpm for 10 min using Heraeus Sepatech Labofuge 200 (Heraeus Instruments, Osterode, Germany). After the supernatant was discarded, pellet was resuspended and washed in phosphate buffer (20 mM phosphate buffer, pH 7.5). The bacteria were then pelleted at 6,000 rpm for 10 min. After the supernatant was discarded, pellet was resuspended with approximately 1.75 mL of 20 mM phosphate buffer at pH 7.2 and transferred to a plastic bead-beater tube filled with one-third of 0.1 mm diameter glass beads. The concentrated cells were broken open using a Mini-Beadbeater (Biospec Products, Bartlesville, OK, USA), run at the maximum speed two times for 20-s disruptions, with 20-s breaks over ice packs between

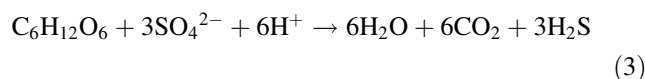
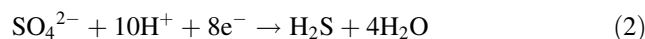
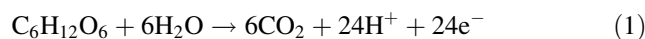
disruptions. The disrupted cells were centrifuged in a microcentrifuge (Eppendorf Centrifuge 5415R, Hamburg, Germany) for 10 min at the maximum power to remove cell debris. The supernatant was the CFE, which was collected and tested for enzyme assays and protein concentration.

A Chebios UV/VIS spectrophotometer (Optimum-One, Chebios s.r.l., Rome, Italy) was used all enzyme assays. The activity of catechol 1,2-dioxygenase (C12DO) was assayed spectrophotometrically by recording the increase in absorbance at 260 nm. Assays were performed in a 1-cm path-length cell. Reaction mixtures (3 ml) containing 2,500 μ l reagent (50 mM Tris-HCl and 10 mM EDTA at pH 7.5) and 250 μ L catechol (10 mM) were equilibrated before addition of CFE (250 μ l) [26]. One unit (U) of enzyme activity was defined as the amount of enzyme that formed 1 mmol of *cis*, *cis*-muconic acid per minute ($\epsilon_{257}=16,800 M^{-1} cm^{-1}$) [27]. The activity of azo reductase was monitored by the change of absorbance at 560 nm with time in mixtures of cell free extract (500 μ L), 1 mM NADH (500 μ L), and enough reagent to make a total volume of 3,000 μ L. The reagent contained a solution of 100 mM K_2HPO_4 , 100 mM KH_2PO_4 , and 0.2 mM RBV-5R [28]. One unit (U) of enzyme activity was defined as the amount of enzyme to decolorize 1 μ mol dye per minute. The protein concentrations were required to calculate specific enzyme activities. Protein concentration was measured using the bicinchoninic acid-copper reaction in which proteins reduce Cu^{+2} to Cu^{+1} in alkaline solution [29]. Bovine serum albumin was used as the standard. A UV-VIS Spectrophotometer (Optimum-One, Chebios s.r.l., Rome, Italy) with a 1-cm light path was used at a wavelength of 562 nm to determine the protein concentration from a standard curve.

Results and discussion

Anaerobic sulfate reduction

The biological sulfate reduction process is mediated by a group of microorganisms known as sulfate-reducing bacteria (SRB). With the supplementation of glucose in the SBR, sulfate is biologically reduced under anaerobic conditions and eight electrons are added to sulfate to make sulfide as shown in the following reactions (Eqs. 1–3).



Sulfate reduction reaction can take part at suitable reduction potentials (E_0). According to the reduction

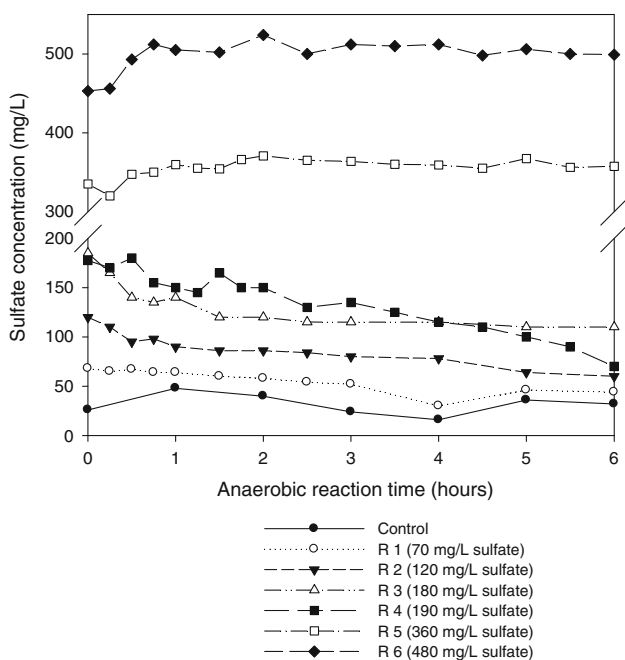


Fig. 1 Sulfate reduction profile in the anaerobic phase

potentials, sulfate is a less favorable electron acceptor than oxygen (O₂) and nitrate (NO₃⁻). To be able to maximize the sulfate reduction, the reduction potential of the environment should be negative. It has been long known that SRBs are strict anaerobes and oxygen has toxic effect on SRBs [30].

Figure 1 summarizes the sulfate removal profiles of the mixed culture exposed to different amount of sulfate concentrations. In our investigation sufficient amount of substrate was supplied to overcome the negative effects of elevated concentrations of sulfate. Control conditions describe the operating conditions of anaerobic phase that receives only azo dye as an electron acceptor (no supplementation of sulfate). During control conditions, however; it was observed that sulfate was released into the anaerobic phase which was probable linked to biodegradation of RBV-5R azo dye (originating from the dye itself). For this reason, sulfate-reducing conditions already took place in the reactor before supplementation of sulfate. The observed sulfate concentration under control conditions was around 20 mg/L as shown in Fig. 1.

Anaerobic sulfate reduction reactions generally perform slowly and need long reaction times. More sulfate addition to the SBR increased the time required for the sulfate reduction. As expected, when sulfate was used as electron acceptor, sulfide and sulfite formation was observed. The maximum sulfate reduction was observed under R4 in which sulfate concentration was about 190 mg/L at the beginning of anaerobic phase. The obtained sulfide and sulfite concentrations were 12 and 75 mg/L; respectively.

The detection of sulfide and sulfite in the same mixed liquor confirmed the occurrence of sulfate reduction. The reactor (R5-R6) showed a poorer sulfate removal capacity than other operational cycles as shown in the Fig. 1. The poor anaerobic sulfate removal yields can be explained by the alternate anaerobic–aerobic phases and adverse effect of aerobic conditions to SRBs. Additionally, sulfides produced under anaerobic conditions can be reoxidize under aerobic conditions leading increased sulfate accumulation at the beginning of the anaerobic phases. In all operational conditions sulfate accumulation was observed, especially when the azo degrading activity out-competed sulfate-reducing activity.

Effect of sulfate on ORP profile

Anaerobic color removal process is based on oxidation reduction reactions in which azo dye acts as final electron acceptor. The sequence of electron accepting processes is differentiated according to the free energy gained in the respective catabolism, which depends on the redox potential difference between electron donors and acceptor. Therefore, to achieve effective color removal, anaerobic conditions with a low redox potential (*E'*₀) is desired. Standard redox potential values of azo dyes are unknown; however; the Dubin and Wright [31] found that redox potential values could be between -430 and -180 mV.

In this study, the electron donor source (Glucose/CO₂; -430 mV) was added at the beginning of the anaerobic reaction phase together with the electron acceptors (azo dye and sulfate). For control conditions azo dye was the only electron acceptor; for the other conditions both sulfate (with increasing concentrations) and the azo dye were the electron acceptors. Standard redox potential of sulfate reduction is *E'*₀ = -220 mV for SO₄²⁻/H₂S, *E'*₀ = -116 mV SO₃²⁻/H₂S for sulfite, and *E'*₀ = -280 mV S⁰/H₂S for elemental sulfur [13, 32]. When compared with the redox potentials of the azo dye and the sulfate it can be concluded that the redox potential level of azo reduction is close to that of sulfate respiration.

As shown in Fig. 2, the ORP values showed variation during anaerobic phase. At the beginning of the anaerobic phase, ORP values obtained between -64 and -166 decreased to between -166 and -290 at the end of the anaerobic reaction phase. Positive ORP values between +30 and +59 were measured during the aerobic phase (results not shown).

As glucose was oxidized at the beginning of the anaerobic reaction phase, sharp decrease in the ORP values were observed and when the sulfate concentration in the reactor was increased, ORP values were decreased (data in 30 min.). ORP values were always low when the sulfate

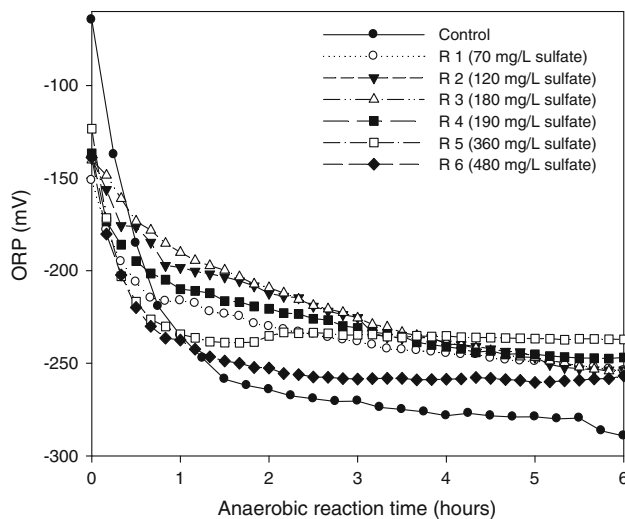


Fig. 2 ORP profile in the anaerobic phase

reduction reactions were dominant in the reactor, especially at the beginning of the anaerobic reaction phase in which glucose oxidation and sulfate reduction were involved. Lourenço et al. [2] observed an apparent relation between ORP level and color removal efficiency. It was also reported that azo dye biodegradation mainly depends on the redox potential of the biochemical environment and that color removal efficiency increases with lower ORP values (< -50 mV) [13, 32, 33]. Since redox potential of the reactor is an indicator of the color removal and the low ORP values are desired, it can be concluded that all study conditions were suitable for the azo dye reduction. It is important to note that the sequence of electron-accepting processes depend on the redox potential difference between the electron donor and electron acceptor.

Effect of sulfate on TOC removal efficiency

The main carbon source of the reactor was glucose with an attribution of 1085 mg/L COD. Electrons released from oxidation of the electron-donating primary substrate (glucose) are transferred to the azo dye which is the terminal electron acceptor of the reduced cofactors, thereby resulting in color removal. In this study, TOC measurement was carried out instead of COD to avoid the interference of sulfide in the COD determination. TOC removal efficiencies (%) of the microorganism exposed to different amount of sulfate concentrations is shown in Fig. 3.

The capacity of a sulfate-reducing sludge for carbon removal in the presence of different concentrations of sulfate was investigated. In this study it was observed that most of the carbon was removed in the first hours of the anaerobic phase in which sulfate and azo dye reductions took place simultaneously. It could be concluded that COD is mainly used by anaerobic organisms for growth and to

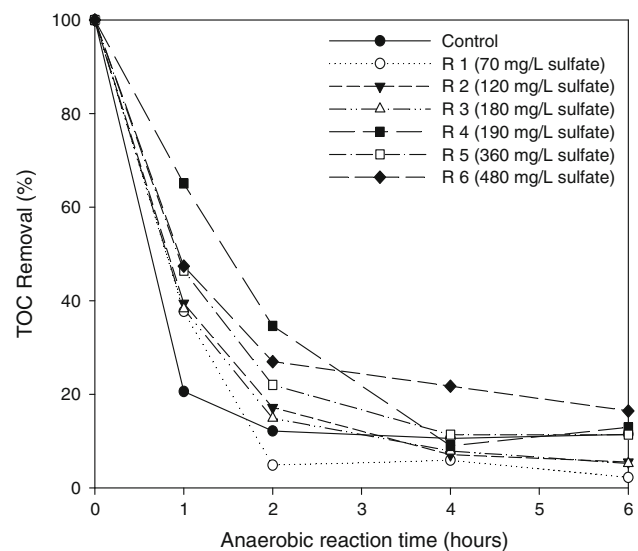


Fig. 3 TOC removal profile in the anaerobic phase

obtain energy. These results are in agreement with previously published data [34].

There was no significant difference observed in the final carbon removal efficiency in operational cycles. Results of percentage carbon reduction at the end of the anaerobic reaction phase were similar for all runs, giving an overall reduction of between 80 and 90 %. This result is in agreement with previous studies. Anaerobic stages of SBR studies have been shown efficient color removal rates mostly higher than 70 % [2, 3, 26, 35]. Meanwhile, COD removal efficiency of anaerobic phase of SBR was found to depend on dyestuff type, amount of initial COD concentration, anaerobic cycle time, etc. Nevertheless, there are also reports about no efficient COD removal in anaerobic cycle of SBR [36, 37].

However, there was a decrease on the rate of carbon removal with increasing sulfate concentrations. When compared with concentration of electron donor (COD equivalent of 1,085) supplied to the sulfate concentration (COD equivalent of -320), it can be seen that electron donor source supplied is around 3.4-fold of the theoretical value. This suggests that decrease in carbon removal rate is not associated with the sufficiency of available carbon source. Hence, it can be hypothesized that biogenic sulfide generated from reduction of sulfate contribute the reducing conditions and may supply electron donor source for biochemical environment.

Effect of sulfate on color removal efficiency and color removal rate

As discussed earlier, sulfate supplementation changed the redox potential of the anaerobic medium proportional to

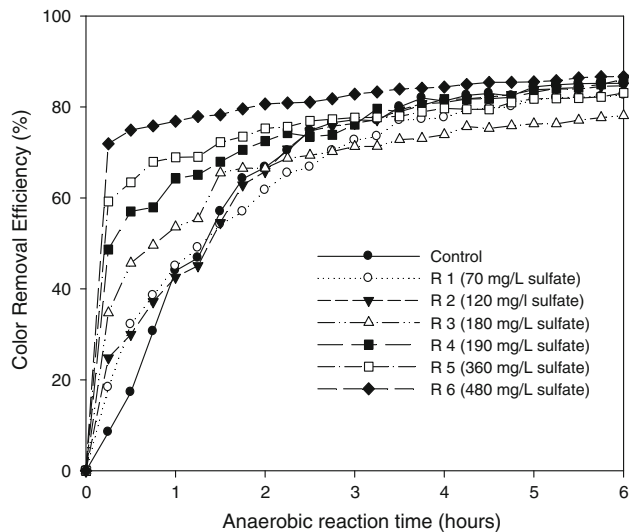


Fig. 4 Effect of sulfate on color removal efficiency

the sulfate concentration used. The proposed scenario was that it could be a competition between dye reducers and sulfate reducers for the reducing equivalents. Anaerobic color removal under sulfate-reducing conditions was investigated and results were shown in Fig. 4.

As shown in Fig. 4, sulfate supplementation to the reactor has no apparent effect on overall color removal efficiency. Obtained color removal efficiency at the end of the anaerobic phase was between 83 and 89 %. However, the application of high sulfate concentration to the anaerobic phase showed important effects on the rate of azo dye biodegradation, especially in the first hour of the reaction phase. For these reasons, another parameter used for evaluating color removal performance within the system was color removal rate. Color removal rate was calculated as the dye concentration removed by unit microorganism in unit time (mg dye/g MLSS/h). Color removal rates for different operating conditions are shown in Fig. 5. Since the azo dye was mostly removed within the first hours of anaerobic conditions, no apparent color removal rate was observed after the 4th hour of the SBR.

As shown in the figure, color removal rate at the 15th minute was increased up to fivefold for R6 (480 mg/L sulfate) by supplying high sulfate concentration compared with the control lacking sulfate. As a result, increase in sulfate concentration in the reactor showed no effect on the overall anaerobic color removal efficiency. However, sulfate accelerated color removal and color removal rate reached around 30 mg dye/g MLSS/h at 15th minute of the anaerobic phase. Since available electron donor source was sufficient, azo dye biodegradation and sulfate reduction took place simultaneously and increased sulfide concentration from the samples was the evidence of sulfate reduction (data not shown). It seems that sulfate was rather accumulated than used but

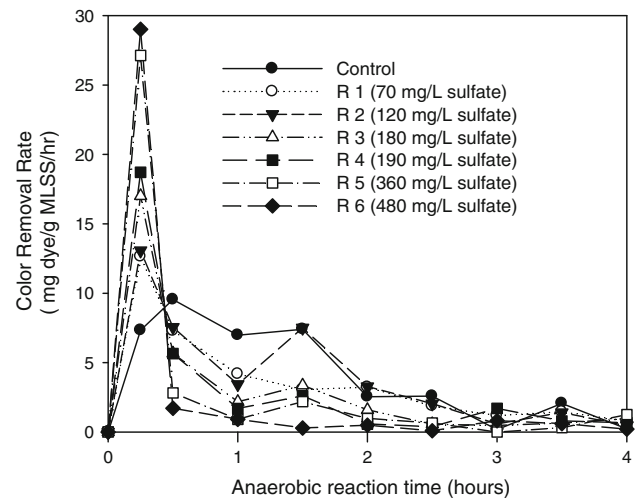


Fig. 5 Effect of sulfate on color removal rate

makes color removal rate increase (see Fig. 1). Azo dye reducing activity out-competed sulfate reduction was one of the main causes of the accumulation of sulfate in the medium. It was probably biogenic sulfide generated via microbial sulfate reduction that contributed color removal process as a reducing agent. The assessment of sulfide on color removal was not in the field of this study but previous studies indicated that sulfide plays an important role on reduction of azo dyes as an electron donor [20, 24, 38–41]. Lourenço et al. [42] suggest that the decolorization of RBV-5R may proceed by two different mechanisms such as direct electron transfer from the cell to the dye or via sulfate reduction. The proposed mechanism may be initiated by (1) oxidation of electron donor, and then (2) simultaneous azo dye reduction and sulfate reduction, (3) sulfate released from azo reduction thereby increasing sulfate concentration in the medium, (4) biogenic sulfide generation from sulfate reduction, and (5) azo dye reduction via biogenic sulfide (rate increasing step). In a study performed by Cervantes et al. [39], adverse effect of sulfate with a concentration of 10 g SO_4/L on azo biodegradation was found. But when electron donor source was increased from 1 to 6.7 g COD/L, adverse effect of sulfate was eliminated. They concluded that insufficient reducing capacity was the cause of these inefficient color removals. It seems that in the presence of sulfate, color removal may be rather stimulated than competitively suppressed [4, 14, 20–22].

It was reported that when inhibiting sulfate-reducing activity of microbial population in SBR by the addition of molybdate, anaerobic azo dye removal efficiency is decreased. Indeed, since sulfate acts as an electron acceptor under anaerobic conditions, it may compete with the dyes for the electrons available, thus causing an adverse effect on the decolorizing process. High sulfate concentrations

are found to adversely affect decolorization unless sufficient amount of substrate is supplied to overcome the negative effects of elevated concentrations of sulfate [40]. Furthermore; when sulfate is reduced under these conditions by sulfate-reducing bacteria (SRB), sulfide, which is known as bulk reductant, is generated and can in turn serve as an electron donor. Sulfide generation is found to also contribute to the reduction of azo dyes. It is also reported that cofactors involved during microbial reduction of sulfate such as cytochrome C_3 (-205 mV) and NADH (-324 mV) have appropriate negative redox potential; therefore, they can channel the electrons to azo dyes. Meanwhile, the redox potentials with more positive of the dye reduction than the redox potential of biological sulfate reduction (-220 mV) can be accelerated by sulfate.

Effect of sulfate on enzyme activity

The anaerobic enzyme used as an indicator of anaerobic RBV-5R biodegradation was azo reductase. Figure 6 shows the maximum observed levels in a cycle of azo reductase in the cultures under different conditions tested. It can be seen that the enzyme responses under different conditions had different features. In the literature it is pointed out that azo reductase activity can be associated with more than one reductase, depending on the microorganism and the environment conditions [43].

It was R3 for the maximum specific azo reductase enzyme activity ($148 \mu\text{g}/\text{mg}$ protein) attained. According to our results, the azo reductase activities for all operational conditions are different from each other. This indicates difference in specificity of the enzyme azoreductase to increased sulfate concentrations. The enzyme azoreductase decolorizes sulfonated azo dye Remazol Brilliant Violet 5R in the presence of sulfate, indicating that a sulfate-insensitive

azoreductase was involved in the decolorization process. Initially, specific enzyme activity in the cultures increased as the amount of the additional sulfate was increased and after R3 it decreased. It can be concluded that high color removal rates after R3 was not associated with the azo reductase enzyme since enzyme levels were gradually decreased but it was still higher from the initial level. Sulfide may be responsible for the residual decolorization process as a reducing agent in the reaction medium. It should be noted that there was no similar studies in the literature that present the azo reductase response in the sulfate-reducing environment. For this reason the enzyme results cannot be compared with those reported in the literature.

To determine how the ability of the cultures to synthesize aromatic amine degrading enzymes under sulfate reducing conditions and probability to degrade aromatic amines under these conditions changed in response to the sulfate-reducing conditions imposed, biomass samples were used to measure their capability in the presence of sulfate as electron acceptor. Only catechol 1,2-dioxygenase was observed throughout this study as key enzyme of aromatic amine biodegradation. Catechol 1,2-dioxygenase (C12O) is the enzyme measured to represent how aerobic enzyme affected anaerobic sulfate reducing conditions. Under R3 maximum C12O enzyme level was observed ($33 \mu\text{g}/\text{mg}$ protein) whereas it was initially 90 % under control conditions. The rapid increase in enzyme level under in R3 is not explicable. Adverse effect of sulfate on enzyme activity was obvious under R4, R5, R6.

Effect of sulfate on aromatic amine formation and removal

To determine how the ability of the cultures to produce aromatic amines under sulfate-reducing conditions and

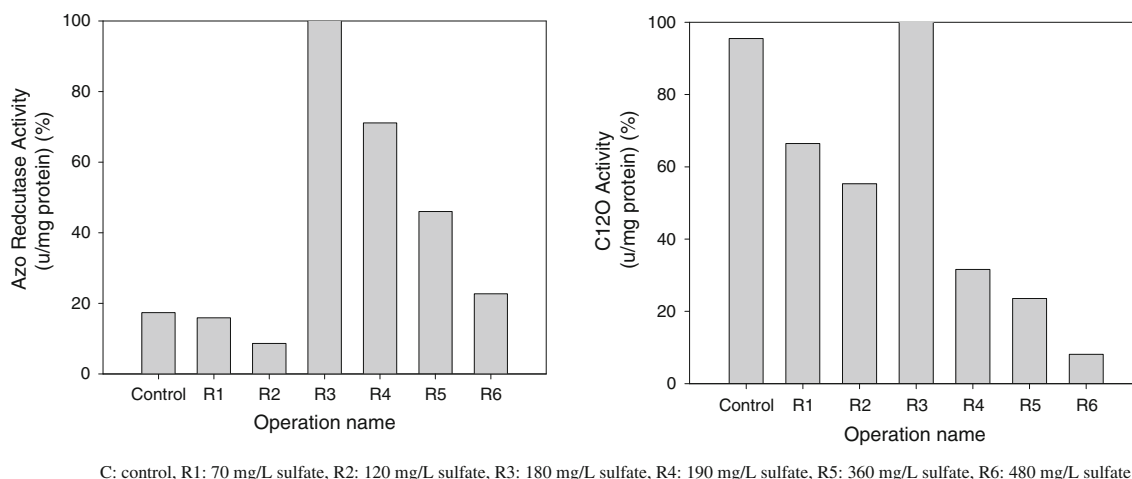


Fig. 6 Effect of sulfate on enzyme activity

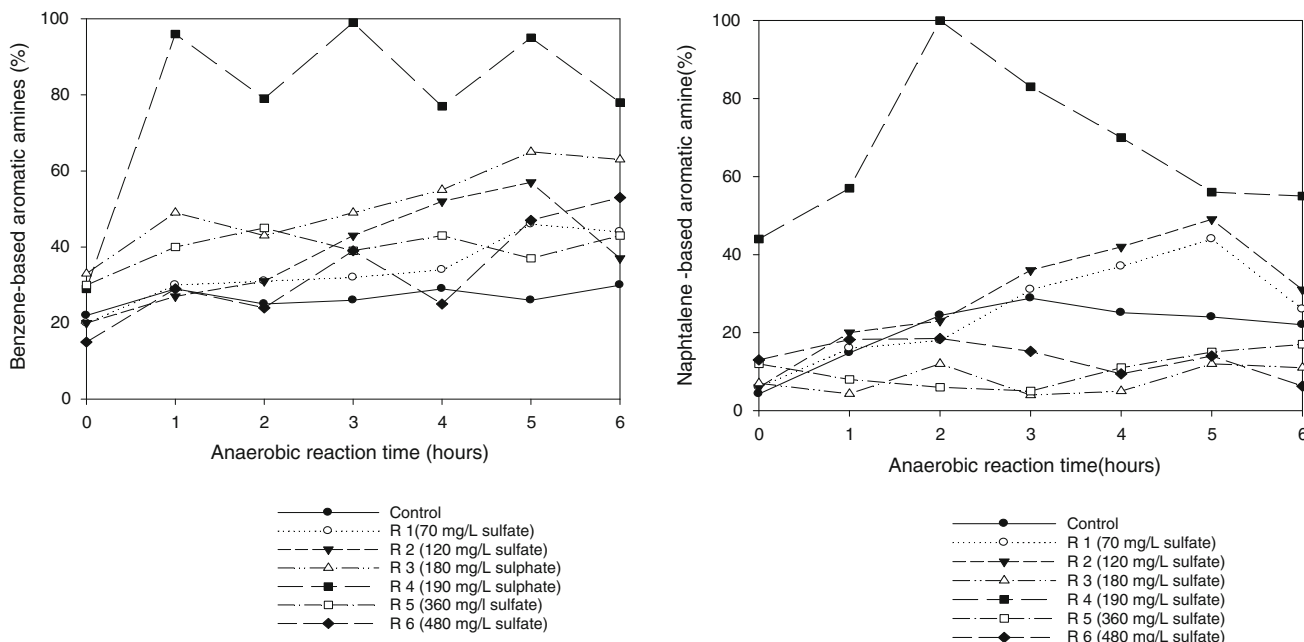


Fig. 7 Effect of sulfate on aromatic amine formation and removal

probability to degrade aromatic amines under these conditions changed in response to the sulfate-reducing conditions imposed, HPLC measurement techniques were used. The chemically reduced Remazol Brilliant Violet 4 R products were used to determine relevant aromatic amines. The results from the HPLC analyses, there were two main peaks (i.e., benzene-based and naphthalene-based aromatic amines) corresponding to the expected amines by comparing chromatograms. It should be noted that due to the unavailability of true standards, the chromatographic peaks appearing in the samples could not be quantified. Chromatographic peak areas from the HPLC analyses were evaluated by assuming the highest peak area as maximum amount of amine (%) produced during SBR operation. The results from the analyses are shown in Fig. 7. Examination of the HPLC analyses results in the figure reveals that formation of aromatic amines under anaerobic periods for all conditions indicates azo dye reduction. This suggests that bacterial cultures can reduce azo dye and produce aromatic amines. Figure 7 presents the benzene- and naphthalene-based aromatic amine data collected under the different conditions studied. The results taken as a whole suggest that cleavage of the azo dyes resulted in aromatic amine accumulation under anaerobic conditions and cleavage products from the RBV-5R were not to be removed aerobically well under sulfate-reducing conditions. However, under aerobic conditions in the anaerobic-aerobic SBR, they could be removed around 40 % (data not shown). It was reported that aromatic amines formed during the anaerobic biodegradation of reactive dyes are

resistant to aerobic biodegradation and not to be removed under these conditions [2, 3, 14, 29].

Typical aromatic amine profiles shown in the figure were not significantly different from those in its control reactor since overall azo reduction efficiencies were almost the same. However, under R4, obtained aromatic amines were surprisingly higher from the others; it is thus reasonable to suggest that obtained peaks confirm the formation of additional aromatic metabolites.

Conclusion

Based on the results of this study, the following conclusions can be made:

- The azo dye Remazol Brilliant R5 was decolorized in the anaerobic mixed culture in the presence of sulfate and glucose. Sulfate-reducing bacteria contributed significantly to the azo dye reduction and organic carbon removal. Aromatic amines are produced under anaerobic conditions as a result of azo dye degradation and tend to accumulate under this condition.
- Sulfate has no adverse effect on azo dye biodegradation and has an inconclusive effect. In this research, the relative contribution of azo reduction mechanisms was investigated with respect to the role of sulfate. There is no deterioration of azo degrading capacity of microorganism over time after the sulfate-reducing conditions in anaerobic-aerobic sequencing batch reactor. There is no competition between dye reducers and sulfate

reducers for the reducing equivalents available. Sulfate reduction significantly accelerates color removal.

- Standard redox potential of sulfate reduction is $E'_0 = -220$ mV for $\text{SO}_4^{2-}/\text{H}_2\text{S}$, $E'_0 = -116$ mV $\text{SO}_3^{2-}/\text{H}_2\text{S}$ for sulfite, and $E'_0 = -280$ mV $\text{S}^0/\text{H}_2\text{S}$ for elemental sulfur. Therefore, addition of different amount of sulfate to the reactor changes the redox potential values as a result of sulfate reduction. Higher amount of sulfate addition results in lower ORP values which has positive impact on azo dye biodegradation. ORP values lower than -150 mV is suitable for color removals.
- Alternate anaerobic–aerobic phases result in poor anaerobic sulfate removal yields since aerobic conditions have adverse effect on SRBs. Additionally, sulfides produced under anaerobic conditions can be reoxidize under aerobic conditions leading increased sulfate accumulation at the beginning of the anaerobic phases. Separate anaerobic–aerobic reactors should be used in full-scale textile wastewater treatment plants to prevent sulfate accumulation problems.

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