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Inhibitory effect and mechanism of azo dyes on anaerobic methanogenic wastewater treatment: Can redox mediator remediate the inhibition?

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ABSTRACT

Inhibitory effect of azo dyes on anaerobic methanogenic wastewater treatment (AMWT) has been studied mainly focusing on biological toxicity in the batch test with simulated sole co-substrate. Detailed information on inhibitory effect and mechanism of azo dyes during the long-term operation with real complex co-substrate is limited. Moreover, whether redox mediator (RM) could remediate the inhibition is still unclear in previous studies, especially under the complex scenario. In this study, the real textile wastewater with alternative concentrations of azo dyes (0–600 mg/L) were used to operate a lab-scale high-rate anaerobic methanogenic bioreactor for 127 days, and 50 μM anthraquinone-2-sulfonate (AQS) as RM was added at the last period of operation. Azo dyes with concentration of 600 mg/L could cause significant inhibition on overall (decolorizing and methanogenic) performance of AMWT. Specific methanogenic activity assays showed that acetoclastic methanogens was more susceptible to high concentration azo dyes than hydrogenotrophic methanogens. The spatial distribution of extracellular polymeric substance in the anaerobic granular sludge (AGS) showed that the high biological toxicity of azo dyes was mainly attributed to enrichment effect in tightly bound-EPS (TB-EPS). The channels of AGS was clogged by azo dyes, which was evidenced by the hard release of aromatic amines in EPSs as well as decreased porosity of AGS and scanning electron microscope images. Meanwhile, the settling ability, particle size and strength of AGS all deteriorated after azo dyes concentration exceeded 450 mg/L. The dosing of AQS could mostly remediate overall performance of the bioreactor even if the recovery of acetoclastic methanogens was slow. However, except for the porosity with a part of recovery, physical characteristics of AGS hardly recovered, and washout of sludge from the bioreactor was still happening. It suggested that additional attention should be paid to prevent sludge from washout if RM was practically used to remediate the anaerobic reactor inhibited by azo dyes.

1. Introduction

The wide application of dyes made our world more colorful, but it also brought us the undesirable visible pollution. It was recorded that the total amount of dye discharged per year had exceeded one million tons since 2007 (Singh and Arora, 2011). Fifty percent of dyes discharged was azo dye, the aromatic compounds that contain one or more azo bonds (R1-N=N-R2) (Meng et al., 2012). The direct discharge of azo dye containing wastewater has adverse effects on the transparency and aesthetic of the aquatic environment. Also, some azo dyes were proven to be stable in soil, in which microbial community structure was significantly impacted (Imran et al., 2015). More importantly, many azo dyes and their cleavage products are toxic, mutagenic and/or carcinogenic to life (Umbuzeiro et al., 2005; Tan et al., 2005). The effective treatment towards azo dye is thus critical for both environmental and ecological concerns.

Combined anaerobic-aerobic biological treatment process has been regarded as the most logical concept for the treatment of azo dyes (Van der Zee and Villaverde, 2005). In the process azo dyes were anaerobically biodegraded to aromatic amines which could be

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further aerobically mineralized. The step of anaerobic treatment is essential because azo dyes were recalcitrant to aerobic biotreatment (Santos et al., 2007), and for some bacterial strains azo dyes were recalcitrant under aerobic condition but easily biodegradable under anaerobic condition (Deng et al., 2008). Unfortunately, preliminary studies have demonstrated that high/medium concentration azo dyes could biologically inhibit the anaerobic methanogenic wastewater treatment (AMWT) (Sponza and Isik, 2004; Hong et al., 2007; Alvarez et al., 2015). In addition, the competition for reducing equivalents between dye reducers and methanogens would happen when azo dyes and co-substrate were existed simultaneously (Santos et al., 2006). As the negative impact of azo dyes on AMWT may lead to low efficiency and/or even breakdown for the AMWT, precise understanding of the impact was particularly crucial for us to remediate the effected AMWT. However, most of studies only included short-term exposure to azo dyes in batch tests with simulated sole co-substrate, while detailed continuous tests with respect to long-term exposure using real complex co-substrate were still limited (Van der Zee et al., 2003; Brás et al., 2005). Meanwhile, the inhibitory mechanism of azo dyes was often studied focusing on biological toxicity. After an extended period of time, the toxic or competitive effect of azo dyes may not only lead to volatile fatty acid (VFA) accumulation, decrease of methane production or low azo dye degradation efficiency, but also may clog channels in anaerobic granular sludge (AGS). Moreover, the azo dyes or their cleavage products may change the physical characteristics or impair the production of extracellular polymeric substance (EPS) of sludge (Sheng et al., 2010), which may lead to disintegration, floating, washout of the granular sludge. For these reasons, we believe that a comprehensive continuous test is important to reveal detailed information about the inhibitory effect and mechanism of azo dyes on the performance of AMWT.

Redox mediators (RMs) (such as anthraquinone-2-sulfonate (AQS)) have been evidenced to catalyze the anaerobic reduction of azo dyes (Van der Zee and Cervantes, 2009). For instance, Santos et al. (2004) found that AQS could accelerate the anaerobic bioreduction of azo dyes for up to 2.67-fold, via decreasing the activation energy for 1.2-fold. The presence of redox mediators may reduce the inhibitory effect of azo dyes on AMWT by catalyzing azo dye biodegradation. But whether RM can remediate the overall performance of the bioreactor and physical characteristics of sludge is unknown up to now.

Within diverse application fields of azo dyes, the textile industry accounted for a large fraction (Santos et al., 2007). Hence the real textile wastewater with alternative concentrations of azo dyes was employed to feed a lab-scale high-rate anaerobic methanogenic bioreactor. The overall performance of the bioreactor as well as methanogenic activity, physical characteristics and EPS of sludge were monitored to assess the inhibitory effect and mechanism of azo dyes on AMWT. The AQS (i.e. RM) was added at the last period of operation to evaluate whether RM could remediate the inhibition.

2. Materials and methods

2.1. Wastewater

The textile dyeing wastewater from the cotton textile industry generally consists of the pre-treatment wastewater (i.e. mixture of wastewaters from desizing, scouring/washing, bleaching and mercerizing processes) and the dyeing wastewater (i.e. mixture of wastewaters from dyeing, rinsing and finishing) (Santos et al., 2007). The wastewater used in this study was the mixture of real pre-treatment wastewater and synthetic dyeing wastewater, with a proportion of 1:1.

2.1.1. Pre-treatment wastewater

Real pre-treatment wastewater was collected from a regulating tank, in which pH of the wastewater was adjusted to 7.0–7.6 by hydrochloric acid, in a cotton textile dyeing wastewater treatment plant at Yixing, Jiangsu Province, China. In the pre-treatment process, the sizing agents discharged were polyvinyl alcohol (PVA, approx. 30%–40%), carboxymethylcellulose (CMC, approx. 10%–20%) and starch (approx. 50%). Auxiliaries used mainly included hydrogen peroxide, disinfectants, surfactants, brighteners, etc. The characteristics of the wastewater were listed in Table 1. The real pre-treatment wastewater was stored at 4 °C before use.

2.1.2. Synthetic dyeing wastewater

Synthetic dyeing wastewater consisted of nutrients (2 mL/L), NaCl (10 g/L) as well as azo dyes, which were diluted from a stock dye solution (50 g/L) that was adjusted by NaOH to pH 12 and then hydrolyzed at 80 °C for 1.5 h (O'Neill et al., 2000a). The adding amount of the stock dye solution depended upon the experimental conditions. Acid orange 7 (AO7, $\lambda_{max} = 484$ nm), reactive red 2 (RR2, $\lambda_{max} = 539$ nm), reactive black 5 (RB5, $\lambda_{max} = 595$ nm), direct yellow 12 (DY12, $\lambda_{max} = 405$ nm) and direct blue 71 (DB71, $\lambda_{max} = 579$ nm) were equally contained in the stock dye solution, and structure formulas of azo dyes used in this study was given in Fig. S1. These azo dyes were bought from Shanghai Jiaying Chemical Engineering Co., Ltd., China. The strength of dyes were 100% except for DY12 (80%). The nutrients included trace element solution I, II and nutrition solution of 1 mL/L, which were prepared according to Tang et al. (2013).

2.2. Experimental set-up, inoculation and reactor operation

A spiral symmetry stream anaerobic bioreactor (SSSAB) with working volume of 18.7 L was used to treat the wastewater. The configured parameters and schematic of the SSSAB were previously detailed (Chen et al., 2016). In the reaction zone (middle part of the reactor) of SSSAB three elliptic plates were set 120° spirally and symmetrically. The sludge bed was then divided into three chambers by the elliptic plates. The reaction zone were provided with an insulation layer for keeping the temperature as 35 ± 1 °C in the reaction zone. Peristaltic pumps were used for pumping the influent and circulating hot water.

The bioreactor was seeded with 9.2 L sludge. The seed sludge was inoculated with methanogenic granular sludge from the bottom of a full-scale internal circulation reactor treating wastewater in a papermaking plant at Wuxi, Jiangsu Province, China. The mean diameter of the seed sludge was 2.2495 mm, and its density and VSS/SS were 1.17 g/cm^3 and 0.77, respectively.

The hydraulic retention time of the bioreactor was 30 h throughout the experiment. Three periods of operation can be distinguished. As shown in Table 2, after inoculation, the reactor

Table 1
Characteristics of real pre-treatment wastewater.

	pH	COD (mg/L)	NH_4^+-N (mg/L)	Total phosphorus (mg/L)	Suspended solids (SS, mg/L)	SO_4^{2-} (mg/L)	Conductivity (mS/cm)
Real pre-treatment wastewater	7.5 ± 0.1	4160 ± 150	80.2 ± 10.1	18.3 ± 3.5	430.5 ± 103.7	42.1 ± 6.2	9.56 ± 0.80

Table 2
Operational strategy of the SSSAB treating azo dye containing wastewater.

Period	Time (day)	Influent azo dye concentration (mg/L)	Influent anthraquinone-2-sulfonate (AQS) concentration (μM)	Remarks
Period I (Start-up)	1–13	0	0	In days 1–13 influent was pretreated by doubling dilution with equal volume glucose synthetic wastewater
	13–33	0		
Period II (Azo dye concentration increasing)	33–49	150		
	49–65	300		
	65–81	450		
	81–97	600		
Period III (Redox mediator dosing)	97–127	600	50	

was firstly fed with mixed wastewater with 0 mg/L azo dyes and COD of 2250 mg/L (organic loading rate (OLR) = 1.81 kg COD/(m^3·d)) for reactor start-up (Period I, days 1–31). Note that in days 1–13 influent was pretreated by doubling dilution with equal volume glucose synthetic wastewater. Then the influent azo dye concentration was increased by 150 mg/L for every 16 days during Period II (days 31–97). Finally the redox mediator AQS (97%, bought from Sinopharm Chemical Reagent Co., Ltd, China) was added and lasted for 30 days during Period III (days 97–127). The overall performance of the reactor was monitored by measuring COD, color of influent and effluent, as well as aromatic amines, VFA, alkalinity (ALK) of effluent. Methane production of the bioreactor was recorded every day.

2.3. Specific methanogenic activity assays

All acetoclastic and hydrogenotrophic methanogenic activity assays were performed in 118 mL serum bottles with duplicate vials. Each bottle received about 1.6 g VSS of sludge (washed by phosphate buffered saline with pH of 7.4) from the bioreactor. The nutrition and trace solutions used were the same as in the bioreactor. The headspaces were flushed with N_2 and the bottles were sealed with butyl rubber stoppers. After a 1-day pre-incubation period (in a rotary shaker with 105 rpm at 36 °C), the headspace of the vials was flushed again. Substrate was added as neutralized sodium acetate (2500 mg/L COD) or H_2 gas. Hydrogen was supplied at an initial pressure of 0.5 atm, applied as an overpressure of H_2/CO_2 (80:20, v/v). The vials were then incubated on rotary shaker with 105 rpm at 36 °C. Headspace samples (100 μL) were obtained regularly and analyzed chromatographically for CH_4 content (GC 7890A, Agilent Technologies, USA) (Zhu et al., 2008). The specific methanogenic activity (SMA) was calculated from the steepest slope (slope with largest value) of cumulative methane production curve (expressed by g CH_4-COD per gram VSS per day).

2.4. Daily analysis

COD, NH_3-N, total phosphorus, sulfate, SS, VSS, pH and ALK were measured following the standard methods (Apha, 2005). Conductivity was measured by a portable conductivity meter (DDBJ-350, Shanghai INESA instrument Co., Ltd., China). Methane production was determined by a wet gas meter after the absorption by NaOH solution. VFA (calculated as acetic acid) of effluents was tested using titration method (Wijetunga et al., 2010).

Since five azo dyes with wide different absorbance peaks (λ_{max} ranged from 405 to 595 nm) were equally contained in the influent of the anaerobic reactor, proper characterizations of color and color removal should be established. The visible spectrum (wavelength of 380–780 nm) of influents (containing 0 mg/L and 300 mg/L) and effluents (Fig. S2) evidenced that both the color and color removal were distributed widely. Conventional peak identification method (Wijetunga et al., 2010) may not be representative enough for color here. Therefore color of wastewater was measured spectrometrically (PerkinElmer Lambda 35, PerkinElmer Inc., USA) at wavelength of 436, 525 and 620 nm in a manner similar to that described in the British Standards (BS 6068 1995) (O'Neill et al., 2000b). Colors and color removals in the following text were all characterized by mean value of absorbance measured at 436, 525 and 620 nm. Before measuring color, the suspended solids of the wastewater sample was removed by centrifugation (4 °C) and the pH was adjusted to 7.6 by using 1 M HCl.

Total aromatic amines were determined colorimetrically at 545 nm following steps in Chinese Standard Methods (GB 11,889–89): diazotization with sodium nitrite, removing excess sodium nitrite by adding ammonium sulphamate and reaction with N- (1-naphthyl) ethylenediamine. To eliminate the interference of color of dyes, controls were tested by replacing N- (1-naphthyl) ethylenediamine with distilled water. Aromatic amine recoveries were calculated from the ratio of total aromatic amines of effluents to expected total aromatic amines for chemical reduction of the equivalent dye (Sponza and Işık, 2005). Sodium dithionite was adopted to chemically reduce the azo dyes, the reduction process was described in detail by Sponza and Işık (2005).

2.5. Evaluation for physical characteristics of anaerobic granular sludge

The test procedures described by Ghangrekar et al. (2005) were used for determinations of strength and settling ability of AGS, which were characterized by integrity coefficient (dimensionless) and settling velocity (m/h), respectively. Particle size distribution was measured by wet sieving method (sizes 0–0.5, 0.5–1.0, 1.0–1.4, 1.4–2.0, 2.0–2.8, 2.8–4.0 mm). Porosity ε of AGS could be calculated with the following equation (Li and Yuan, 2002):

$$\varepsilon = 1 - \frac{6fW_d}{\pi\rho_c d^3} \quad (1)$$

where f, W_d, ρ_c and d were the ratio of the wet mass to the dry mass of the AGS, wet mass, wet density and mean particle sizes of the AGS, respectively. The determination of W_d, ρ_c followed Mu et al. (2006). The ratio (f) was estimated according to Li and Yuan (2002).

Morphological characteristic of the AGS was tested by a field emission scanning electron microscope (FESEM, S-4800 (Hitachi, Japan)), the pre-treatment of the AGS followed Chai et al. (2015).

2.6. Extracellular polymeric substances

The extraction of the EPSs [including soluble-EPS (S-EPS), loosely bound-EPS (LB-EPS) and tightly bound-EPS (TB-EPS)] from the AGS referred to Lu et al. (2015). Color and total aromatic amines in EPSs were determined according to methods described above. Total organic carbon of EPSs were measured by a TOC meter (SHIMADZU TOC-VCPN, SHIMADZU Co., Ltd., Japan). The protein (PN)

concentration in EPSs was determined by the BCA method (Smith et al., 1985). To eliminate the interference of color of dyes, controls were tested by replacing BCA working solution with distilled water. The polysaccharide (PS) concentration in EPSs was measured using the anthrone method (Zhu et al., 2015). To eliminate the interference of color of dyes, controls were tested by replacing anthrone-ethanol-75% sulphuric acid solution with distilled water.

3. Results

3.1. Overall bioreactor performance

The bioreactor was operated in three different periods described in Fig. 1. In the whole operation lasted for 127 days the OLR were kept stable unless the azo dye concentration increased. During the start-up period (period I), both COD removal rate and volumetric methane production (VMP) increased during days 1–13 and decreased sharply as the influent was not diluted by synthetic glucose wastewater anymore. The COD removal rate and VMP recovered gradually from day 19–31. The COD removal rate and VMP on day 31 reached up to 65.8% and 0.386 $m^3/(m^3 \cdot d)$, respectively, lower than those on day 13 (76.1% and 0.440 $m^3/(m^3 \cdot d)$).

Azo dyes were added into the influent during the azo dye concentration increasing period (period II). Fig. 1 showed the overall performance of the bioreactor declined significantly as soon as azo dye concentration increased, including the reductions of COD removal rate, VMP and mean color removal rate, as well as the increment of VFA. The overall performance after azo dye

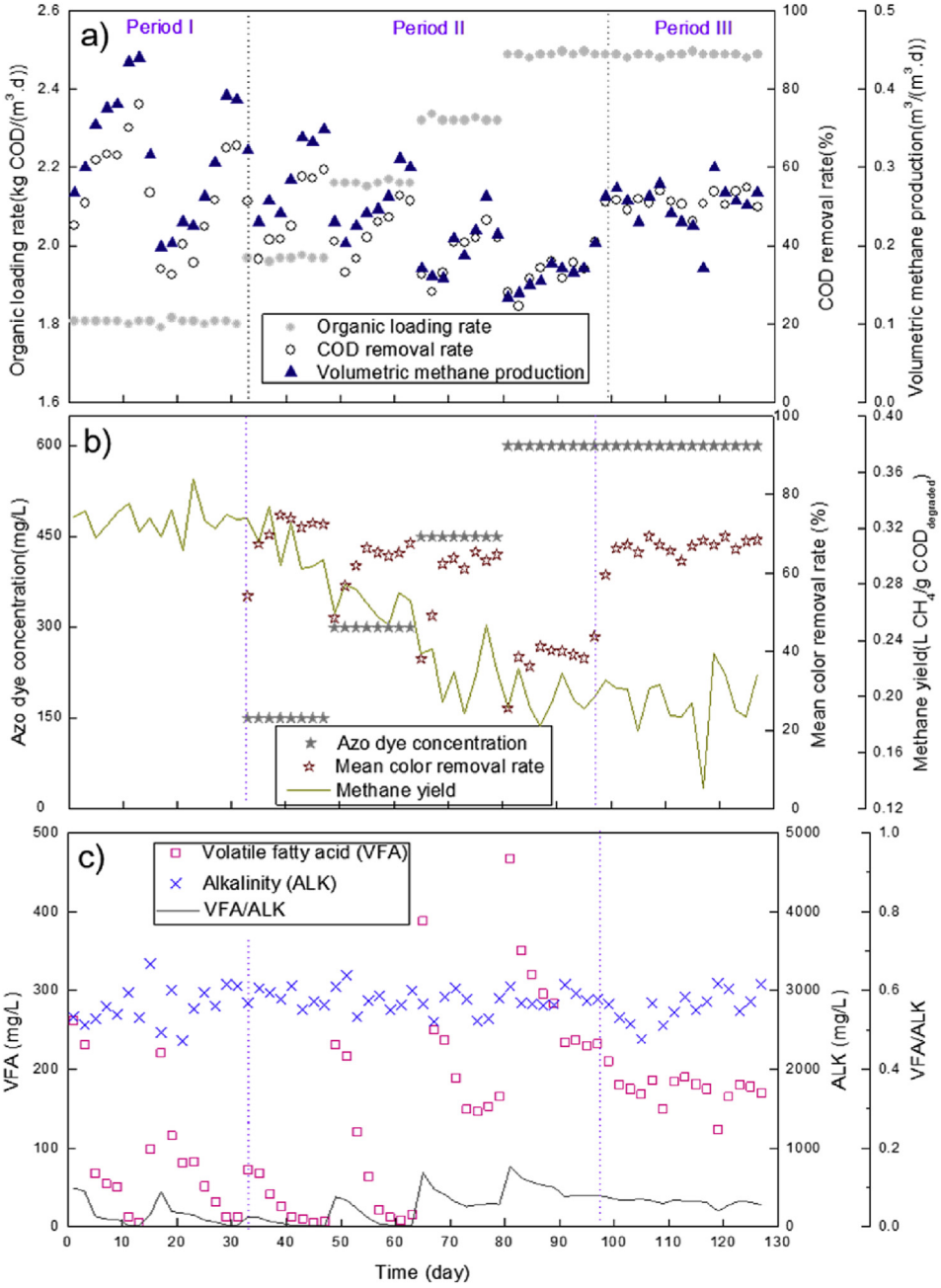

Fig. 1. Overall performance of the anaerobic bioreactor treating wastewater containing azo dyes during different phases: (a) OLR, COD removal rate and VMP; (b) azo dye concentration, mean color removal rate and methane yield; (c) VFA, ALK and VFA/ALK.

concentration increasing, although recovered subsequently, was worse than that before, particularly when azo dye concentration was increased to 600 mg/L. For example, the COD removal rate, VMP and mean color removal rate on day 95 (600 mg/L azo dyes) were 35.9%, 0.080 $m^3/(m^3 \cdot d)$ and 38.3%, lower than that on day 79 (450 mg/L azo dyes) of 42.1%, 0.214 $m^3/(m^3 \cdot d)$ and 64.6%, respectively. VFA accumulation had been happening since azo dye concentration exceeded 450 mg/L. Moreover, the methane yield performed a tendency of decreasing with the increase of azo dye concentration (averagely 0.219 and 0.309 L CH_4/g $COD_{degraded}$ on days 81–97 and 33–49, respectively). In addition, after azo dyes were applied, the values of recovery of aromatic amines ranged from 80% to 85% (Fig. S3), indicating that the reduction was the major mechanism of dye removal. The recovery lower than 100% might be attributed to a part of further anaerobic biotransformation for the aromatic amines (Van der Zee and Villaverde, 2005; Tan et al., 1999).

In order to evaluate if the overall performance of the inhibited bioreactor could be remediated by dosing redox mediator, 50 μM AQS was added into the influent on day 97 (period III). After this modification, the mean color removal rate increased distinctly. The average value of the mean color removal rate on days 97–127 was 65.1%, much higher than that on days 81–97 of 37.5%. The COD removal rate and VMP increased while the VFA decreased, but not significantly. ANOVA analysis by SPSS 22.0 showed that the COD removal rate, VMP, mean color removal rate and VFA had significant difference before and after dosing of AQS at azo dye concentration of 600 mg/L. Nevertheless, the methane yield remained low or even lower with redox mediator dosing (averagely 0.197 L CH_4/g $COD_{degraded}$).

Throughout the bioreactor operation, the values of VFA/ALK were all lower than 0.2 (Fig. 1(c)) though the VFA accumulation occurred during some periods. Meanwhile, the effluent pH was observed to be stable ranging from 7.5 to 8.0, illuminating that the overall performance of bioreactor was not inhibited by the possible low pH caused by VFA accumulation.

3.2. Specific methanogenic activity

To obtain additional information of inhibitory effect of azo dyes on the sludge methanogenic activity, AGS from the bioreactor was determined for acetoclastic and hydrogenotrophic methanogenic activity at selected times of the bioreactor operation. Fig. 2 showed the results of SMA tested using acetate or hydrogen as sole substrates. The acetoclastic and hydrogenotrophic SMA measured during the period II decreased stepwise down to 16% and 59% of that measured on day 47, respectively. Additionally, hydrogenotrophic methanogenic activity recovered fast after redox mediator dosing. The SMA of hydrogenotrophic methanogens on day 111 was 1.22 times higher than that on day 79. But the SMAs of acetoclastic methanogens on day 111 and 127 were only 69.6% and 95.7% of that on day 79, respectively.

3.3. Distribution of azo dyes and aromatic amines in AGS

The distributions of azo dyes and aromatic amines in AGS were determined as it may provide critical information for inhibitory mechanism of azo dyes on AMWT. The distributions were characterized by the color and aromatic amines distributions in S-EPS, LB-EPS and TB-EPS. They were normalized basing on the concentrations in the corresponding S-EPS (Fig. 3), in order to be directly compared for the distributions at different times. Fig. 3(a) showed that the azo dyes concentration in the LB-EPS and TB-EPS increased along with the increase of azo dyes concentration (days 47–95, 150–600 mg/L). The azo dyes concentration in the LB-EPS and TB-EPS exceeded that in the liquid after azo dyes concentration reaching 450 mg/L. Then the distribution of azo dyes performed a tendency of azo dyes in the TB-EPS > LB-EPS > S-EPS. This tendency reached a peak (concentration in TB-EPS: LB-EPS: S-EPS = 2.52: 1.78: 1) when azo dyes concentration was 600 mg/L. Fortunately, such tendency was alleviated after redox mediator dosing (days 111 and 127 in Fig. 3(a)).

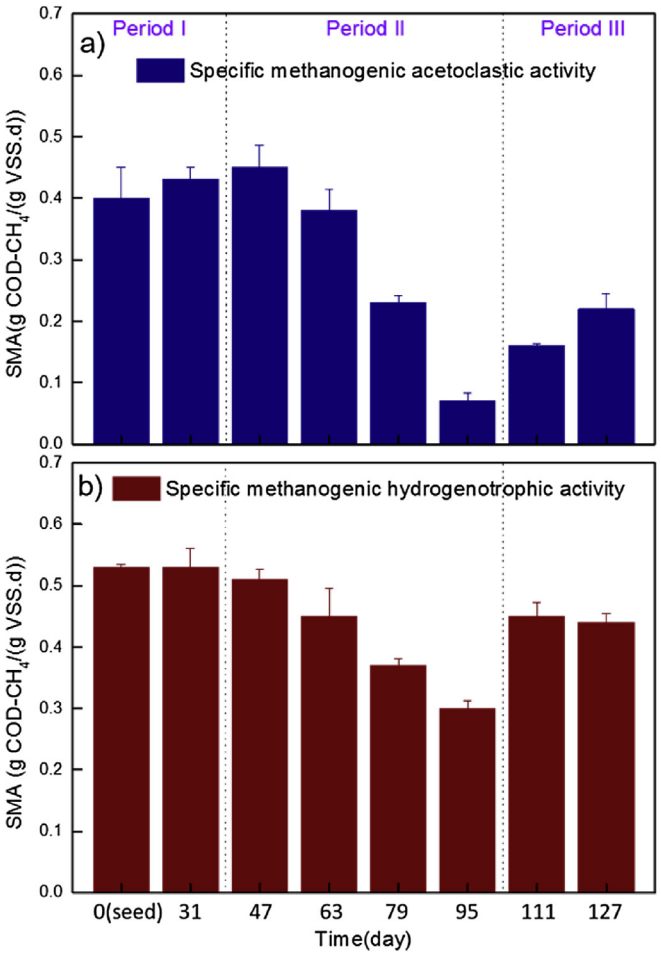

Fig. 2. Specific methanogenic acetoclastic (a) and hydrogenotrophic (b) activity of sludge from the bioreactor at different times of operation.

Fig. 3(b) illustrated that the aromatic amines concentrations in the S-EPS at different times were higher than that in the LB-EPS and TB-EPS. The distribution of aromatic amines in sludge exhibited a tendency of that in the S-EPS > LB-EPS > TB-EPS (e.g. 1: 0.60: 0.41 on day 79), except on day 95 (1: 0.81: 0.80).

3.4. Physical characteristics of AGS

Physical characteristics of AGS from the bioreactor at different times of operation were evaluated in terms of porosity, settling velocity, particle size and integrity coefficient (Table 3). Porosity of AGS was observed to decrease significantly (from 68.53% to 32.35%) with azo dye concentration increasing (from 0 mg/L to 600 mg/L), and increased to 56.94% after dosing of RM. We assumed that channels inside AGS may be clogged by azo dyes, so the SEM test of sludge samples was carried out (Figs. 4 and 5). It is presented that the surface of AGS on day 31 (Fig. 4(a)) was much rougher than that on day 95 (Fig. 4(b)), and much more channels can be observed on day 31. In Fig. 4(c and d) with magnification × 2,000, the surface of

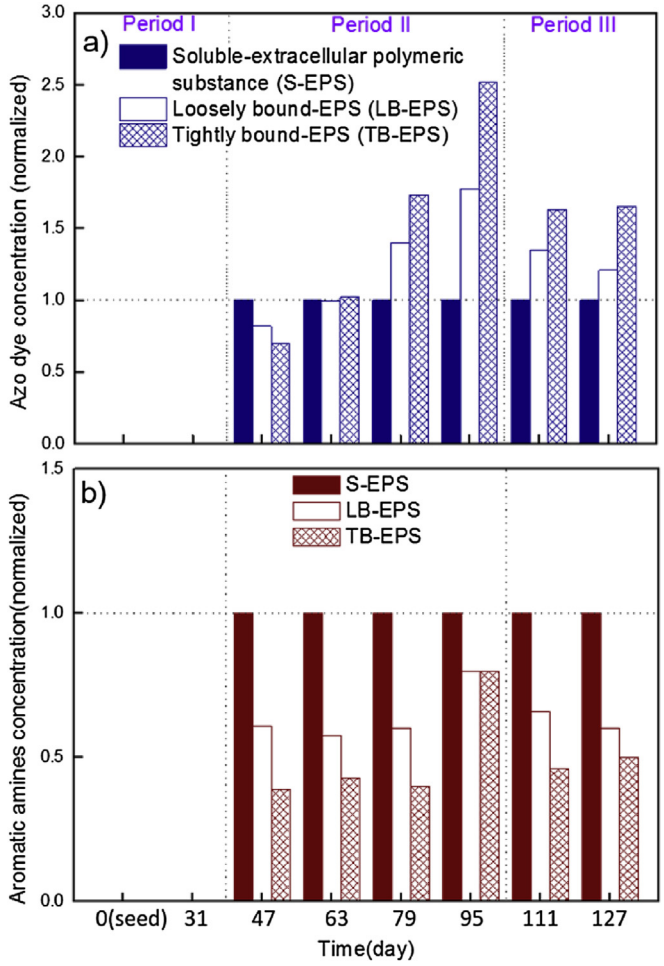

Fig. 3. Normalized azo dyes (a) and aromatic amines (b) concentration distribution in liquid, LB-EPS and TB-EPS of sludge from the bioreactor at different times of operation.

sludge sample from the bioreactor on day 31 exhibited stripes-like network with clear texture and pattern (Fig. 4(c)), while the surface on day 95 showed a laminated structure (Fig. 4(d)). As the surface of AGS in Fig. 4(d) seemed to have possible channels as well, the biggest possible channel in Fig. 4(d) was magnified and Fig. 4(f) was obtained. In Fig. 4(f), channels were hardly observed for the sample on day 95, while microorganisms seemed to be bonded by azo dye-like or EPS-like substances. On the contrary, channel-like structure for the sample on day 31 can still be noticed (Fig. 4(e)). Additionally, cavities of AGS were likely to be clogged by substance with structure different from AGS (Fig. 5(b–f)). We deduced that azo dyes could be bonded to form aggregates (Sakong et al., 2011) to clog cavities of AGS (Fig. 5(a)).

Table 3 illustrated that the settling velocity, mean particle size and integrity coefficient of AGS were relatively stable when influent azo dyes concentration was lower than 450 mg/L, indicating that low concentration of azo dyes and substances in pre-treatment wastewater did not significantly impact the physical characteristics of AGS. However, the settling velocity of AGS became unfavorable when azo dyes concentration exceeded 450 mg/L, and it hardly recovered after dosing of redox mediator. Meanwhile, the mean particle size decreased from 2.2730 mm to 1.4930 mm. Particle size distribution of sludge (Fig. S4) provided that the dominant particle sizes changed from 2.8 to 4.0 mm on day 63 to 1.0–1.4 mm on day 127. It also presented that the sludge sized 0–0.5 mm increased apparently from day 63 to day 127, corresponding to the increase of integrity coefficient (from 11 ± 5 to 38 ± 7, the lower the integrity coefficient is, the greater the strength of AGS is (Ghangrekar et al., 2005)).

3.5. EPS distribution and PN, PS in EPS

The physical characteristics of AGS related to the EPS and PN, PS in it (Sheng et al., 2010). Thus, the amount of LB-EPS and TB-EPS of sludge from the bioreactor at different times, as well as the ratio of LB-EPS/TB-EPS, were determined (Fig. 6(a)). The total EPS (=LB-EPS + TB-EPS) increased from 10.6 to 23.0 mg/g VSS during period I and was stable during period II and III, averaging 20.8 mg/g VSS. Nevertheless, the ratio of LB-EPS/TB-EPS increased fast (from 0.57 to 1.31) after day 63 (azo dye concentration reached 450 mg/L), then became relatively constant during period III (after dosing of redox mediator). Fig. 6(b) presented that the amount of PN, PS and PN/PS increased during period I (from 5.21 mg/g VSS, 1.75 mg/g VSS and 2.99–15.50 mg/g VSS, 2.19 mg/g VSS and 7.08, respectively). Then the PN/PS decreased to 3.74 after day 63 during period II. After dosing of redox mediator, the ratio of PN/PS became stable.

4. Discussion

4.1. Inhibition of azo dyes on overall performance of AMWT: mostly recovered by redox mediator dosage

The COD removal rate, VMP and mean color removal rate of the AMWT did not recover at azo dye concentration of 600 mg/L, different from those at azo dye concentration of 150, 300 and 450 mg/L. Therefore, azo dye concentration reaching 600 mg/L was regarded as the significant inhibition of overall (decolorizing and methanogenic) performance of the AMWT. Van der Zee et al. (2001) found that an up-flow anaerobic sludge blanket reactor (UASBR) was severely inhibited by 200 mg/L RR2. The relative high inhibitory threshold of azo dyes in this study could be attributed to the pre-hydrolysis of azo dyes. After hydrolysis of azo dyes' reactive groups (such as vinylsolfone in RB5, triazyl in RR2 shown in Fig. S1), the toxicity of azo dyes to anaerobic biomass would reduce (Van der Zee and Villaverde, 2005). However, the decline of bioreactor performance (Fig. 1) and inhibition of methanogenic activity (Fig. 2) illustrated that the hydrolyzed azo dyes still had strong toxicity on the anaerobic biomass, as their cleavage products aromatic amines had been demonstrated having low toxicity on anaerobic microorganisms (Isik and Sponza, 2007).

The dosing of AQS on day 97 (period III) enabled the color removal rate immediately recovered within 2 days. By changing the

Table 3
Physical characteristics of anaerobic granular sludge from the bioreactor at different time of operation.

Time (day)	Period	Porosity	Settling velocity (m/h)	Mean particle size (mm)	Integrity coefficient
0 (Seed sludge)		75.80%	46.61 ± 17.78	2.2495	10 ± 3
31	I	68.53%	44.57 ± 9.81	2.2995	12 ± 1
63	II	59.21%	42.32 ± 12.61	2.2730	11 ± 5
95		32.35%	33.91 ± 12.87	1.7292	38 ± 1
127	III	56.94%	32.13 ± 6.30	1.4930	38 ± 7

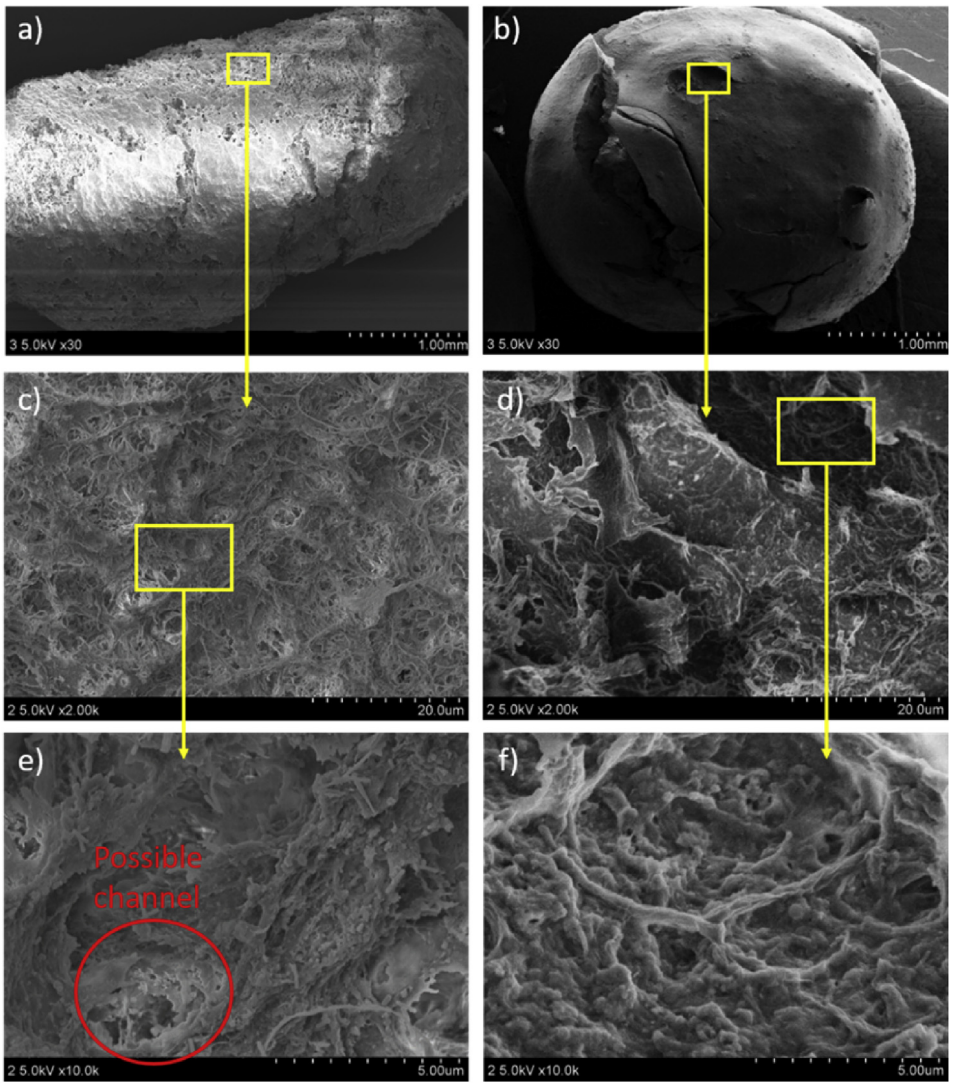

Fig. 4. SEM images of surface of AGS from the bioreactor at different times and with different magnifications: a) day 31 with magnification × 30; b) day 95 with magnification × 30; c) day 31 with magnification × 2000; d) day 95 with magnification × 2000; e) day 31 with magnification × 10,000; f) day 95 with magnification × 10,000.

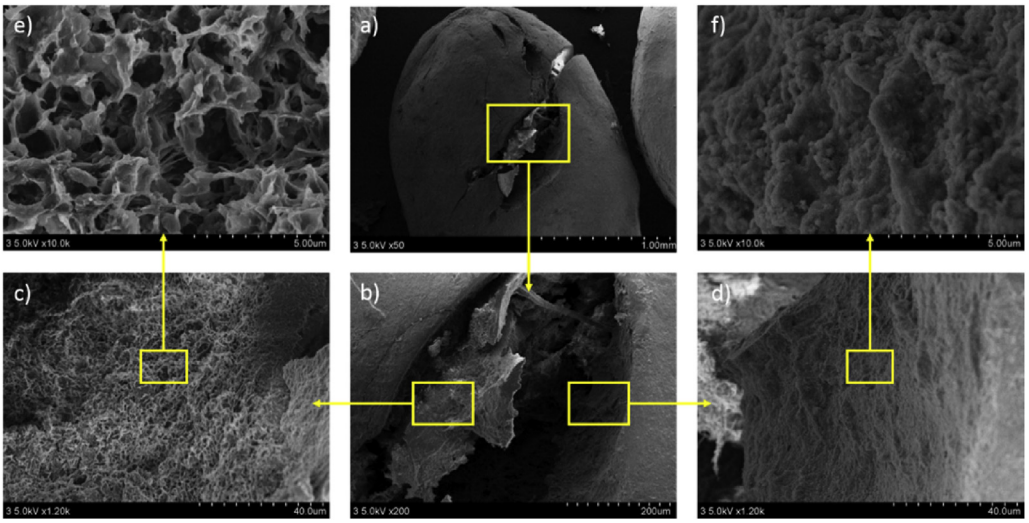

Fig. 5. SEM images of blocking phenomenon of AGS caused by possible aggregates of azo dyes: (a) magnification × 50; (b) magnification × 200; (c, d) magnification × 1200; (e, f) magnification × 10,000.

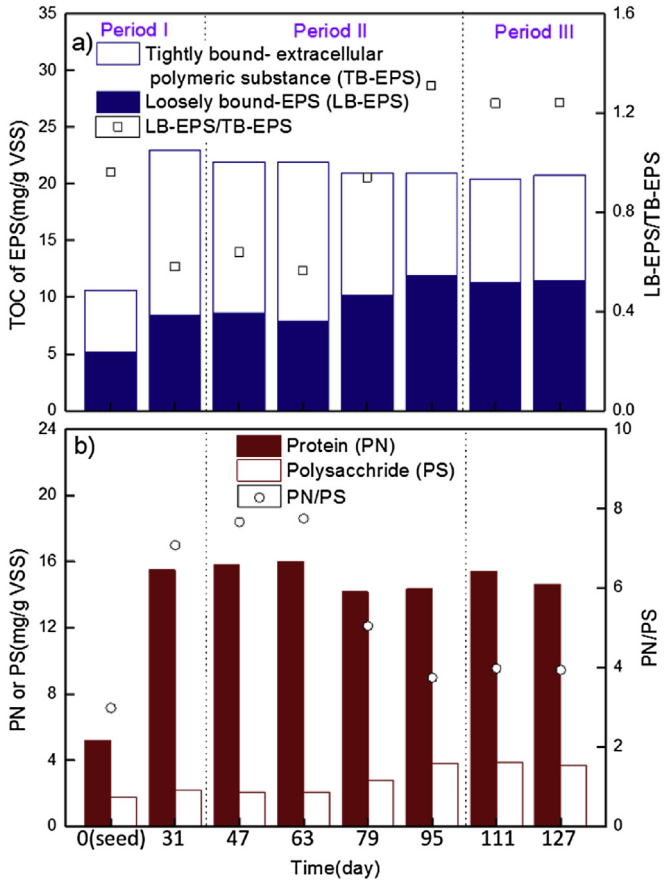

Fig. 6. EPS distribution (a) and PN, PS in EPS (b) of sludge from the bioreactor at different times of operation.

redox state of itself, the RM could efficiently transfer the reducing equivalent from co-substrate to the azo dye (Kazuya et al., 2009), which resulted in the acceleration of the reduction of azo dye. Therefore, the increase of color removal rate of the bioreactor after dosing of AQS is mainly due to the catalyzing effect of RM. Previous researchers have also tried to use the RM to remediate the AMWT inhibited by azo dyes. Van der Zee et al. (2003) added activated carbon as RM into a UASBR inhibited by RR2 and dye removal efficiency of the UASBR increased to 76% from the prior value of 32%, despite the declining of dye removal efficiency to 50% in following three weeks (due to a exhaustion of activated carbon for the dye adsorbing capacity). Though the RMs used in our and their studies were different, the RMs were both evidenced to remediate the inhibitory effect of azo dyes on the bioreactor decolorizing performance. However, since the COD removal rate, VMP, mean color removal rate and VFA after dosing of AQS are near or even a little lower than those at last phase of 450 mg/L, the AQS is defined to mostly remediate the overall performance.

The COD removal rate and VMP of the bioreactor increased while the VFA decreased after dosing of AQS, indicating that the methanogenic performance of the bioreactor somewhat recovered. However, the phenomenon of VFA accumulation in the bioreactor was still existed, suggesting that the methanogenic ability of the reactor did not recover completely. This was also demonstrated by the decrease of SMA of sludge in Fig. 2. Both acetoclastic and hydrogenotrophic methanogenic activity of sludge were evidenced to be inhibited by azo dyes. And the hydrogenotrophic methanogens were less susceptible to inhibition of azo dyes. This could be due to the susceptibility of acetoclastic methanogens (compared to hydrogenotrophic methanogens) to toxic or inhibitory compounds, which was also reported by other studies including to CuO nanoparticles (Otero-González et al., 2014), to As^{III} (Rodriguez-Freire et al., 2015), etc. Additionally, we considered that the relatively slow growth for acetoclastic methanogens in a co-metabolic system could also be another possible reason. In comparison to fermentative bacteria and hydrogenotrophic methanogens, acetoclastic methanogens has been believed to not participate effectively on dye reduction (Santos et al., 2007), and H_2 has been proven to be effective electron donor for azo dye reduction (Santos et al., 2006). Hydrogenotrophic methanogens can thus obtain energy from azo dye reduction while acetoclastic methanogens cannot, leading to the relatively slow growth of acetoclastic methanogens. These reasons could also explain why the recovery of acetoclastic methanogens was much slower than that of hydrogenotrophic methanogens.

Methane yield was observed to decline gradually during phase II (azo dye concentration increasing), and scarcely recovered after dosing of AQS. It might be due to the following: 1) inhibition of methanogenic activity by toxicity of azo dyes; 2) competition for reducing equivalents between dye reducers and other microorganisms, electrons which should have been transferred to acetate or H_2 (to form CH_4) were transferred to azo dyes (Santos et al., 2006).

4.2. Inhibitory mechanism of azo dyes on AWMT

According to the results in Figs. 2–4, we proposed a possible inhibitory mechanism of azo dyes on AGS wastewater treatment (Fig. 7). Biologically speaking (the right part of Fig. 7), the distribution of azo dyes in S-EPS, LB-EPS and TB-EPS suggests that the azo dyes were concentrated in TB-EPS and LB-EPS when concentrations of azo dyes in influent exceeded 450 mg/L. High concentration azo dyes in TB-EPS might be beneficial to their degradation because the driving force of mass transfer enhanced. However, the decrease of overall performance of the bioreactor implied that concentrated azo dyes in TB-EPS (or called enrichment effect) caused more negative effect. Compared to azo dyes in S-EPS and LB-EPS, azo dyes in TB-EPS would cause more severe inhibition because of their direct and tight contact with cells as well as increment of the effective toxic compounds concentration around the microorganisms.

In addition, the results of porosity and SEM of AGS reflected that

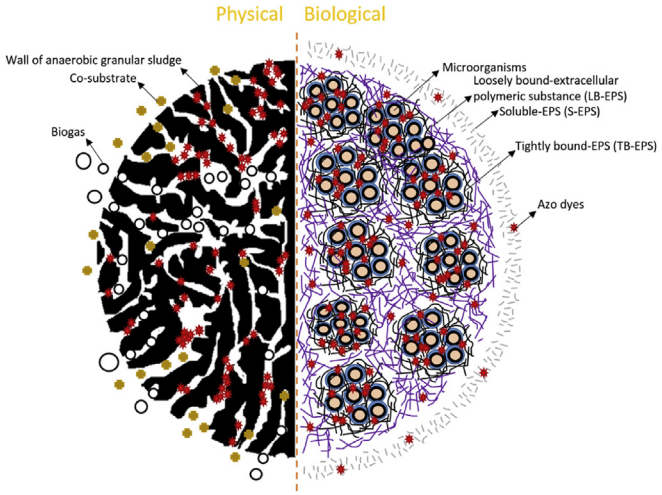

Fig. 7. Proposed schematic diagram of inhibitory mechanisms of azo dyes on anaerobic granular sludge at physical and biological aspects.

the inhibition of azo dyes on granular wastewater system should be considered physically, i.e. the channels of AGS were clogged by azo dyes (the left part of Fig. 7). The relative high aromatic amines concentration in LB-EPS and particularly TB-EPS of sludge on day 95 also evidenced that some channels of AGS were clogged by azo dyes, which hindered aromatic amines to release from inside of AGS. The clogging of channels in AGS would reduce the effective reaction volume of AGS. It means that the space of substrate in contact with microorganisms decreases. Likewise, the release of metabolic products (e.g. CH_4) and entering of co-substrate would be hindered, which slows down the degradation rate at the view of chemical kinetics. Furthermore, since the anaerobic bio-reduction of azo dyes requires electrons from co-substrate (Pereira et al., 2015), the hard entering of co-substrate into granules may limit the bio-reduction rate of azo dyes.

After dosing of AQS, the bio-reduction of azo dyes was accelerated. Subsequently the sludge channels clogged by azo dyes were dredged, resulting in increase of porosity of AGS and recovery of distributions of azo dye and aromatic amines in granules (Fig. 3(a and b)). It demonstrates again that the transport of co-substrate or products (CH_4, aromatic amines, etc.) was hindered by the block of channels in AGS.

4.3. Impact of azo dye inhibition on physical characteristics of AGS: hardly recovered by redox mediator dosage

As the high concentration sludge is the basic of high-rate anaerobic bioreactor to treat wastewater efficiently (Lettinga et al., 1997), much attention should be paid on the retention of AGS. Settling velocity of AGS is one of the main control factor regarding the sludge retention (Ghangrekar et al., 2005). Unfortunately, a sustained decline of settling velocity of AGS was appreciable after adding of azo dyes. More severely, sludge washout phenomenon was observed when azo dye concentration exceeded 450 mg/L. According to the formula of settling velocity in the Stokes range (Chen et al., 2013), the decrease of settling velocity could be related to the particle size and density of AGS. Since densities of AGS were relatively stable (fluctuated from 1.17 to 1.20 g/cm^3, data not shown), the decline of particle size of AGS could be the main cause. Meanwhile, the strength of AGS became worse during period II, which would lead AGS more susceptible to hydraulic and gaseous scour. As a consequence, the disintegration would happen, which was evidenced by the switch of dominant particle size in Fig. S4 and distinct decrease of mean particle size (Table 3). In addition, the settling ability, particle size and strength of AGS from the bioreactor seemed to be more unfavorable during phase III. It illustrates that the physical characteristics of AGS (except for porosity) cannot be resumed by dosing of RM (at least within a month).

EPS, a complex high-molecular-weight mixture of polymers secreted by multiple microbial communities, has great influence on physical characteristics and microbial function of sludge aggregates (Sheng et al., 2010). The total EPS increased considerably during period I, which could be due to protective response (Kokabian et al., 2013) of microorganisms towards the toxic compounds in the pretreatment wastewater. With increase of EPS, better adsorption and complexation of aggregates to substrates could be obtained (Kokabian et al., 2013). Then the total EPS kept stable after increasing concentration of azo dyes. It could be explained by the fact that the toxic substances in wastewater might go over a threshold, thus their effects on the stimulation of EPS production became less obvious (Sheng et al., 2005). The ratio of LB-EPS/TB-EPS was observed to increase when azo dye concentration exceeded 450 mg/L, indicating that the amount of EPS tightly bounding the microorganisms declined, which means the protective effect of EPS on microorganisms weakens. The increase of LB-EPS has a negative effect on the settling ability of sludge (Yang and Li, 2009). In the meantime, the PN/PS ratio of the EPS decreased. Microbial aggregates incline to deflocculate when their surface proteins were removed (Higgins and Novak, 1997). Given this, we believe that the decrease of PN content is somewhat responsible for the disintegration and strength attenuation of sludge. Furthermore, increase of PS content in EPS can induce adherence properties for AGS (Lu et al., 2015), leading to production of foam and easily adherence to biogas-bubbles. Subsequently floating and washout of sludge would happen. It is noteworthy that the total EPS and its distribution, PN, PS in EPS were stable but not recovered after dosing of RM, explaining why the physical characteristics of AGS did not recover during period III.

4.4. Implications of this work

Generally, inhibition of azo dyes on AMWT was studied from the view of biological toxicity. Our results showed that the methanogenic AGS was inhibited not only by concentrated azo dyes in TB-EPS, but also by the clogging of channels in AGS caused by azo dyes. Thus the remediation of the inhibition should be considered both biologically and physically.

Azo dye concentration in the wastewater discharged by textile dyeing industry was always higher than 500 mg/L (Van der Zee and Villaverde, 2005; Santos et al., 2007; O'Neill et al., 2000a), which had the risk of causing inhibition on AMWT. In this study, the dosing of RM could mostly recover the overall (decolorizing and methanogenic) performance of the bioreactor, though the recovery of methanogenic performance was slow. However, physical characteristics of AGS did not revive and washout phenomenon of sludge was still happening after dosing of RM. Thus it could be concluded that the RM could be practically adopted to remediate the overall performance of a bioreactor inhibited by azo dyes, but meanwhile the control strategy for preventing sludge washout should be employed carefully.

Though the overall performance of the bioreactor could be mostly remediated when the AQS dosage level is low (μM level), continuous dosing of AQS would not only be economically unavailable (in the practical engineering) but also cause secondary pollution because AQS is recalcitrant to biodegradation (Van der Zee et al., 2003). Since insoluble RMs were widely developed these years and they were proven to have favorable adsorption capacities (which would be beneficial to the sludge retention) (Van der Zee et al., 2003; Wang et al., 2009), insoluble RMs might have the potential to remediate the AMWT inhibited by azo dyes.

5. Conclusions

Azo dyes with high concentration (600 mg/L) could cause significant inhibition on overall performance of AMWT. The high biological toxicity of azo dyes was attributed to the enrichment effect in TB-EPS. The channels of AGS was clogged by azo dyes so that the transport of substrate and products in AGS was limited. The settling ability, particle size and strength of AGS all became worse after azo dyes concentration exceeded 450 mg/L. The dosing of RM could mostly remediate overall (decolorizing and methanogenic) performance of the bioreactor even if the recovery of acetoclastic methanogens was slow. However, most physical characteristics of AGS did not recover (except for porosity with partial recovery), indicating more attention should be paid to prevent sludge from washout if RM was practically used to remediate the AMWT inhibited by azo dyes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2016.08.046.

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