Two-stage anaerobic process benefits removal for azo dye orange II with starch as primary co-substrate

Jingang Huang, Binfang Shi, Zhenjiang Yin, Kangyin Guo, Chen Fu and Junhong Tang

ABSTRACT

Two-stage anaerobic system (S1: R1 (acidogenic phase) + R2 (methanogenic phase)) and the one-stage control (S0) were established to investigate the effect of phase separation on the removal of an azo dye orange II, i.e., Acid Orange 7 (AO7), with starch as the primary co-substrate. Although final AO7 removal from two systems showed no statistical differences, the first-order rate constants for AO7 removal (k_{AO7}) and sulfanilic acid (SA) formation (k_{SA}) were higher in S1. Kinetic analysis showed that k_{AO7} and k_{SA} in S1 were 2.7-fold and 1.7-fold of those in S0, respectively, indicating the benefit of phase separation to the AO7 reduction. However, this benefit only appeared in the period with influent AO7 concentrations higher than 2.14 mM. Otherwise, this advantage would be hidden due to the longer HRT (5 d) and sufficient electron donor (1.0 g starch L^{-1}). Within S1, R1 only contributed about 10% of the entire AO7 removal, and k_{AO7} in R1 (0.172 h^{-1}) was much lower than in R2 (0.503 h^{-1}). The methanogenic phase rather than acidogenic phase was the main contribution to AO7 removal, because the influent of R2 had more available electron donors and suitable pH condition (pH 6.5–7.0) for the bio-reduction process.

Key words | azo dye, phase separation, reduction, starch, two-stage

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HIGHLIGHTS

- Effect of phase separation on azo dye removal was investigated.
- Phase separation benefits azo dye removal with starch as co-substrate.
- k_{AO7} and k_{SA} were higher in the two-stage anaerobic system.
- The advantage of phase separation would be hidden by longer HRT.
- Methanogenic phase was the main contribution to AO7 removal.

INTRODUCTION

China and other Asian countries have been suffering from heavy pollutions caused by textile and dyeing industries in the past decades. Azo dyes and other chemical additives contained in textile dyeing wastewater are not readily degradable, resulting in the potential environmental and health risks. Thus, an adequate treatment is required before textile dyeing wastewater discharges to the water environment. Many chemical processes, including Fenton- or UV-assisted advanced oxidation processes (AOPs), have been widely used due to their quick elimination of azo dyes from wastewater (Shokri *et al.* 2017; Shokri 2018). However, biological process is still the cheap and energy-saving option (Xu *et al.* 2018). Traditional biological systems, which are composed of anaerobic and series-connected aerobic processes, were mostly employed in engineering applications (da Silva *et al.* 2012a; Abiri *et al.* 2017). The anaerobic reaction in the above combined process contributed to a large part of colour removal and could improve the removal of bio-refractory organic pollutants, finally enhancing the chemical oxygen demand (COD) removal in the following aerobic process (Frijters *et al.* 2006; Hameed & Ismail 2020). However, the slow anaerobic process limited the treatment process because

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of the electrophilic property of the –$N=$N– bond associated in azo dye molecules. Therefore, to fully exploit the potential advantages of the combined bio-process for textile wastewater treatment, it is necessary to upgrade the anaerobic process.

Many anaerobic systems, such as the up-flow anaerobic sludge blanket (UASB) and anaerobic sequencing batch reactor (ASBR), have been successfully applied in textile wastewater treatment (Huang *et al.* 2015). In these systems, the acidogenic and methanogenic microorganisms co-exist to contribute the azo dye reduction (da Silva *et al.* 2012b), where the removal of COD and colour were only 20–30% and 40–60%, respectively. To avoid the imbalance between acidogenic and methanogenic microorganisms, a two-stage anaerobic process was always used in treating wastewaters with high organic load rate (OLR) and/or toxicity (Ráduly *et al.* 2016; Tanikawa *et al.* 2016). Two-stage anaerobic process was assembled with two series-connected reactors, separating the acidogenic and methanogenic phases. In this way, the conditions for different microbial consortia in two separated phases were optimized, and finally enhance the pollutant removal (Silva *et al.* 2020). Firmino *et al.* (2010) have reported that azo dye removal in two-stage anaerobic system was more stable than in a one-stage system. Some substrates, such as Fe^0 and redox mediators (RMs), were introduced to the acidogenic phase to enhance the key enzyme activities and the pollutant removal, thence hiding the benefits of phase separation (da Silva *et al.* 2012b; Liu *et al.* 2012).

The above studies were all fed with ethanol or easily biodegradable electron donors for azo dye decolouration. However, starch is usually used as a sizing agent in textile industry (Franca *et al.* 2015), resulting in real textile wastewater always containing starch but not ethanol. Due to higher molecular weight, the conversion of starch to ethanol and/or other readily biodegradable intermediates needs special conditions such as longer hydraulic retention time (HRT) (Bai *et al.* 2008). Therefore, the characteristics of the two-stage process applied in real textile dying wastewater might be more complex than a laboratory-scale study with readily biodegradable ethanol and/or volatile fatty acids (VFAs) as co-substrates. This would in turn affect the azo dye removal. Thus, the scrutinizing investigation of phase separation for anaerobic treatment of real textile wastewater has a remarkable engineering significance.

In this study, two-stage ASBR systems were established to study the effect of phase separation on azo dye removal with soluble starch as a primary electron donor. It aims to: (1) investigate the advantage of two-stage anaerobic process for azo dye removal by both long-time operation and kinetic analysis; and (2) reveal the associated mechanism relevant to azo dye reduction and starch conversion.

MATERIALS AND METHODS

Experimental set-up

Two laboratory-scale systems (S0 and S1) composed of plexiglass- made ASBRs were set up in this study. The schematic diagram of the two-stage ASBR system were designed as shown in Figure 1. System S0 was designed as a one-stage anaerobic system (control), which consisted of only one reactor (R0), while system S1 was designed as a two-stage anaerobic system consisting of two series-connected ASBRs (R1: acidogenic phase; R2: methanogenic phase). The working volumes of R0, R1 and R2 were 5.0 L, 2.2 L and 4.4 L, respectively. Water seals were used to keep the ASBR in airtight condition. The ASBRs were continuously stirred (80 rpm) by an electric mixer (HD 2004 W, Sile Co., Shanghai, China) in a water bath at 35 ± 1 °C. The two systems were operated with one cycle per day. Each cycle contained the following operations: 22 h stirring, 1.5 h settling, 10 min decanting, and 20 min feeding. The inoculated sludge was obtained from a secondary clarifier at Qige municipal wastewater treatment plant in Hangzhou, China. Inoculated sludge in any ASBR was 3 g VSS L^{-1}. Artificial textile wastewater was employed as the influent in this study. Azo dye orange II; that is, Acid Orange 7 (AO7), and soluble starch, were the main composition of the feed water. Macro and trace metals and required vitamins were supplemented as described according to the previous study (Huang *et al.* 2015).

Continuous operation

To ensure the two systems were comparable in azo dye removal, the HRT in both S0 and S1 was designed at 5 d. This was determined according to a previous study when starch was employed as a co-substrate for azo dye removal (Manu & Chaudhari 2003). To bring out a phase separation in S1, the designed HRTs of R1 and R2 were 1.67 and 3.33 d, respectively; the designed pH in R1 ranged from 5.5 to 6.5, while that in R2 was 6.5 to 7.0, which was the same as that in S0 (R0). The designed pH in each ASBR was maintained by adding stocked $NaHCO_3$ buffer solution (50 g L^{-1}). During the entire operation, soluble starch concentrations in the influent of S0 and S1 were maintained at 1,000 mg L^{-1}; that is, the COD equivalent was

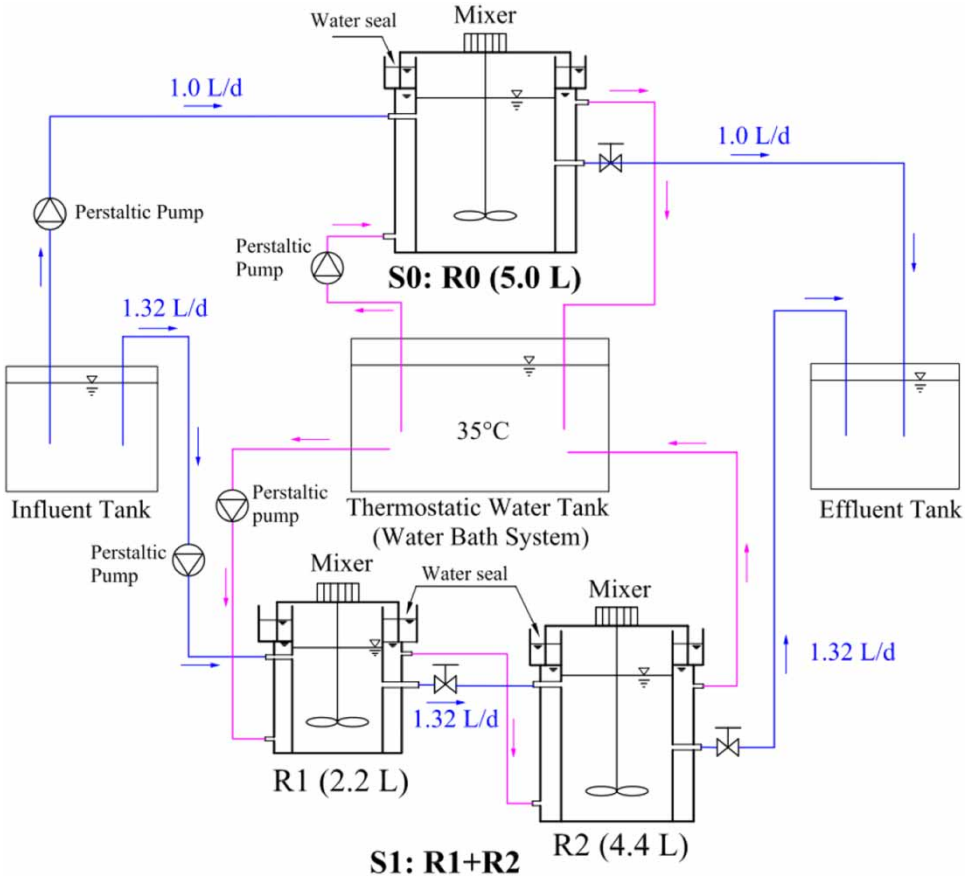

Figure 1 | Schematic diagram of the one-stage (S0) and two-stage (S1) systems.

1,067 mg COD L^{-1}, resulting in starch loading rates of 0.2 kg m^{-3} d^{-1}. During the start-up period (day 1 to day 42), the influent AO7 concentrations were gradually increased from 0.43 to 2.57 mM, and finally stabilized at 2.14 mM after day 43, lasting to the end of the operational period (day 77). $NaHCO_3$ and AO7 in the influent of each system at different operating times are shown in Table 1.

To obtain the designed HRT of S0, 1.0 L of artificial wastewater was fed to R0 (working volume 5.0 L) per day. However, it contained two series-connected ASBRs in S1. That is, R1 and R2. R1 was fed with fresh-prepared artificial wastewater, while R2 was fed with the effluent of R1. An HRT of 1.67 d in R1 (working volume 2.2 L) was obtained by replacing 1.32 L of fresh artificial wastewater per day; and an HRT of 3.33 d in R2 (working volume 4.4 L) was obtained by removing 1.32 L of the effluent in R2 and adding 1.32 L of the effluent in R1 per day. To monitor the operating performance of these systems, pH, VFAs, AO7 and its reduction product of sulfanilic acid (SA) in the effluent were analysed.

Batch assay

Batch assays were conducted on day 74 to better illustrate the decolourization processes for the one-stage and two-stage anaerobic process in reducing AO7. The kinetics of AO7 removal and SA yield were determined by varied concentrations as a function of time expanding. Within the batch assay cycle, 5 mL of mixture were withdrawn from R0, R1 and R2 at appropriate time intervals (0, 2, 4, 6, 8, 10, 12, and 24 h). Pseudo first-order kinetics were usually employed for the degradation rate analysis of bio-refractory pollutants (Shokri *et al.* 2016; Shokri & Mahanpoor 2017), and also well described the azo dye reduction process (Yang *et al.* 2016; Olivo-Alanis *et al.* 2018). The equations of modified pseudo first-order kinetic models were as follows.

$$C_{AO7(t)} = a + b\,e^{(-k_{AO7}t)} \quad (1)$$

$$C_{SA(t)} = a + b\,e^{(-k_{SA}t)} \quad (2)$$

Table 1 | $NaHCO_3$ and AO7 concentrations in the influent of each ASBR during different periods

		$NaHCO_3$ (mg L^{-1})*			
Marking	**Time (d)**	**R0**	**R1**	**R2**	**AO7 (mM)****
a	1	300	0	300	0.43
b	5	400	400	400	0.57
c	6	500	300	400	0.57
d	7	600	300	400	0.57
e	8	600	300	400	0.71
f	9	750	500	500	0.71
g	10	1000	600	500	0.71
h	11	1000	600	400	0.86
i	12	1100	600	400	0.86
j	16	1100	600	400	1.14
k	20	1100	600	600	1.14
l	28	1100	600	600	2.14
m	34	1100	600	600	2.57
n	43	1100	600	600	2.14

Note: *$NaHCO_3$ concentrations in each reactor were different.
**Indicates the same influent AO7 concentrations in systems S0 and S1.

where $C_{AO7(t)}$ and $C_{SA(t)}$ were the AO7 and SA concentrations (mM) at the time of t (h) during batch assays; k_{AO7} and k_{SA} (h^{-1}) were the first-order rate constants for AO7 removal and SA formation, respectively; a is the possible minimum and b is the possible maximum concentration of AO7 or SA (mM) during the batch assay.

Chemical analysis

All collected samples were centrifuged at 4,000 × g for 20 min, and then membrane filtered (0.45 μm). AO7 was spectrophoto-metrically measured at the wavelength of 484 nm. VFAs were composed of acetate, propionate, *n/iso*-butyrate, and *n/iso*-valerate. SA and VFA components were simultaneously measured by a high performance liquid chromatography unit (HPLC, Agilent 1200, USA) equipped with a UV detector at 210 nm. Shodex RSpak KC-G + RSpak KC-811 columns (Showa Denko, Japan) was used in assembly. The mobile phase was phosphoric acid solution (0.05%) at a flow rate of 0.7 mL min^{-1}. The concentrations of each VFA component were converted into COD with a coefficient as follows: acetate (1.067), propionate (1.512), butyrate (1.818) and valerate (2.039).

Statistical analysis

To compare the average levels of effluent AO7, SA and VFAs during the operational period between two experimental sets of S0 and S1, an analysis of Student's unpaired t-test was used and $P < 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

AO7 reduction in continuous operation

The effluent concentrations of AO7 and SA in the one-stage system S0 (R0) and two-stage system S1 (R1 + R2) are shown in Figure 2. Under an HRT of 5 d, the effluent AO7 concentrations of both systems were at very low levels, and the final AO7 removal achieved 90% and 95%, respectively (Figure 2(a)). During the entire period, the maximum effluent SA concentrations of S0 and S1 were 1.79 and

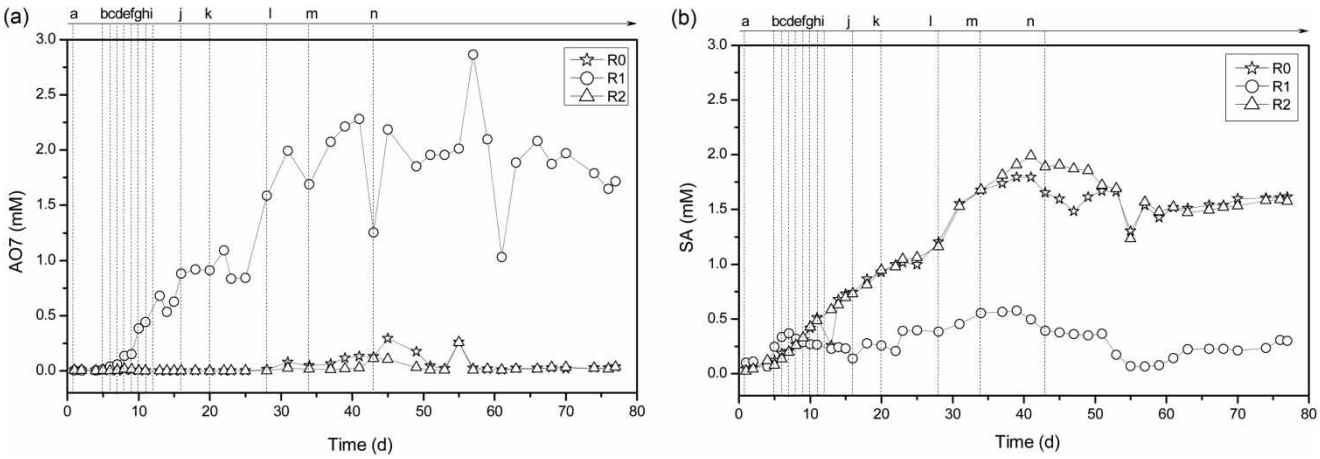

Figure 2 | Effluent concentrations of AO7 (a) and SA (b) in system S1 (R0) and S2 (R1 + R2) during the entire operational period.

2.00 mM (day 41), respectively; and during the stable period (after day 50), they kept at 1.6 ± 0.1 and 1.7 ± 0.2 mM, respectively (Figure 2(b)). Because SA was one of the reductive products of AO7 decolouration, bio-reduction was the main pathway for AO7 removal in this study. The statistical analysis of the above results suggested that better AO7 removal and SA production in S1 were only found during days 34–49 ($P < 0.05$); whereas no significant differences were observed during the entire and the stable periods ($P > 0.05$). After influent AO7 was resumed to 2.14 mM on day 43, final AO7 removal in S0 declined from 94.0% to 88.7%, while it still kept at ~95% in S1. Therefore, S1 was more efficient than S0 in removing AO7 when influent concentration was higher than 2.14 mM. However, under lower influent concentration, the advantage of phase separation on AO7 removal would be sheltered from the long HRT (5 d) and sufficient electron donor supply (1,067 mg COD L^{-1}). Firmino *et al.* (2010) reported a similar result, that the stable azo dye (Congo Red) removal in a two-stage anaerobic process (UASB) was dependent on the molar ratio of available electron donor to azo dye. Under sufficient electron donor condition, the competition between azo dye reduction and other electron-consuming biological processes such as methanation could be ignored, resulting in comparable azo dye removal in both one- and two-stage processes (da Silva *et al.* 2013). On day 34, influent AO7 increased to the maximum of 2.57 mM, which might need more electron donor for its reduction process. In this condition, the electron competition resulted in less electron flow shift to the azo dye reduction process (Firmino *et al.* 2010). In this study, although the required electron donor for a complete reduction of maximum 2.57 mM of AO7 was just 82 mg COD L^{-1}, much lower than the added electron donor (1,067 mg COD L^{-1}), it might limit the reduction kinetics by the smaller concentrations of available substrates. The two-stage anaerobic process separated the acidogenic and methanogenic phases, which would optimize the constituents of available electron donors and reaction kinetics for AO7 reduction, promoting more electrons shifting from the available donor to the final acceptor (AO7). Apart from this way, the addition of RMs has also been reported to improve the reaction kinetics and finally make a sound impact of phase separation on azo dye decolourization (da Silva *et al.* 2012b; Liu *et al.* 2012). Thus, a kinetic study within one batch cycle should be conducted to clearly state the AO7 reduction process in one- and two-stage systems.

As mentioned for two-stage system S1, it was found that effluent AO7 in the acidogenic phase (R1) was much higher than that in the methanogenic phase (R2). R1 only accounted for about 10% of the entire AO7 removal of S1, implying that the methanogenic phase had a greater contribution to AO7 removal in this study. This in turn led to a higher SA production in R2 than that in R1. The result was in contrast with the previous publication, in which the fermentation process rather than the methanogenic process was considered as the main contribution to the azo dye decolourization (Dos Santos *et al.* 2006). It was noticed that the used co-substrates for azo dye reduction in the previous study were all efficient electron donors such as glucose, methanol, acetate, formate and H_2/CO_2 (Hong *et al.* 2007). Whereas starch, which is widely used for the sizing process of the textile dyeing industry, was used in this study. As compared to those readily bio-available electron donors, starch has a much higher molecular weight and complex structure; it should be first hydrolysed to monosaccharide and then be acidified to VFAs and H_2 in R1. This lagged process of available electron donor production might hide the advantage of AO7 reduction in the acidification phase (R1). The detailed characteristics of reaction kinetics and electron donor conversion in the two-stage anaerobic system and their effects on AO7 reduction will be discussed below.

AO7 reduction in batch assay

To compare the reaction kinetics of the one-stage system S0 (R0) and two-stage system S1 (R1 + R2), concentrations of AO7 and SA in R0, R1 and R2 at different times during one batch cycle on day 74 were analysed. The obtained results were fitted by Equations (1) and (2) (Figure 3). The rate constants for AO7 removal (k_{AO7}) and SA formation rate (k_{SA}) are shown in Table 2.

Figure 3(a) shows that AO7 was completely removed within 6 h in the methanogenic phase (R2) of S1, while that in S0 (R0) needed 12 h. Figure 3(b) shows that SA was simultaneously formed during the batch assays. k_{AO7} and k_{SA} from R2 were 2.7-fold and 1.7-fold of those from R0, respectively (Table 1). The above kinetic analysis from batch assays indicated that the two-stage process of S1 was more efficient in removing AO7 than S0, suggesting the benefit of phase separation to the AO7 reduction process. However, the longer HRT (5 d) used in this study has hidden this advantage, resulting in comparable AO7 removal during the continuous operation as described in Figure 1. Under suitable HRT and pH conditions, two-stage anaerobic processes have been previously reported to be more effective in pollutant removal and bio-energy production than those in one-stage systems (Boonsawang

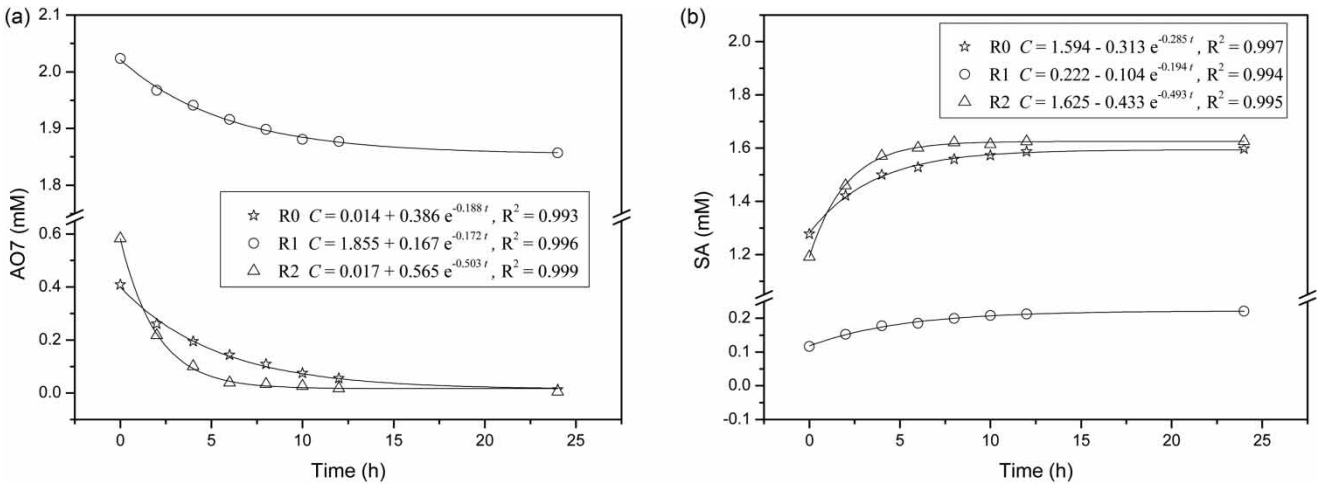

Figure 3 | AO7 (a) and SA (b) concentrations at different times, and first-order kinetic fitting curves in one-stage system S0 (R0, control) and two-stage system S1 (R1, R2).

Table 2 | Pseudo first-order rate constants of AO7 removal (k_{AO7}) and SA formation (k_{SA}) during the stable period (day 74) in systems S0 (R0) and S1 (R1, R2)

	R0		R1		R2	
Operational stage	k_{AO7} (h^{-1})	k_{SA} (h^{-1})	k_{AO7} (h^{-1})	k_{SA} (h^{-1})	k_{AO7} (h^{-1})	k_{SA} (h^{-1})
Day 74	0.188	0.285	0.172	0.194	0.503	0.493

et al. 2015). If the applied HRT was shortened, the beneficial effect of phase separation on AO7 removal during the long operation period could also appear. This was a promising way and needed further studies, because the shortened HRT would decrease the reactor size and save the cost for textile wastewater treatment.

As mentioned for the AO7 reduction process within the acidogenic phase (R1) of S1, it was noticed that the AO7 concentration declined from 2.02 mM to 1.86 mM during the batch assay, and k_{AO7} and k_{SA} were 0.172 h^{-1} and 0.194 h^{-1}, respectively. Therefore, AO7 reduction in this study was mainly finished in the methanogenic phase (R2). The result of the batch assay was consistent with the performance of continuous operation, but was in contrast with the previous publication (Dos Santos *et al.* 2006). The possible reasons for these results have been hypothesized in the above section, and the related mechanisms responsible for electron donor conversion will be discussed below.

VFAs accumulation in continuous operation

Soluble starch (1.0 g L^{-1}) was used as a co-substrate and primary electron donor in this study. Under anaerobic conditions, starch can be hydrolysed, fermented to VFAs, and then used for AO7 reduction, methanation and biomass yield. To elucidate the electron donor conversion in this study, intermediates of VFAs in the effluent of the one-stage system S0 (R0) and two-stage system S1 (R1 + R2) during the entire period are shown in Figure 4.

In the initial period (days 1–15), influent AO7 concentrations were at a lower level (<0.86 mM, 300 mg L^{-1}), and VFAs were not detected in both effluents. After then, influent AO7 gradually increased to the maximum of 2.57 mM, along with the increased VFAs in the final effluent of both systems. On day 45, the final effluent VFAs from S0 (R0) and S1 (R2) achieved the maximum of 685 and 522 mg COD L^{-1}, respectively. Afterwards, influent AO7 resumed to 2.14 mM until the end of operation (day 77); accordingly, a decreased and relatively stable VFAs accumulation in both systems was observed (150–300 mg COD L^{-1}). Therefore, the electron shift from the donor VFAs to the acceptor AO7 hardly contributed to the variation of effluent VFAs. This could also be explained by the stoichiometric requirement that the maximum reduction of 2.57 mM was only 82 mg COD L^{-1}, less than 10% of the addition. Thus, the VFAs accumulation was mainly attributed to the balance of hydrolysis-acidification and methanation processes. It was observed that the acidogenic phase (R1) in S1 was always in higher VFAs accumulation; even in the initial stage, the VFA concentration appeared as nearly 600 mg COD L^{-1}. Therefore, the slow AO7 reduction in R1 was not due to insufficient electron donor; the higher influent AO7 concentration would inhibit the methanogenic process but weakly to the acidogenic process. Thus, the consumption of the produced

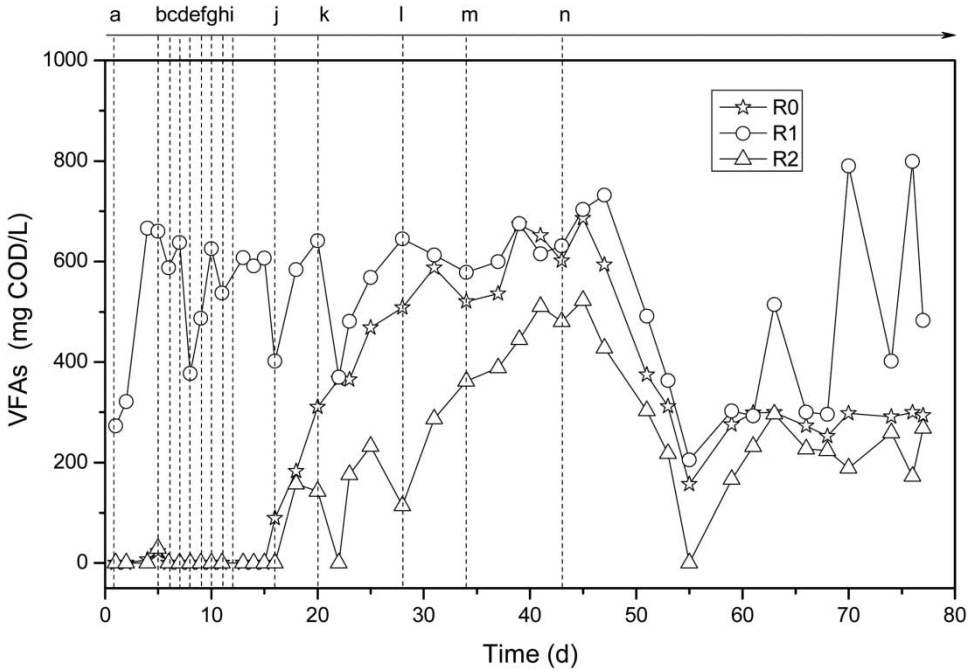

Figure 4 | Effluent VFA concentrations in system S0 (R0, control) and S1 (R1, R2) during the entire operational period.

VFAs by methanogenic microorganisms will decline along with the increase of influent AO7 concentrations, resulting in an increased VFAs accumulation in the effluent. This finding was consistent with a previous study indicating that a certain azo dye concentration (above 200 mg L^{-1}) would inhibit the methanogenic processes (Işik & Sponza 2005); as a consequence, the accumulated VFAs were observed proportionally at a higher azo dye concentration.

Overall, it was further found that the final effluent VFAs of S0 (R0) were significantly higher than those of S1 (R2), especially during the middle stage (days 20–50) of the operation ($P < 0.05$). The phase separation in the two-stage anaerobic system balanced the metabolisms of acidogenic and methanogenic processes (Ráduly *et al.* 2016). As a result, VFAs were favourably produced from starch fermentation in R1, and were then efficiently converted to methane in R2, leading to a lower VFAs accumulation in the final effluent of S1.

Starch is one of the polysaccharides with higher molecular weight. Albuquerque *et al.* (2005) reported that starch itself could not be easily utilized as an electron donor for the bio-reduction of azo dyes. When starch is used as a primary electron donor, it should be first chemically or biologically hydrolysed to reducing substrates such as glucose, which could be easily fermented to C_2–C_5 VFAs. Among which, C_3–C_5 VFAs (propionate, butyrate and valerate) could be further acetified to H_2 and acetate, and then be used for methanogenic process and/or for azo dye reduction (Manu & Chaudhari 2002). The proposed two-stage anaerobic process of AO7 reduction with starch as a primary electron donor is shown in Figure 5.

This complex process might be the reason for the long HRT (5–10 d) used in semi-continuous reactors with starch (1.0 g L^{-1}) as an electron donor in treating cotton dyeing wastewater (Manu & Chaudhari 2003). The relatively lower HRT (1 d) with tapioca starch as an electron donor only resulted in less than 60% removal of azo dye, much lower than that in this study and the above report (Chinwetkitvanich *et al.* 2000). Thus, the microorganisms in R1 mainly participated in the hydrolysis-acidification process, and the operating conditions such as weak acid pH (5.5–6.5) were not suitable for AO7 removal. Whereas in R2, effluent from R1 containing readily available electron donors was fed as influent, and it was operated in neutral condition (pH 6.5–7.0), in which AO7 was easier to reduce than that in R1. This could be the explanation for the contrasting result with ethanol as a primary electron donor, in which the acidogenic phase was considered as the main contribution to azo dye removal (Dos Santos *et al.* 2006; da Silva *et al.* 2013).

CONCLUSIONS

During the continuous operation, the benefit of phase separation in the two-stage anaerobic system only appeared in the

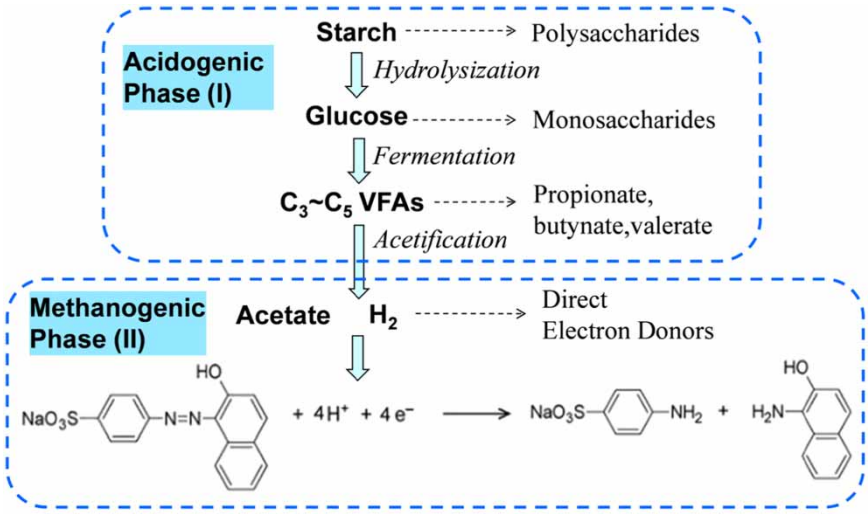

Figure 5 | The proposed two-stage anaerobic process for AO7 reduction with starch as a primary electron donor.

period with higher influent AO7 concentrations (>2.14 mM); while no statistical differences between two systems were observed in the entire operational period. However, k_{AO7} and k_{SA} in the two-stage system were 2.7-fold and 1.7-fold of those in one-stage system, indicating the positive effect of phase separation on AO7 reduction. The longer HRT (5 d) and sufficient electron donor supply (1.0 g starch L^{-1}) could hide the advantage of phase separation in AO7 reduction during the continuous operation. Within the two-stage system, the methanogenic phase accounted for about 90% of the entire AO7 removal, and the obtained k_{AO7} was 2.93-fold of that in the acidogenic phase. Methanogenic phase rather than acidogenic phase was the main contribution to AO7 removal. Effluent from the acidogenic phase containing readily available electron donors was fed as the influent for the methanogenic phase, in which AO7 was preferred to be reduced.

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