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ORIGINAL PAPER

A. B. dos Santos · F. J. Cervantes · J. B. van Lier

Azo dye reduction by thermophilic anaerobic granular sludge, and the impact of the redox mediator anthraquinone-2,6-disulfonate (AQDS) on the reductive biochemical transformation

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Abstract Azo dye reduction at 55°C by thermophilic anaerobic granular sludge was investigated distinguishing between the biotic and abiotic mechanisms. The impact of the redox mediator anthraquinone-2,6-disulfonate (AQDS) on colour removal and co-substrate oxidation was also investigated. Metabolic activities of the thermophilic inoculum induced a fast azo dye reduction and indicated a biotic predominance in the process. The addition of co-substrate enhanced the decolourisation rates 1.7-fold compared with the bottles free of co-substrate. Addition of AQDS together with co-substrate enhanced the *k* value 1.5-fold, compared with the incubation containing co-substrate in the absence of AQDS. During a comparative study between sludge samples incubated under mesophilic (30°C) and thermophilic (55°C) conditions, the decolourisation rate at 55°C reached values up to sixfold higher than at 30°C. Biological treatment at 55°C showed a fast initial generation of reducing compounds via co-substrate oxidation, with AQDS increasing the azo dye reduction rate in all the incubations tested. Nevertheless, high concentrations of AQDS showed severe inhibition of thermophilic acetate and propionate oxidation and methane production rates. These promising results indicate that there may be good prospects for thermophilic anaerobic treatment of other reductive transformations such as reduction of nitroaromatics and dehalogenation.

A. B. dos Santos (✉) · J. B. van Lier
Sub-department of Environmental Technology, Wageningen University,
Bomenweg 2,
PO Box 8129, 6700EV Wageningen, The Netherlands
e-mail: andre.dossantos@wur.nl
Tel.: +31-317-484993

F. J. Cervantes
Departamento de Ciencias del Agua y del Medio Ambiente,
Instituto Tecnológico de Sonora,
5 de Febrero 818 Sur Cd. Obregón,
85000 Sonora, Mexico

Introduction

Almost 10^9 kg of dyes are produced annually in the world, of which azo dyes, characterised by an azo-bond (R_1–N=N–R_2), represent about 70% on a weight basis (Zollinger 1987). 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%). Since textile industry wastewaters are generally discharged at high temperatures (40–70°C), thermophilic anaerobic treatment could serve as an interesting option for azo dye reduction, especially when closing process water cycles is considered.

Anaerobic microorganisms maintain low redox potential conditions (<50 mV) and generate the reducing equivalents required for azo dye reduction (Beydilli et al. 1998; Bromley-Challenor et al. 2000). The exact mechanism, occurring either intracellularly or extracellularly, is still a subject of investigation, as is the role of biogenic water-soluble electron carriers, e.g. flavins. Reduced flavins can act as electron shuttles from NADPH-dependent flavoproteins to azo dyes as the electron acceptors (Gingell and Walker 1971). Intracellular azo dye reduction cannot be used for the conversion of all types of azo dyes, especially sulfonated azo dyes, which have limited membrane permeability (Stolz 2001). An increase in colour removal rates of sulfonated azo dyes by cell-free extracts, as well as after addition of toluene, i.e. a membrane-active compound which increases cell lysis, demonstrate the limited membrane permeability (Kudlich et al. 1997). The current hypothesis is that azo dyes are mostly reduced by extracellular or membrane-bound enzymes (Stolz 2001). Reduced cytoplasmic cofactors, such as reduced flavins, do not contribute to the chemical azo dye reduction because of their inability to cross cell membranes of living cells (Russ et al. 2000). However, cell fractionation experiments have demonstrated that a quinone reductase activity located in the cell membrane enhanced the azo dye reduction rates of a sulfonated azo compound, and cross-membrane dye transport was not

required (Kudlich et al. 1997). Recently, a NADH-dependent lawsone reductase activity located in the cytosolic fraction of *Escherichia coli* also showed the capacity for azo dye reduction (Rau and Stolz 2003).

Quinones are the electron accepting moieties of humic substances. Such compounds have been shown to play an important role not only as final electron acceptors for many recalcitrant organic compounds, but also facilitating electron transfer from an electron donor to an electron acceptor, e.g. azo dyes (Cervantes et al. 2000; Field et al. 2000). The first step is the non-specific enzymatic reduction of quinone to hydroquinone, and the second step is the chemical reoxidation of hydroquinone by azo dyes (Keck et al. 1997). The chemical regeneration of hydroquinone is sometimes the rate-limiting step, e.g. using lawsone as redox mediator at 30°C during reduction of amaranth (Rau et al. 2002). In general, the chemical reaction follows the Arrhenius equation, indicating that an increase in temperature decreases the activation energy requirements, thus improving the kinetics of the reaction. Therefore, the use of redox mediators to accelerate azo dye reduction under thermophilic conditions might be advantageous, not only for the expected faster enzymatic reduction of quinone to hydroquinone compared to mesophilic conditions, but also for the faster regeneration of hydroquinone by chemical reaction with azo dyes.

The main objective of this study is to investigate in batch assays the feasibility of applying thermophilic anaerobic treatment for colour removal of coloured wastewaters. Therefore, the importance of biotic and abiotic activities is distinguished at 55°C. Additionally, the initial generation of reducing compounds after co-substrate oxidation is assessed by comparing sulfide-free incubations with those supplied with sulfide as the reducing agent. Finally, the impact of the redox mediator anthraquinone-2,6-disulfonate (AQDS) on colour removal efficiency rates and co-substrate oxidation is evaluated.

Materials and methods

Chemicals

Reactive Red 2 (RR2), a sulfonated reactive azo dye, was selected as model compound for this study (Procion Red MX-5B, ~50% 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 (after Beydilli et al. 1998). This procedure aims to simulate the hydrolysed dye structure found in real textile wastewaters. Fig. 1 shows the chemical structure before and after hydrolysis.

AQDS (Aldrich, Gillingham, UK) was used as redox mediator model compound, without additional purification (Fig. 2).

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 acclimatised for 3 months at 55°C in an expanded granular sludge bed (EGSB) reactor (5.6 l) operating at a hydraulic retention time (HRT) of about 6 h and an organic loading rate of 2.5 kg chemical oxygen demand (COD) m^{-3} day^{-1}. The 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.

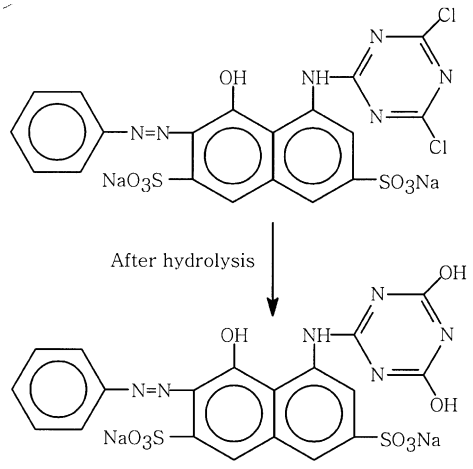

Fig. 1. Chemical structure of the reactive azo dye Reactive Red 2 (RR2) used as a model compound, before and after hydrolysis

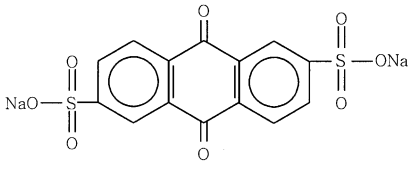

Fig. 2. Chemical structure of the external redox mediator anthraquinone-2,6-disulfonate (AQDS) used as a redox mediator model compound

For batch tests at 30°C the same mesophilic granular sludge was acclimatised in an EGSB reactor (30°C) with the same co-substrate described previously until reaching steady-state conditions.

The basal medium consisted of (mg l^{-1}): NH_4Cl (280), K_2HPO_4 (250), $MgSO_4.7H_2O$ (100), $CaCl_2.2H_2O$ (10) and 1 ml l^{-1} trace elements containing (mg l^{-1}): H_3BO_3 (50), $FeCl_2.4H_2O$ (2,000), $ZnCl_2$ (50), $MnCl_2.4H_2O$ (500), $CuCl_2.2H_2O$ (38), $(NH_4)_6Mo_7O_{24}.4H_2O$ (50), $AlCl_3.6H_2O$ (90), $CoCl_2.6H_2O$ (2,000), $NiCl_2.6H_2O$ (92), $Na_2SeO_3.5H_2O$ (162), EDTA (1,000) and HCl 36% (1), buffered with 6.21 g l^{-1} sodium bicarbonate at a pH of around 7.1. Resazurin was not included in the trace elements solution because of its mediating properties (Van der Zee et al. 2001).

Activity test

Inoculation took place by adding 1.3±0.1 g volatile suspended solids (VSS) l^{-1} of EGSB sludge into 117-ml serum bottles with 50 ml of pre-heated basal medium 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}(mixture of glucose and VFA at a COD ratio of 1:3) of co-substrate was added to the bottles as the electron donor and carbon source. RR2 (0.3 mM), AQDS (variable) and sulfide (variable) were also added. 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, azo dye and sulfide were added to the bottles from sterile stock solutions under sterile conditions. The pH and the amount of VSS were determined after completion of the experiment.

Biotic and abiotic azo dye reduction at 55°C

A pseudo first-order reaction with respect to the dye concentration was used, whereas the first-order rate constant k was determined using the following equation:

$$A_t = A_0 e^{-kt} \quad (1)$$

A_t is the absorbance at time t, A_0 is the initial absorbance at t=0, k is the first-order rate constant (per day) and t is the accumulated time of the experiment (days). Time was plotted against $\ln(A_t/A_0)$ and the k value was estimated by the slope of a linear regression.

Autoclaved sludge was used as a control of abiotic azo dye reduction by reducing compounds and adsorption in the sludge, and sludge-free controls were used to monitor the stability of the azo dye at 55°C. The effects of co-substrate and AQDS (0.012 mM) were also evaluated for living and autoclaved sludges.

To assess the electron transfer capacity in terms of the redox potential (ORP), a comparative study was conducted between sludge samples stabilized under mesophilic (30°C) and thermophilic (55°C) conditions. The ORP values of the serum bottles with living sludge were measured in an anaerobic chamber under a N_2:H_2 (95%:5%) atmosphere, after an incubation time of 48 h. The ORP values were reported in terms of the reference electrode (+207 mV at 25°C). The serum bottles contained RR2 and co-substrate, either in the presence or absence of AQDS (0.012 mM).

Effect of VSS concentration on the azo dye reduction at 55°C

The impact of AQDS (0.012 mM) on the colour removal at 55°C for different VSS concentrations was assessed. The VSS concentrations tested were 1.3, 3.0, 6.0 and 12.0 g l^{-1}. A mixture of glucose and VFA at a COD ratio of 1:3 (2 g COD l^{-1})was used as co-substrate.

Initial generation of reducing equivalents at 55°C

The biological generation of reducing equivalents from co-substrate oxidation at 55°C was investigated. For this purpose, different concentrations of sulfide as reducing compound were added to living sludge, autoclaved sludge and sludge-free media. The k values were determined in a sulfide-free medium, sulfide at sub-stoichiometric concentration (≤2 mM) and excess of sulfide (>2 mM). AQDS (0.012 mM) was used to evaluate the impact of the external mediator on azo dye reduction. The concentrations of sulfide were measured at the start and completion of the experiment.

Effects of AQDS dosage on the azo dye reduction and co-substrate oxidation

To assess the catalytic potential of redox mediators on colour removal at 55°C, different AQDS concentrations were tested in batch assays. The effect of AQDS on co-substrate oxidation and methane production, after 2 days incubation, was also observed. For this purpose, an AQDS gradient of 0, 0.006, 0.012, 0.024, 0.120, 0.600, 1.2, 5.0 and 10.0 mM, was tested with both living and autoclaved sludge. A mixture of glucose and VFA at a COD ratio of 1:3 (2 g COD l^{-1})was used as co-substrate. A sludge-free control was used to monitor the azo dye stability at 55°C.

Analysis

Colour removal was determined photometrically (Spectronics 60, Milton-Roy Analytical Products, Belgium) according to Van der Zee et al. (2001), reading the absorbance at the maximum absorbance wavelength, i.e. 539 nm. After hydrolysis, 1.72 mM of RR2 was equivalent to 57.2 absorbance units (AU) cm^{-1}, yielding a molar extinction coefficient of 34.31 AU cm^{-1} M^{-1}.

Methane production was determined on a gas chromatograph model 438/S (Packard-Becker, Delft, The Netherlands), according to Cervantes et al. (2000). The methane standard was stored at 55°C.

VFA, methanol and ethanol were measured on a Hewlett Packard 5890 gas chromatograph (Palo Alto, USA), according to Cervantes et al. (2000).

Sucrose, fructose, glucose, lactate and formate were measured on a high pressure liquid chromatograph equipped with an Ion-300 column and a refractive index detector, according to van Lier et al. (1997).

Sulfide was measured photometrically as described by Trüper and Schlegel (1964). The pH was determined using a Schott Gerate N32A double electrode (Hofheim, Germany) connected to a Knick 511 pH meter (Berlin, Germany).

The ORP was measured using a Sentix ORP 0–100°C combination electrode (platinum–silver/silver chloride) (WTW, Weilheim, Germany), using a KCl solution (3 M) as the electrolyte.

VSS were analysed according to APHA standard methods (1998).

Results

Biotic and abiotic azo dye reduction at 55°C

An anaerobic granular sludge originating from a mesophilic full-scale UASB reactor treating paper mill wastewater was tested for its capacity to reduce an azo dye, RR2, under thermophilic conditions. The sludge was previously stabilised at 55°C in a laboratory-scale EGSB reactor prior to the assays. Azo dye reduction by thermophilic living sludge in a sulfide-free medium followed a first-order reaction with respect to the dye concentration. For the incubation supplied with co-substrate, the k value was enhanced 1.7-fold compared with the endogenous control, i.e. the incubation free of co-substrate (Fig. 3). Addition of AQDS together with co-substrate enhanced the k value 1.5-fold, compared with the incubation supplied with co-substrate in the absence of AQDS. Addition of AQDS had no effect on the k values observed in endogenous controls.

During the incubation time of 6 days, negligible (<1%) colour removal occurred in sludge-free controls in the presence of AQDS. However, incubations conducted with autoclaved sludge in AQDS-supplied assays achieved about 10% of colour removal during the incubation time of 6 days (results not shown), and a complete decolourisation after prolonged periods (>90 days).

The anaerobic granular sludge under study was also stabilised at 30°C in a laboratory-scale EGSB reactor in a parallel experiment, which followed the same protocol described for the thermophilic (55°C) EGSB reactor. During a comparative study between sludge samples incubated under mesophilic (30°C) and thermophilic (55°C) conditions, in the presence of the azo dye RR2 (0.3 mM), the decolourisation rate at 55°C (k=0.93 d^{-1}) was about sixfold higher than at 30°C (k=0.15 d^{-1}) in the absence of AQDS. Meanwhile, enhanced decolourisation rates were obtained in the presence of AQDS (0.012 mM) both at 30°C (k=0.77 d^{-1}) and 55°C (k=1.52 d^{-1}), which

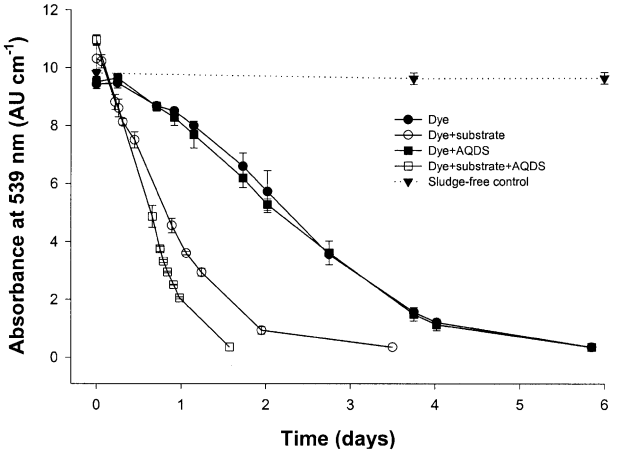

Fig. 3. Colour removal of RR2 (0.3 mM), in the presence and absence of co-substrate [2 g chemical oxygen demand (COD) l^{-1} as a mixture of glucose and volatile fatty acids (VFA) at a ratio of 1:3] and AQDS (0.012 mM). Endogenous controls (in the absence of co-substrate) were used to assess the improvement of colour removal by addition of external co-substrate and AQDS. Sludge-free incubations were used to assess the stability of the basal medium at 55°C and contained RR2, AQDS and co-substrate. The results are means of triplicate incubations and the *bars* indicate the standard deviations

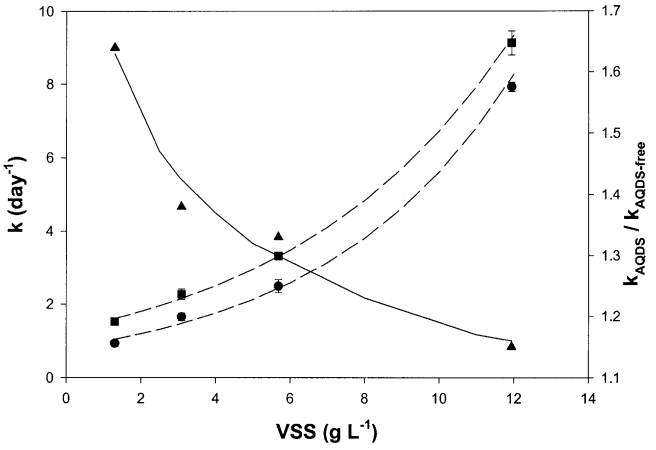

Fig. 4. First-order rate constant (k, *left y-axis*) for different sludge concentrations (volatile suspended solids, VSS), during azo dye reduction of RR2 (0.3 mM), either in the presence (■) or absence (●) of AQDS (0.012 mM). The ratio $k_{AQDS}/k_{AQDS\text{-}free}$ decrease (*right y-axis*) was also plotted against VSS concentration (▲). The *dashed line* represents an exponential approximation and the *continuous line* represents a pseudo-exponential decrease. Co-substrate (2 g COD l^{-1} as a mixture of glucose and VFA at a ratio of 1:3) was present in all incubations. The results are means of triplicate incubations and the *bars* indicate the standard deviations

represent increments of fivefold and 1.6-fold, respectively, compared with the AQDS-free controls. Furthermore, comparing the rate of decolourisation achieved in the AQDS-supplied incubations, it can be deduced that there is a twofold higher rate for the thermophilic (55°C) controls compared with the mesophilic (30°C) assays. In the same experiment, a variation of the redox potential values at 30°C and 55°C was verified after 48 h incubation, in which AQDS (0.012 mM) stimulated more negative values. For AQDS-free incubations the redox values were −148 mV at 30°C and −204 mV at 55°C. For the AQDS-supplied incubations the redox values were −158 mV at 30°C and −214 mV at 55°C. Therefore, AQDS lowered redox potential values by around −10 mV at both 55°C and 30°C.

Effect of VSS concentration on azo dye reduction at 55°C

The k value was enhanced by increasing the VSS concentration for both AQDS-supplied and AQDS-free incubations (Fig. 4). A pseudo-exponential approximation curve fitted well in both cases, in which AQDS stimulated the rate of decolourisation in all of the cases. Nevertheless, the ratio $k_{AQDS}/k_{AQDS\text{-}free}$ was reduced with the increase in VSS concentration (Fig. 4). A pseudo-exponential approximation curve could approximately represent the fall in the ratio $k_{AQDS}/k_{AQDS\text{-}free}$.

Table 1. First-order rate constant (k) values (per day) for incubations with living sludge, autoclaved sludge and sludge-free control, during azo dye reduction in the presence of sulfide. Co-substrate (2 g COD l^{-1} as a mixture of glucose and volatile fatty acids, ratio 1:3) was present in all incubations. Reactive Red 2 (0.3 mM) was used as an azo dye model compound. Anthraquinone-2,6-disulfonate (AQDS, 0.012 mM) was used as a redox mediator model compound. The results are means of triplicate incubations and the standard deviation was less than 10% in all cases

Sulfide (mM)	Living sludge		Autoclaved sludge		Sludge-free control	
	+AQDS	−AQDS	+AQDS	−AQDS	+AQDS	−AQDS
0	1.5	0.9	[a]	[a]	[b]	[b]
0.5	1.5	0.9	[a]	[a]	[b]	[b]
1	2.1	1.2	0.8	0.6	0.6	0.4
2	2.7	1.9	1.6	1.4	1.7	1.4
5	6.2	3.9	5.5	3.8	5.4	3.7
10	9.9	7.1	9.8	7.2	10.0	7.2

[a]Complete colour removal after prolonged periods (>90 days) of incubation

[b]Incomplete colour removal even after prolonged period of incubation

Initial generation of reducing equivalents at 55°C

Sulfate is a common pollutant in textile wastewaters and it can readily be reduced to sulfide by sulfate reducing bacteria. The biogenic sulfide may play an important role as a reducer of azo dyes (Van der Zee et al. 2003), thus contributing to the decolourising processes. Therefore, an experiment was conducted to assess the impact of different sulfide concentrations on the reduction of RR2. In a sulfide-free medium, no colour removal was observed in the absence of sludge, whereas incubations with living as well as autoclaved sludge achieved total colour removal. AQDS enhanced the decolourisation rate in all of the incubations tested. In serum bottles with living sludge, no difference in the decolourisation rates for a sulfide gradient up to 0.5 mM was observed (Table 1). Nevertheless, for sulfide concentrations above 0.5 mM, there were increments in the decolourisation rates, which were dependent on the sulfide concentration (Table 1). For instance, incubations with living sludge and 2 mM sulfide showed a *k* value increase of 1.8-fold in the presence of AQDS and twofold in the absence of AQDS, compared with the sulfide-free incubation (Table 1). For sulfide concentrations above 0.5 mM, a complete colour removal was achieved in the sludge-free incubation (Table 1). When sulfide concentrations were ≥1 mM, the same decolourisation rates were obtained for both autoclaved sludge and sludge-free incubations (Table 1).

For living sludge incubations with sulfide concentrations up to 2 mM, there was a biological predominance in the process (Fig. 5). Namely, the *k* values obtained with living sludge incubations were significantly higher compared with those obtained with autoclaved and sludge-free incubations. Thus, the reducing equivalents generated via co-substrate oxidation by living sludge and endogenous respiration were the predominant mechanisms for colour removal. With excess of sulfide (>2 mM) the *k* values for living sludge, autoclaved sludge and sludge-free bottles were almost the same, indicating the prevalence of chemical azo dye reduction, in which the reducing equivalents generated via co-substrate oxidation and endogenous respiration were masked by the reducing equivalents provided by sulfide (Fig. 5).

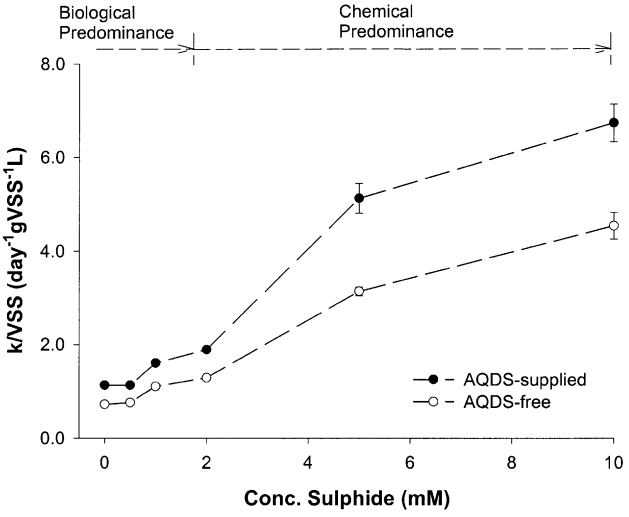

Fig. 5. The *k* value for azo dye reduction of RR2 (0.3 mM), normalised by the VSS of living sludge, in the presence of sulfide (variable) and living sludge. AQDS (0.012 mM) was supplied to some of the bottles. Co-substrate (2g COD l^{-1} as a mixture of glucose and VFA at a ratio of 1:3) was present in all incubations. The results are means of triplicate incubations and the *bars* indicate the standard deviations

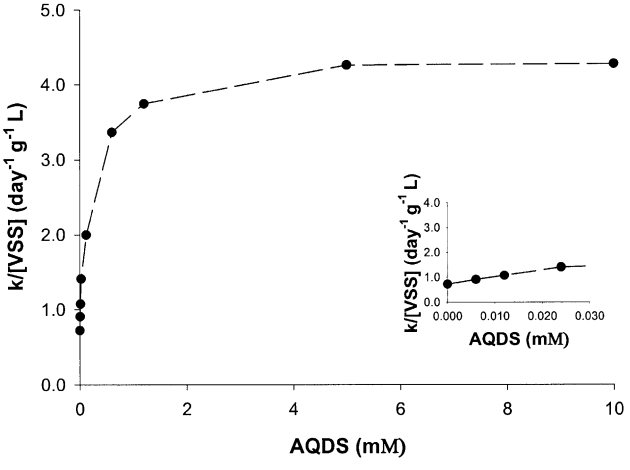

Fig. 6. The *k* values for azo dye reduction normalised by the VSS of living sludge. Different concentrations of AQDS were used in the incubations. Co-substrate (2 g COD l^{-1} as a mixture of glucose and VFA at a ratio of 1:3) was present in all incubations. The results are means of triplicate incubations

Effects of AQDS concentration on the azo dye reduction and co-substrate oxidation

Figure 6 presents the normalised *k* values as a function of the supplied AQDS concentration. A linear correlation was found for AQDS concentrations up to 0.024 mM. The *k* value increased 1.9-fold for the incubation with 0.024 mM AQDS compared with the AQDS-free incubation (Fig. 6). At incubations with AQDS concentrations exceeding 1.2 mM, only a small increase in the rate of decolourisation was observed, e.g. 10 mM AQDS increased the *k* value 1.1-fold compared with 1.2 mM AQDS. At incubations with AQDS concentrations of 10 mM, the *k* value was 5.9-fold higher than in AQDS-free incubations.

The use of high concentrations of AQDS, however, showed severe inhibition of thermophilic acetate oxidation, since acetate accumulated (Fig. 7A) and methane production rates concomitantly decreased (Fig. 7B). Propionate oxidation was also affected by an increase in the AQDS concentration, in which propionate conversion almost ceased at AQDS concentrations exceeding 1.2 mM. On the other hand, glucose fermentation was not affected by high AQDS concentrations as demonstrated by the negligible detection of this substrate at the end of the incubation period. After 2 days incubation, the final co-

substrate (glucose–VFA mixture) mass balance presented about a 20% deficit. The glucose fermentation products pyruvate, formate, lactate and alcohols were not detected. Thus, such a deficit was attributed mainly to the high maintenance energy requirements of the inoculum, as well as production of soluble microbial products and non-identified products.

AQDS at low concentrations (<0.024 mM) had no influence in generating reducing compounds and further conversion to methane, via external co-substrate oxidation (Fig. 7B). AQDS was just accelerating the shuttle of electrons to the dye (Fig. 6). For instance, the addition of co-substrate and AQDS (0.024 mM) increased the *k* value 1.9-fold compared with the AQDS-free control with co-substrate (Fig. 6); however, no difference in methane production rate was observed under these conditions (Fig. 7B).

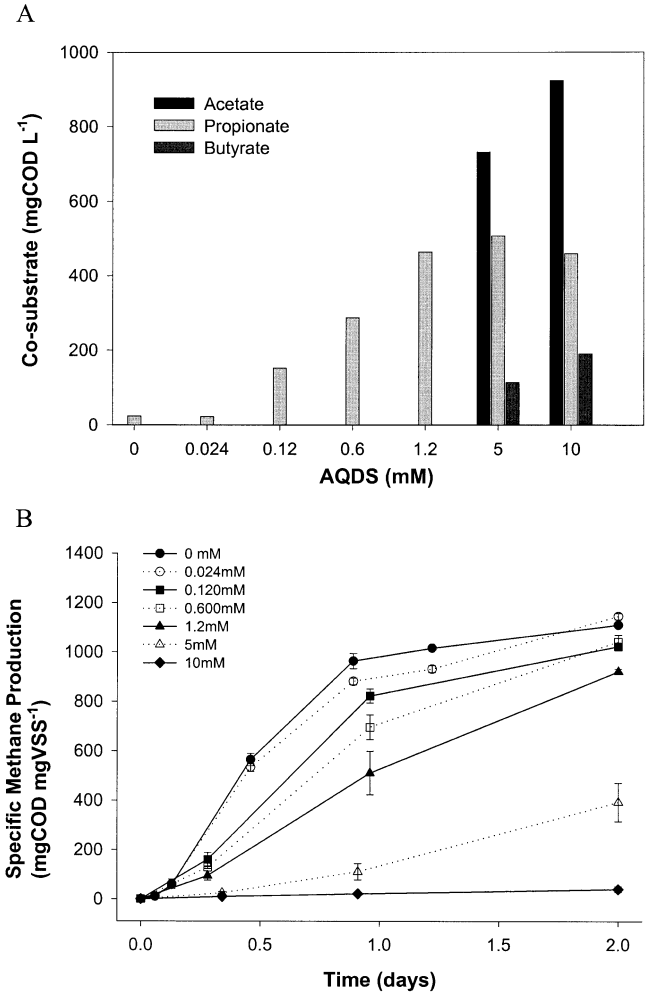

Fig. 7. A Increase in VFA concentrations after 2 days incubation showing the inhibitory effect of high concentrations of AQDS on acetate and propionate oxidation. **B** Concomitant decrease of methane formation rates for high AQDS concentrations. Co-substrate (2g COD l^{-1} as a mixture of glucose and VFA at a ratio of 1:3) was present in all incubations. Glucose, pyruvate, formate, lactate and alcohols were not detected at the end of the experiment. The results are means of triplicate incubations and the *bars* indicate the standard deviations

Discussion

The aim of this paper was to investigate the feasibility of applying thermophilic anaerobic treatment for colour removal of coloured wastewaters. Metabolic activities of the thermophilic inoculum used in this study induced a fast reduction of RR2 (Fig. 3), indicating a biotic predominance in the process. However, addition of high concentrations of sulfide (≥5 mM) revealed the potential contribution of this reducing agent to chemical decolourisation of azo dyes (Table 1). Co-substrate, acting as a primary electron donor, was important not only for improving the colour removal rates, but also for supplying the high maintenance requirements of the thermophilic microorganisms. Biological treatment at 55°C showed a fast initial generation of reducing compounds via co-substrate oxidation, which could be verified comparing the *k* values of sulfide-free with 2 mM-sulfide incubations (Fig. 5). The pseudo-exponential increase at 55°C on the decolourisation rates by increasing VSS concentration (Fig. 4) is another example of the fast initial generation/transfer of reducing compounds via co-substrate oxidation at 55°C. In addition, the more negative values of redox potential obtained at 55°C compared with those at 30°C, after 48 h incubation, confirmed a better electron transfer capacity at 55°C. As a result, increases of sixfold and twofold on the decolourisation rates were verified in the absence and presence of AQDS, respectively, between thermophilic (55°C) and mesophilic (30°C) conditions by using the same sludge source. Therefore, the initial generation/transfer of reducing equivalents at 30°C was probably the rate-limiting step of the process.

AQDS (0.012 mM) in the presence of co-substrate increased the colour removal rates (Fig. 3), but it had no effect on the *k* values observed on the endogenous controls (Fig. 3), suggesting that co-substrate oxidation coupled to electron transfer through AQDS was required for the enhanced decolourisation. Moreover, the impact of AQDS as a redox mediator at high VSS concentrations was less evident than at low VSS concentrations (Fig. 4). Therefore, it was suggested that intracellular components, with more superior mediating properties than those of AQDS, were released to the medium by the addition of biomass. In general, compared with mesophilic microorganisms, thermophilic microorganisms present higher metabolic rates (2–3 times), maintenance energy requirements and sludge turnover rates (van Lier et al. 1993a). Due to the expected higher sludge turnover rates of thermophilic microorganisms compared with mesophilic microorganisms, it is expected that the concentration of reducing and mediating compounds in the reactor bulk would be higher. Keck et al. (2002) reported that during aerobic degradation of naphthalene-2-sulfonate at 30°C for a *Sphingomonas xenophaga* strain BN6, redox mediators were produced, which increased the efficiency of the strain to reduce azo dyes anaerobically. Furthermore, Van der Zee et al. (2003) showed that riboflavin, present in flavin coenzymes, had a more superior capacity as a redox mediator than AQDS during anaerobic reduction of Acid Orange 7 at 30°C in

the presence of sulfide. This reducing/mediating effect seems to be more evident at 55°C than at 30°C based on the higher *k* values obtained under thermophilic conditions. Probably such an effect is due to the higher sludge turnover rate and cell lysis at 55°C than at 30°C, in which reducing/mediating compounds, previously inactive due to inability to cross the cell membrane, can actively participate in the azo dye reduction. Cell lysis and sludge disruption can provide the medium with reducing agents, e.g. sulfide, iron and other reduced cofactors, which reduce azo dyes chemically, and also increase the concentration of naturally mediating and quinone-based compounds (Yoo et al. 2000).

The negligible increase in colour removal at high AQDS concentrations (Fig. 6) is in accordance with previous studies using redox mediators to accelerate azo dye reduction (Keck et al. 1997; Kudlich et al. 1997; Rau et al. 2002). As with high AQDS concentrations, there was an inhibitory effect on acetate and propionate oxidation (Fig. 7A) and methane production (Fig. 7B), so the enzymatic generation of hydroquinone was probably the rate-limiting step of the process. Apparently, the consortium used in the experiment had a poor AQDS reducing capacity via acetate and propionate oxidation. This is in accordance with the fact that to date the literature has reported only a few microorganisms that can couple the oxidation of acetate or propionate to quinone reduction under thermophilic or hyperthermophilic conditions (Kashefi et al. 2002). On the other hand, acetate-oxidizing and propionate-oxidizing quinone-reducing microorganisms are more abundant under mesophilic conditions (Field et al. 2000). Hydrogen-oxidizing rather than acetate-oxidizing bacteria seem to be more actively involved on quinone respiration under thermophilic or hyperthermophilic conditions (Field et al. 2000; Lovley et al. 2000). Considering the increasing role of H_2 at high temperatures (Zinder 1990; van Lier et al. 1993b; Stams 1994), thermophilic anaerobic treatment seems to be advantageous for hydroquinone generation when redox mediators are involved. Saturation kinetics could also explain the negligible increase on colour removal for high AQDS concentrations. Field and Brady (2002) used riboflavin as a redox mediator at 30°C for the reduction of mordant yellow 10. They reported that there was a small variation in the *k* values for riboflavin concentrations higher than 0.055 mM, which they attributed to saturation kinetics of the enzymatic reduction of riboflavin. Nevertheless, in the present experiment at 55°C, the generation of hydroquinone rather than saturation kinetics of AQDS, seems to explain better the constant colour removal rates for the high concentrations of AQDS tested. The latter is in accordance with Rau et al. (2002) who demonstrated at 30°C that the reduction of anthraquinone-2-sulfonate to the hydroquinone form by *Sphingomonas xenophaga* strain BN6 was the rate-limiting step in the reduction of amaranth.

The promising results achieved in this study suggest good prospects for the application of thermophilic anaerobic treatment not only to azo dye reduction, but also to other reductive transformations such as nitroaromatic reduction and dehalogenation.

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