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Effect of redox mediator, AQDS, on the decolourisation of a reactive azo dye containing triazine group in a thermophilic anaerobic EGSB reactor

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Abstract

The feasibility of thermophilic (55 °C) anaerobic treatment applied to colour removal of a triazine contained reactive azo dye was investigated in two 0.53 l expanded granular sludge blanket (EGSB) reactors in parallel at a hydraulic retention time (HRT) of 10 h. Generally, this group of azo dyes shows the lowest decolourisation rates during mesophilic anaerobic treatment. The impact of the redox mediator addition on colour removal rates was also evaluated. Reactive Red 2 (RR2) and anthraquinone-2,6-disulfonate (AQDS) were selected as model compounds for azo dye and redox mediator, respectively. The reactors achieved excellent colour removal efficiencies with a high stability, even when high loading rates of RR2 were applied (2.7 g RR2 l^{-1} per day). Although AQDS addition at catalytic concentrations improved the decolourisation rates, the impact of AQDS on colour removal was less apparent than expected. Results show that the AQDS-free reactor R2 achieved excellent colour removal rates with efficiencies around 91%, compared with the efficiencies around 95% for the AQDS-supplied reactor R1. Batch experiments confirmed that the decolourisation rates were co-substrate dependent, in which the volatile fatty acids (VFA) mixture was the least efficient co-substrate. The highest decolourisation rate was achieved in the presence of either hydrogen or formate, although the presence of glucose had a significant impact on the colour removal rates.
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Keywords: Azo dye; Triazine group; Colour removal; Thermophilic; Anaerobic; Redox mediator; AQDS

1. Introduction

Almost one million tons of dyes are annually produced in the world [1], of which azo dyes, characterised by an azo-bond (R_1–N=N–R_2), represent about 70% on a weight basis [2]. Reactive azo dyes, i.e. dyes with reactive groups that form covalent bonds with OH–, NH–, or SH– groups, are extensively used in the textile industry, despite the fact that they have a low degree of fixation into the fibres (efficiency 10–50%). Reactive azo dyes are highly water-soluble. Therefore, wastewater treatment processes such as activated sludge systems or by using coagulation methods, may not be efficient to treat them [3].

Biological treatment applied to azo dye treatment has been extensively researched. Under aerobic conditions low colour removal efficiencies are achieved (10–30%), because oxygen is a more effective electron acceptor, therefore having more preference for reducing equivalents than azo dye [4]. In contrast, anaerobic treatment generally gives good colour removal efficiencies [5–7]. Since textile industry wastewaters are generally discharged at high temperatures (40–70 °C), thermophilic anaerobic treatment could serve as an interesting option, especially when closing process water cycles is considered.

Some reactive azo dyes containing triazine as reactive group presented the lowest colour removal rates during mesophilic anaerobic colour removal [8]. The required high hydraulic retention time (HRT) in the reactors could be lowered by using quinone compounds to accelerate the electron transfer from the micro-organisms to the dye [5,6]. Quinones are the electron accepting moieties of humic substances. Such compounds have been shown to play an important role not only as final electron acceptor for many recalcitrant organic compounds, but also facilitating electron transfer from an electron donor to an electron acceptor, e.g. azo dyes [9,10]. The first step is the non-specific enzymatic reduction of quinone to hydroquinone, and the second step is the chemical reoxidation of hydroquinone by the azo dye [11]. The redox potential prevailing in the medium is an important factor determining the rate of electron transfer when redox mediators are involved [12]. Either the mediator

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reduction or its regeneration can be the process rate-limiting steps. Therefore, compared with mesophilic anaerobic treatment, thermophilic anaerobic treatment seems to be advantageous not only for the expected faster enzymatic reduction of the mediator, but also for its chemical regeneration.

The main objective of this paper is to evaluate the colour removal efficiency of a continuous flow EGSB reactor at 55 °C treating an azo dye containing triazine as reactive group. The impact of AQDS as a redox mediator on the decolourisation rate is also studied. Co-substrate and temperature dependency were researched by using standardised activity tests.

2. Materials and methods

2.1. Chemicals

Reactive Red 2 (RR2), a sulfonated reactive azo dye, was selected as model compound in this study (Procion Red MX-5B, ∼50% of purity) (Aldrich, Gillingham, UK). RR2 was used without additional purification. Prior to utilisation, RR2 was hydrolysed by increasing the pH to 11 with NaOH, heating at 80 °C for 1 h, and by decreasing the pH to 7 with HCl [13]. This procedure aims to simulate the hydrolysed dye structure found in real textile wastewaters. Fig. 1 shows the chemical structure before and after hydrolysis.

Anthraquinone-2,6-disulfonate (AQDS) (Aldrich) was used as redox mediator model compound, without additional purification.

2.2. Batch and continuous experiments

2.2.1. Seed inoculum and basal medium for decolourisation assays

Granular anaerobic sludge was collected from a full-scale mesophilic upflow anaerobic sludge blanket (UASB) reactor treating paper mill wastewater (Eerbeek, The Netherlands). The mesophilic sludge was acclimated for 3 months at 55 °C in an expanded granular sludge bed (EGSB) reactor (5.6 l) operating at a HRT of about 6 h and an organic loading rate (OLR) of 2.5 kg COD m^{-3} per day. The chemical oxygen demand (COD) consisted of a mixture of glucose and volatile fatty acids (VFA) at a COD ratio of 1:3. The neutralised VFA solution contained acetate, propionate and butyrate at a COD ratio of 1:1:1.

The glucose–VFA mixture simulates the organic compounds normally present in textile wastewaters. The influent stock solution was free of dye and AQDS during the whole period.

The basal medium consisted of (mg l^{-1}): NH_4Cl (280), K_2HPO_4 (250), $MgSO_4{\cdot}7H_2O$ (100) and $CaCl_2{\cdot}2H_2O$ (10) and 1 ml l^{-1} of trace elements containing (mg l^{-1}): H_3BO_3 (50), $FeCl_2{\cdot}4H_2O$ (2000), $ZnCl_2$ (50), $MnCl_2{\cdot}4H_2O$ (500), $CuCl_2{\cdot}2H_2O$ (38), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (50), $AlCl_3{\cdot}6H_2O$ (90), $CoCl_2{\cdot}6H_2O$ (2000), $NiCl_2{\cdot}6H_2O$ (92), $Na_2SeO_3{\cdot}5H_2O$ (162), EDTA (1000) and HCl 36% (1). Resazurin was not included in the trace elements solution due to its mediating properties [5].

2.2.2. Batch experiments

2.2.2.1. Activity tests. Inoculation took place by adding 1.3 ± 0.1 g of volatile suspended solids (VSS) per litre, of EGSB sludge in 117 ml serum bottles with 50 ml of basal medium pre-heated to 55 °C and sealed with butyl rubber stoppers. After anaerobic conditions were established by flushing the headspace with N_2/CO_2 (70%:30%), 2 g COD l^{-1} of co-substrate (variable) and RR2 (0.3 mM) were added to the bottles. To some of the bottles AQDS (variable) was added to control the impact of an external redox mediator. Sludge-free and autoclaved sludge were used as a control for abiotic colour removal. The incubations were conducted under non-static conditions by applying 50 shakes per minute.

Sterile controls were autoclaved for 240 min at 122 °C, pre-incubated for 5 days and autoclaved again at 122 °C for 240 min. Afterwards, co-substrate, AQDS, and azo dye were added to the bottles from sterile stock solutions under sterile conditions. The pH and the amount of VSS were determined on completion of the experiment.

2.2.2.2. Effect of different co-substrates. VFA mixtures of acetate:propionate:butyrate (1:1:1) on COD basis, glucose–VFA mixtures (1:3) on COD basis, glucose and H_2/CO_2 (80%:20%) were used as co-substrates (2 g COD l^{-1}), during azo dye reduction at 55 °C either in the presence or absence of AQDS (12 μM). When H_2/CO_2 was used as co-substrate, the shaker rotation was increased from 50 to 100 shakes per minute, to facilitate the hydrogen transfer to the liquid phase. The incubation with endogenous substrate was used to control the impact of co-substrate.

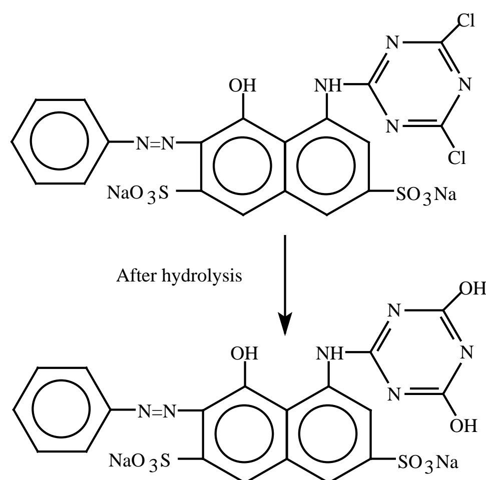

Fig. 1. Chemical structure of the reactive azo dye RR2 used as model compound, before and after hydrolysis.

2.2.2.3. Effect of temperature on thermophilic range. The incubation temperatures (°C) were 45, 55, 60, 65 and 75, for the serum bottles with both living and autoclaved sludge. The co-substrates used were VFA mixture of acetate:propionate:butyrate (1:1:1), and glucose–VFA mixture (1:3), either in the presence or absence of AQDS (120 μM).

2.2.3. Continuous experiment

2.2.3.1. Reactor. Two 0.53 l EGSB reactors were operated in parallel, in which reactor R1 was AQDS-supplied and reactor R2 was AQDS-free, i.e. control. The reactors were seeded to a final concentration of 30 g VSS l^{-1}. The reactors were operated at a HRT of 10 h and an up-flow velocity of 4 m h^{-1} was imposed recirculating the flow by peristaltic pumps (505S, Watson–Marlow, Cornwall, UK). The reactors were operated at 55 °C using a thermostat bath. Biogas was collected in a gas–solid–liquid separator, and afterwards washed in a NaOH (10% w/v) solution with timol blue as indicator. Methane was measured in a Mariotte Flask at 30 °C and 1 atm. Fig. 2 shows a schematic set-up of the continuous flow experiment.

2.2.3.2. Stock solution. The 10 l influent stock solution was composed of a glucose–VFA mixture at a COD ratio of 1:3 as co-substrate, azo dye and demineralised water. The stock solution of reactor R1 also contained AQDS. Different concentrations of co-substrate, RR2 and AQDS were added to the medium (Table 1). To prevent acidification of the reactor medium, the medium was buffered with sodium bicarbonate, i.e. 1 g $NaHCO_3$ for 1 g COD–glucose. The VFA stock solution at a final concentration of 150 g COD l^{-1} was neutralised with NaOH. The influent stock solution had a final pH adjustment to 7 by adding some drops of NaOH or HCl. The influent stock solutions and basal media were stored at 4 °C.

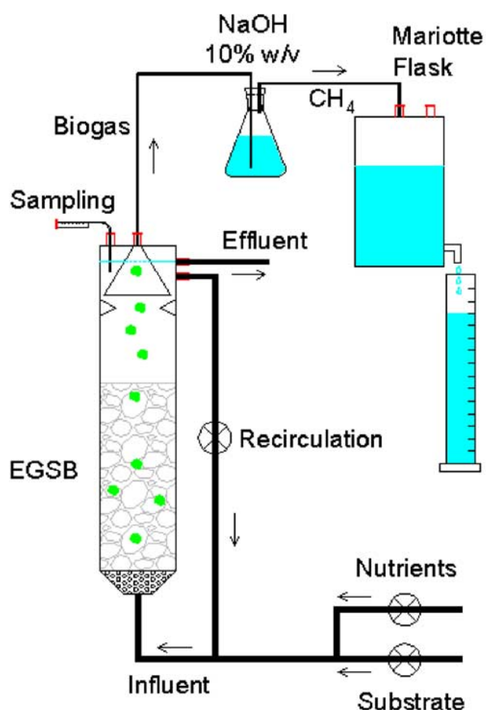

Fig. 2. Experimental set-up of the EGSB reactor system.

2.3. Analysis

Colour removal was determined photometrically (Spectronics 60, Milton-Roy Analytical Products Division, Belgium) according to Van der Zee et al. [8], reading the absorbance at the maximum absorbance wavelength, i.e. 539 nm. After hydrolysis 1.72 mM of RR2 was equivalent to 57.2 a.u. cm^{-1}, yielding a molar extinction coefficient of 34.31 a.u. cm^{-1} M^{-1}.

The daily methane production was measured by using a Mariotte Flask. The conversion factor used was 2.58 g COD l^{-1} CH_4 at 30 °C and 1 atm according to Kato et al. [14].

VFA, methanol and ethanol were measured on a Hewlett-Packard 5890 gas chromatograph (Palo Alto, USA), according to Cervantes et al. [9].

Sucrose, fructose, glucose, lactate and formate were measured on a High Pressure Liquid Chromatograph (HPLC) equipped with an Ion-300 column and a refractive index detector according to Van Lier et al. [15].

Soluble COD was analysed photometrically on a Spectronic 20 Genesys model 4001/4 (Spectronic Instruments, NY) using a micro method according to APHA [16]. Samples were centrifuged for 3 min at 13,000 rpm and absorbance was read at a wavelength of 600 nm.

The pH was determined using a Schott Gerate N32A double electrode (Hofheim, Germany) connected to a Knick 511 pH meter (Berlin, Germany).

The redox potential (ORP) was measured using a Sentix ORP 0–100 °C combination electrode (platinum—silver/silver chloride) (WTW, Weilheim, Germany), using as electrolyte a KCl solution (3 M). ORP values were corrected to the redox values of reference electrode (+207 mV at 25 °C).

VSS were analysed according to APHA standard methods [16].

3. Results

3.1. Batch experiments

3.1.1. Effect of different co-substrates

Fig. 3 shows that the addition of 2 g COD l^{-1} as co-substrate accelerates the rate of colour removal at 55 °C, even though the endogenous control showed reasonable rate. During the continuous flow experiment, the impact of co-substrate on the colour removal rates was more pronounced, probably owing the fact that the reducing equivalents from

Table 1
Operational parameters and performance of the reactor R1 (AQDS-supplied) and reactor R2 (control) during the continuous flow experiment using EGSB reactors at 55 °C

	Period									
	I	II	III	IV	V	VI	VII	VIII	IX	X
	End of period (days)									
	5	14	35	54	76	96	107	117	123	129
Operational parameters										
Co-substrate ($g COD l^{-1}$)	1.25	1.25	1.25	1.25	2.50	2.50	1.25	1.25	–	1.25
RR2 ($g l^{-1}$)	–	0.10	0.17	0.17	0.34	0.70	0.70	1.35	1.35	1.35
OLR substrate ($g COD l^{-1}$ per day)	2.5	2.5	2.5	2.5	5.0	5.0	2.5	2.5	–	2.5
OLR RR2 ($g l^{-1}$ per day)	–	0.2	0.3	0.3	0.7	1.4	1.4	2.7	2.7	2.7
Co-substrate/azo dye ($g COD g^{-1}$ RR2)	–	12.5	7.4	7.4	7.4	3.6	1.8	0.9	–	0.9
AQDS (μM) R1	–	6	6	24	24	24	24	24	24	24
Performance of the reactors										
COD removal R1 (%)	96 ± 4.4	92 ± 0.4	86 ± 0.5	86 ± 0.2	88 ± 0.8	80 ± 0.8	60 ± 11.8	63 ± 2.6	21 ± 0.9	62 ± 0.0
COD removal R2 (%)	96 ± 4.0	91 ± 1.0	86 ± 0.4	87 ± 0.6	88 ± 0.8	79 ± 0.8	62 ± 10.1	64 ± 2.5	29 ± 10.2	61 ± 0.0
Colour removal R1 (%)	–	93 ± 1.0	93 ± 1.3	92 ± 1.2	93 ± 0.4	97 ± 0.5	97 ± 0.6	98 ± 0.2	54 ± 13.9	97 ± 0.8
Colour removal R2 (%)	–	92 ± 1.2	90 ± 1.2	87 ± 0.7	91 ± 0.3	93 ± 1.2	91 ± 0.9	87 ± 0.6	46 ± 7.7	91 ± 4.5
Methane R1 ($g COD l^{-1}$)	1 ± 0.0	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.2 ± 0.0	0.8 ± 0.1
Methane R2 ($g COD l^{-1}$)	1 ± 0.0	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	0.1 ± 0.0	0.7 ± 0.1
ORP R1 (mV)	−248	–	–	–	–	–	–	−215	−53	−210
ORP R2 (mV)	−248	–	–	–	–	–	–	−205	−53	−203

ORL: organic loading rate. Co-substrate, RR2 and AQDS concentrations in the influent. $1 g l^{-1}$ RR_2 is about $1.1 g COD l^{-1}$. Redox potential (ORP) values were corrected to the redox values of reference electrode (+207 mV at 25 °C).

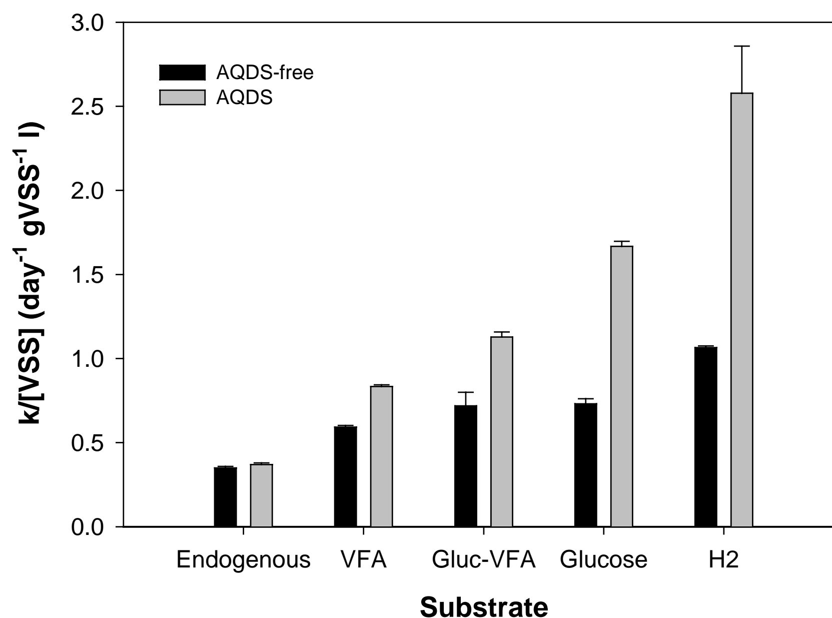

Fig. 3. First-order rate constant (k) value for colour removal of RR2 (0.3 mM) at 55 °C, normalised by the VSS of living sludge, in the presence of different co-substrates (2 g COD l^{-1}). AQDS (0.012 mM) was added to some of the incubations. The results are means of triplicate incubations with the standard deviations being indicated by vertical bars.

endogenous respiration were limited. Results also show different rates for the different types of co-substrates tested, even though a complete colour removal and good decolourisation rates were achieved in all cases. The highest rate of colour removal was achieved when hydrogen was used as co-substrate for both AQDS-free and AQDS-supplied medium, whereas VFA mixture was the least efficient co-substrate. Interestingly, in the presence of AQDS, colour removal rates increased by increasing the glucose concentration, i.e. comparing the incubations when glucose–VFA mixture or glucose solely was used as co-substrate, whereas the rates for both co-substrates were the same in the absence of AQDS.

Colour removal rate in the presence of formate as co-substrate (results not shown) achieved a close value than when hydrogen was present as co-substrate, suggesting that both interspecies formate and interspecies hydrogen could be involved upon electron transfer in the system.

During the course of experiment, the colour removal was lower than 10% in the autoclaved sludge controls and lower than 1% in the sludge-free controls.

3.1.2. Effect of temperature

The temperature dependency was assessed in the range of 45–75 °C, showing a maximum rate of colour removal at 60 °C (Fig. 4). The temperature response at 60 °C was similar for both VFA and glucose–VFA mixture, enhancing 1.1-fold the first-order rate constant "k" (day^{-1}), compared with the incubations at 55 and 65 °C. The lowest k values were found for the incubations at 75 °C.

Results again show the stimulatory effect of glucose on the decolourisation rates. For instance, at the optimum temperature of 60 °C and 120 μM-AQDS, the k value was 1.3-fold higher using glucose–VFA mixture as co-substrate compared to the vials supplied with solely VFA mixture.

AQDS stimulated colour removal in all of the incubations and the impact on the decolourisation rates varied with the substrate and the temperature tested. For instance, for the AQDS-supplied incubations at 60 °C with glucose–VFA mixture as co-substrate, the k value enhanced 2.4-fold compared with the AQDS-free incubations. Doing the same comparison but considering VFA mixture as co-substrate, the k value increased 1.9-fold.

During the course of experiment the colour removal was lower than 10% in the autoclaved sludge controls and lower than 1% in the sludge-free controls.

3.2. Continuous experiment

The operating data of the continuous flow EGSB experiments are presented in Table 1.

3.2.1. Colour removal

Both reactors were started and stabilised with a dye-free and AQDS-free medium (period I). In period II, RR2 (0.10 g l^{-1}) was introduced in both reactors media at sub-stoichiometric concentration, i.e. in a low concentration to prevent some toxicity during the start-up, and AQDS (6 μM) was supplied to reactor R1. Results reveal a similar performance of reactors R1 (Fig. 5A) and R2 (Fig. 5B), probably due to the initial adsorption of RR2 in the sludge bed. In period III, the RR2 concentration was increased to 0.17 g l^{-1} to exhaust the adsorption capacity of the sludge bed, which was reached around 30 days, i.e. 25 days after

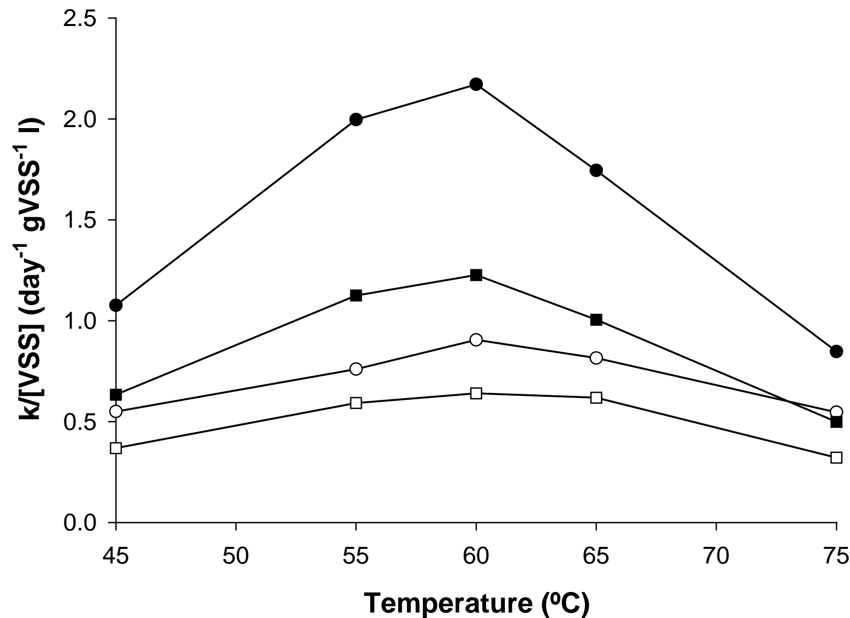

Fig. 4. First-order rate constant (k) value for colour removal of RR2 (0.3 mM) at different incubation temperatures, normalised by the VSS of living sludge: (○) glucose–VFA AQDS-free, (●) glucose–VFA AQDS-supplied, (□) VFA AQDS-free, (■) VFA AQDS-supplied. The co-substrate concentration was $2\,g\,COD\,l^{-1}$. AQDS (0.12 mM) was added to some of the incubations. The results are means of triplicate incubations, in which the standard deviations were lower than 10% in all cases.

RR2 was introduced in the medium. At the same period the colour removal in R2 started to drop. At the beginning of period IV, the efficiency of R2 has dropped to $87 \pm 0.7\%$, and was kept around this value until day 54. For the reactor R1 however, the efficiency values were the same as in period III. In period V, the loading rate of co-substrate and RR2 was doubled, which resulted in an increase in colour removal efficiency for both reactors. In this period, the ratio co-substrate/dye was not changed. In the following periods co-substrate/azo dye ratio was decreased from 7.4 to $3.6\,g\,COD\,g^{-1}$ RR2 (period VI) and $1.8\,g\,COD\,g^{-1}$ RR2 (period VII), limiting the availability of reducing equivalents. It was observed that the reduced ratios affected the colour removal efficiency in R2. In period VIII, a further decrease in the ratio co-substrate/azo dye to $0.9\,g\,COD\,g^{-1}$ RR2 was imposed. In contrast to R1, reactor R2 responded with a significant drop in the colour removal to $86 \pm 0.5\%$. In period IX, both reactors were refrained from co-substrate, which resulted in a sharp decrease in the colour removal efficiency values in both reactors to about 30% (Fig. 5A and B). Once the feeding with co-substrate was resumed to the loading rate of $2.5\,g\,COD\,l^{-1}$ per day (period X), the reactor R1 reached the same colour removal efficiency observed during period VIII and in reactor R2 the efficiency values increased to higher values to those of period VIII (Fig. 5B).

Addition of AQDS resulted in a different colour fixation in the sludge bed. For the AQDS-supplied reactor R1, almost no red colour was attached to the granules, which contrasted the strongly red-coloured sludge bed of the AQDS-free reactor R2. The latter sludge was apparently reversibly coloured since all colour easily disappeared after washing the granules.

3.2.2. *Co-substrate removal and methane production*

The system efficiency for co-substrate removal was constantly evaluated in terms of soluble COD. In addition, the products formed from glucose fermentation and VFA oxidation, were also monitored, but in a low frequency. The products of glucose pyruvate, formate, lactate and alcohols were not detected in the effluent, but only traces of VFA, i.e. acetate ($<5\,mg\,COD\,l^{-1}$) and butyrate ($<19\,mg\,COD\,l^{-1}$). Propionate accumulation was not observed.

The COD influent was composed by co-substrate and RR2, and COD effluent by traces of co-substrate, RR2 not removed and aromatic amines. The decrease in COD removal (Fig. 6) can be attributed to the increase in RR2 and aromatic amines (mainly aniline) concentrations in the effluent. Interestingly, the colour removal efficiencies and co-substrate conversion were not affected even when high RR2 concentrations were imposed to the system.

Aromatic amines are known to be extremely toxic to methanogens even in small concentrations. Nonetheless, during the continuous flow experiments even when a high load rate of RR2 ($2.7\,g\,l^{-1}$ per day) was applied, the methane production remained stable. The aromatic amine aniline, which was detected as product of azo-bond cleavage (results not shown), was probably not converted since it is known that aniline is a rather stable compound anaerobically [17].

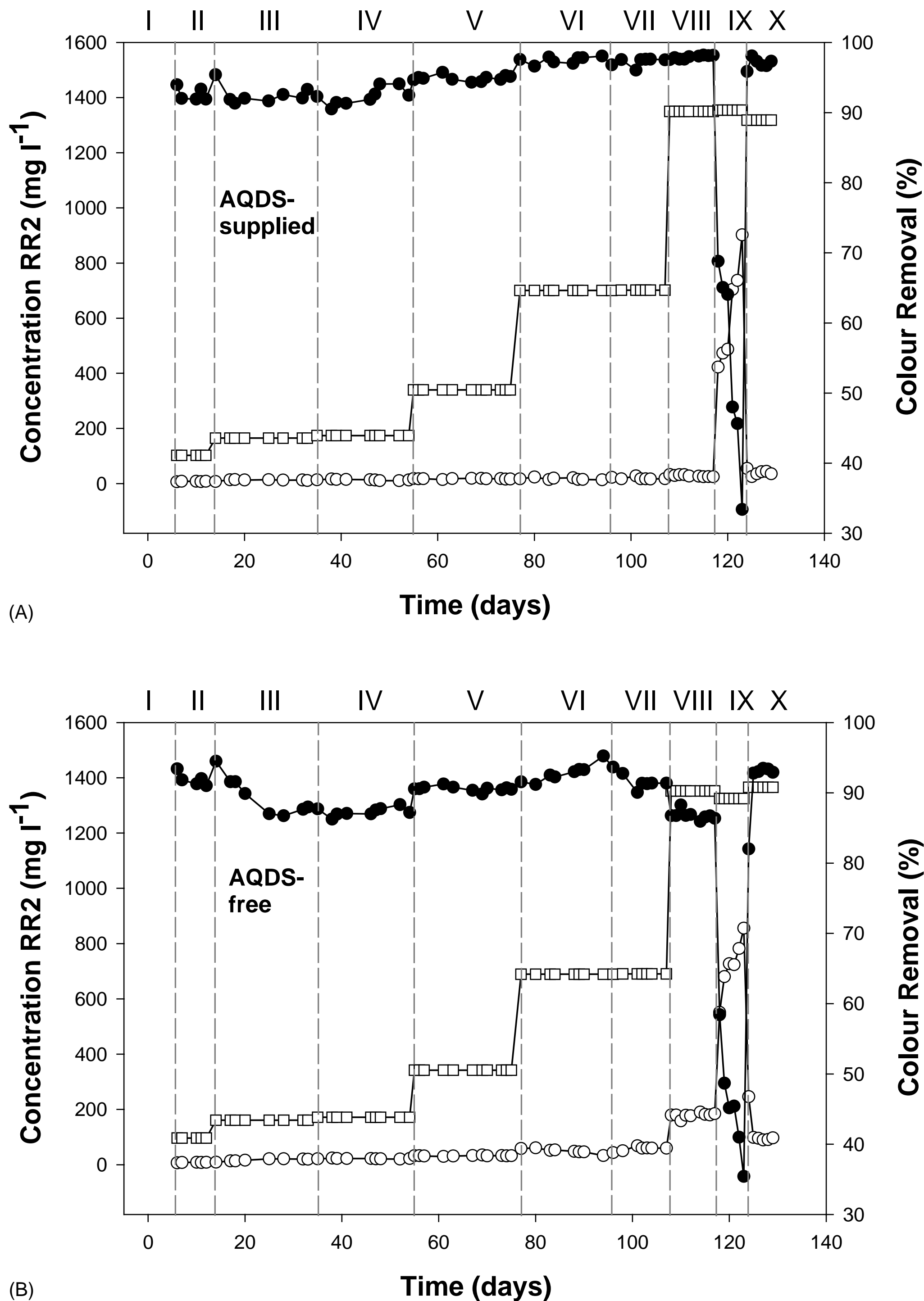

Fig. 5. Performance of the continuous flow EGSB reactors at 55 °C indicating the concentration of RR2 on the left *Y*-axis and the colour removal on the right *Y*-axis. (A) Reactor R1 AQDS-supplied and (B) reactor R2 AQDS-free (control). (□) Influent RR2 concentration, (○) effluent RR2 concentration, (●) colour removal efficiency. (– – – – –) corresponds to the different periods of the experiment (Table 1).

4. Discussion

The results of the continuous flow experiments clearly show the potentials of thermophilic anaerobic treatment on colour removal of an azo dye containing triazine as the reactive group. The results also show that the decolourisation rate improves by using AQDS at catalytic concentrations. The reducing equivalents generated after co-substrate oxidation biologically reduce AQDS to the hydroquinone form, AH_2QDS, with consequent chemical reoxidation of

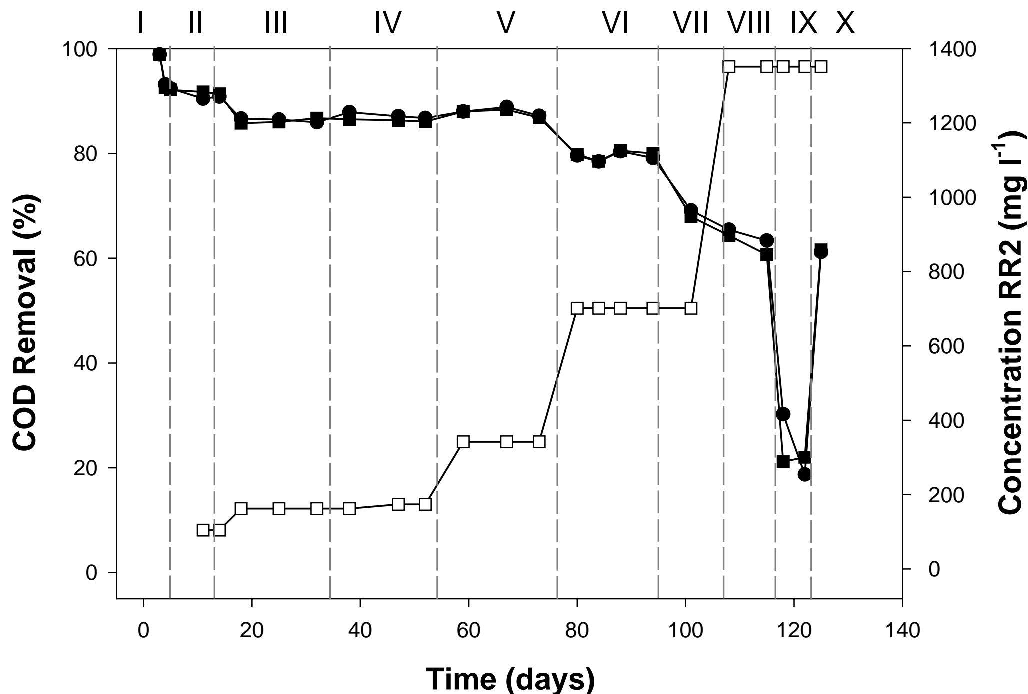

Fig. 6. Performance of the continuous flow EGSB reactors at 55 °C in terms of COD removal: (□) COD-efficiency for R1, (■) COD-efficiency for R2, (●) RR2 concentrations. (– – – – –) corresponds to the different periods of the experiment (Table 1).

hydroquinone by the azo dye [10]. So far, there are only a few publications on anaerobic azo dye reduction at high temperatures. Furthermore, differences in type and loading rates of azo dye, co-substrate and redox mediator, as well as the temperature and sludge concentration in the reactor hamper a sound comparison with previously reported results under mesophilic conditions.

The EGSB reactors achieved excellent colour removal efficiencies with a high stability (Fig. 5A and B), even when high loading rates of azo dye were applied (2.7 g RR2 l^{-1} per day). However, the impact of AQDS on colour removal was less apparent than expected, based on the results achieved under mesophilic conditions. Long-term experiments show that the AQDS-free reactor R2 achieved excellent colour removal rates with efficiencies around 91% (Table 1), compared with the efficiencies around 95% (Table 1) for the AQDS-supplied reactor R1. This was in contrast to the findings of Cervantes et al. [6], who reported a considerable impact of AQDS on the anaerobic colour removal at 30 °C, using Acid Orange 7 as azo dye model compound. When AQDS was absent, the efficiency values were around 86%, but increased to about 99% after 30 μM-AQDS was introduced in the system. The stability of the system in our experiment, in terms of colour removal and co-substrate oxidation, was also in contrast to the results of Van der Zee et al. [5]. They reported a decrease in the colour removal efficiencies to around 25% after 40 days of feeding with non-hydrolysed RR2 in the absence of AQDS, during anaerobic treatment at 30 °C. Furthermore, the co-substrate (VFA) removal efficiencies dropped to around 10%. Nevertheless, the system capacity for colour removal and co-substrate oxidation was recovered after the reactor was supplied with 19 μM-AQDS. In our experiments, no toxicity effects were observed, which might be attributed to the following differences with the above-cited work. Firstly, Van der Zee et al. [5] used non-hydrolysed RR2 in their experiments (Fig. 1), whereas we used hydrolysed RR2. The toxicity effect of the former RR2 could be more pronounced as indicated by the results of Chung and Stevens [18] who reported microbial respiration inhibition due to the presence of chloride or bromide as functional groups. The azo dye concentrations in and around the biofilm could be lower under thermophilic conditions, owing to the fact that at 55 °C the decolourisation rates are higher compared to those at 30 °C. As a result, a much lower fraction of the sludge is exposed to the toxic concentrations of RR2. Azo dyes are known to be more toxic to methanogens than their cleavage products, i.e. aromatic amines [19].

The decolourisation rate showed to be co-substrate dependent (Fig. 3), in which the VFA mixture was the least efficient co-substrate. The highest decolourisation rate was achieved in the presence of hydrogen. Also glucose has a significant impact on the colour removal rates, probably because of the high amounts of H_2 (or formate) that are likely produced in glucose oxidation, i.e. maximum of 4 mol H_2 per mol glucose. The findings are similar to Donlon et al. [20] who reported that interspecies hydrogen resulting from the oxidation of substrates such as butyrate, propionate, and ethanol, could provide the medium with reducing equivalents, and stimulation of the nitrophenol reduction. In the latter work, the direct methane precursors

acetate and methanol did not stimulate the nitrophenol reduction rates.

AQDS has a considerable impact on the decolourisation rate either when glucose–VFA mixture or glucose solely is used as co-substrate, whereas the rates for both co-substrates were the same in the absence of AQDS (Fig. 3). This is an indication that AQDS was facilitating the electron transfer to the dye reducing micro-organisms, most probably interspecies hydrogen or interspecies formate that is generated via glucose fermentation. The redox couple AQDS/AH_2QDS (reduction potential E_0' equal to -0.18 V) is thermodynamically more favourable than the redox couple CO_2/CH_4 (E_0' equal to -0.24 V) indicating the preference of AQDS as an electron acceptor [9,21]. Recently, a NADH-dependent lawsone reductase activity located in the cytosolic fraction of *Escherichia coli* also showed the capacity for azo dye reduction [22]. Hydrogen-oxidising rather than acetate-oxidising micro-organisms seem to be more actively involved in quinone-respiration under thermophilic and hyperthermophilic conditions [10]. Considering the increasing role of H_2 (or formate) at high temperatures [23,24], thermophilic anaerobic treatment seems to be advantageous for the hydroquinone generation, which is sometimes the process rate-limiting step, when the low-molecular weight redox mediators are externally added to accelerate the decolourisation rates [12].

The highest decolourisation rate at 60 °C (Fig. 4) agrees with the generally observed optimum temperature for thermophilic methanogenic consortia [25], and the resulting highest production of reducing equivalents. During continuous flow experiment however, it was decided to operate the reactors at 55 °C following the recommendations of Ahring [26] and Van Lier et al. [27] who reported a dramatic decrease in the VFA oxidising populations (particularly C_3 oxidisers) of temperatures higher than 55 °C.

The promising results achieved in this study, suggest good prospects for the application of thermophilic anaerobic treatment for treating reactive azo dyes, in which high efficiency values on colour removal were obtained either in the absence or presence of AQDS at catalytic concentrations.

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