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The transformation and toxicity of anthraquinone dyes during thermophilic (55 °C) and mesophilic (30 °C) anaerobic treatments

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Abstract

We studied in batch assays the transformation and toxicity of anthraquinone dyes during incubations with anaerobic granular sludge under mesophilic (30 °C) and thermophilic (55 °C) conditions. Additionally, the electron shuttling capacity of the redox mediator anthraquinone-2-sulfonic acid (AQS) and subsequent increase on decolourisation rates was investigated on anthraquinone dyes. Compared with incubations at 30 °C, serum bottles at 55 °C presented distinctly higher decolourisation rates not only with an industrial wastewater containing anthraquinone dyes, but also with model compounds. Compared with batch assays at 30 °C, the first-order rate constant "*k*" of the Reactive Blue 5 (RB5) was enhanced 11-fold and 6-fold for bottles at 55 °C supplemented and free of AQS, respectively. However, the anthraquinone dye Reactive Blue 19 (RB19) demonstrated a very strong toxic effect on volatile fatty acids (VFA) degradation and methanogenesis at both 30 °C and 55 °C. The apparent inhibitory concentrations of RB19 exerting 50% reduction in methanogenic activity (IC_{50}-value) were 55 mg l^{-1} at 30 °C and 45 mg l^{-1} at 55 °C. Further experiments at both temperatures revealed that RB19 was mainly toxic to methanogens, because the glucose oxidizers including acetogens, propionate-forming, butyrate-forming and ethanol-forming microorganisms were not affected by the dye toxicity.

Keywords: Anthraquinone dyes; Colour removal; Textile wastewater; Anaerobic treatment; Mesophilic; Thermophilic; Redox mediators; Toxicity

1. Introduction

Anthraquinone dyes constitute the second largest class of textile dyes, after azo dyes (Baughman and

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Weber, 1994). Normally to obtain a target colour, a mix of dyes, e.g., red, yellow and blue, is applied in dyebaths. These dyes might contain different chromophores, of which azo, anthraquinone and phtalocyanine dyes are the most important groups (Hao et al., 2000). Anthraquinone dyes give a wide range of colours in almost the whole visible spectrum, but they are most commonly used for violet, blue and green colours (Fontenot et al., 2002; Christie, 2001). Azo dyes, on the other hand, are mostly used for yellow, orange and red colours (Christie, 2001). Because anthraquinone dyes are widely applied, they might be present in considerably or relatively high concentrations in wastewaters originating from textile factories. Despite this, biological and physical–chemical investigations applied on decolourisation of anthraquinone dyes are not that frequent in comparison with those carried out with azo dyes. A large portion of textile wastewaters, particularly those coming from dyebaths, is discharged at high temperatures (40–70 °C). However, thermophilic anaerobic decolourisation of anthraquinone dyes by granular sludge was never studied before.

Quinone-based redox mediators have been shown to accelerate the transfer of reducing equivalents from a primary electron donor to an azo dye, and a distinct improvement on decolourisation rates has been observed in bioreactors (Cervantes et al., 2001; Dos Santos et al., 2003, 2004a, 2004b). These redox mediators are very effective for azo dye reduction very likely due to the nature of the azo chromophore –N=N–, which is electronically unstable and has the capacity to receive electrons from the reduced form of the mediator. However, anthraquinone dyes are electronically stable and as a result, the reduced form of the above-mentioned mediator will likely be less effective in transferring electrons to the dye. In the present paper, the impact of a quinone-based redox mediator on colour removal of anthraquinone dyes was evaluated. Additionally, we investigated in batch assays the transformation and toxicity of anthraquinone dye model compounds in incubations with anaerobic granular sludge under mesophilic (30 °C) and thermophilic (55 °C) conditions. Finally, a comparative study between mesophilic and thermophilic decolourisation of anthraquinone dye-containing wastewater originating from a textile factory was performed.

2. Materials and methods

2.1. Chemicals

Reactive Blue 5 (RB5, C.I. 61210) and Reactive Blue 19 (RB19, C.I. 61200) were selected as anthraquinone dye model compounds (Fig. 1). Anthraquinone-2-sulfonate (AQS) was selected as redox mediator model compound.

All chemicals used were of analytical grade and purchased from Aldrich (Gillingham, UK), Sigma (Bornem, Belgium) or Acros (Geel, Belgium).

2.2. Seed inoculum and basal medium for decolourisation assays

Anaerobic granular 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 hydraulic retention time (HRT) of about 6 h and an organic loading rate (OLR) of 2.5 kg COD m^{-3} day^{-1}. The chemical oxygen demand (COD) consisted of a mixture of glucose and volatile fatty acids (VFA) at a COD ratio of 1:3. The neutralized VFA solution contained acetate, propionate and butyrate at a COD ratio of 1:1:1.

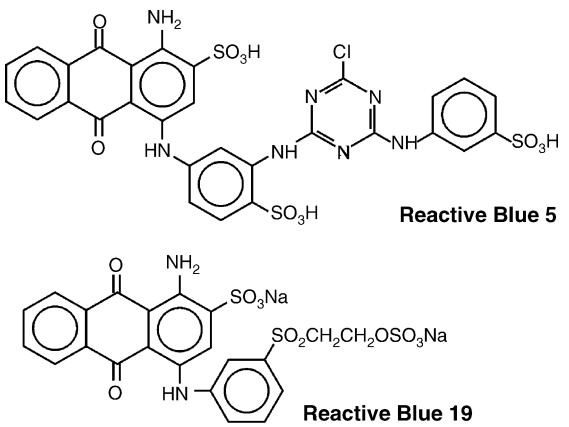

Fig. 1. Molecular structure of the anthraquinone dye model compounds used in the current investigation.

For batch tests at 30 °C the same mesophilic granular sludge was acclimated in an EGSB reactor (30 °C) following the protocol previously described. Stable performance efficiencies in terms of COD removal for the mesophilic and thermophilic reactors were 95 and 85%, respectively. The influent stock solution was free of dye and AQS during the whole period.

The basal medium for the tests with model compounds 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), buffered with 6.21 g l^{-1} of sodium bicarbonate at a pH of around 7.1.

In decolourisation assays with the industrial wastewater, the wastewater was tested undiluted and without addition of nutrients or trace elements. The pH was adjusted to 7 with NaOH or HCl.

2.3. Activity tests

In the activity tests, 1.3 ± 0.1 g VSS l^{-1} of the previously described stabilized sludge were added to 117 ml serum bottles with 50 ml basal medium. The bottles were then sealed with butyl rubber stoppers and aluminium crimp caps. Anaerobic conditions were established by flushing the headspace with N_2/CO_2 (70:30%) and 2 g COD l^{-1} co-substrate (VFA mixture or glucose), dyes (variable) and AQS (variable) were added. The neutralized VFA solution contained acetate, propionate and butyrate at a COD ratio of 1:1:1. Sterile controls were autoclaved once at 122 °C for 240 min and again following a 5-day incubation period, after which sterile co-substrate, mediator and dye stock solutions were added. The pH and the amount of VSS were determined after completion of the experiment.

2.3.1. Decolourisation assays with wastewater originating from a textile industry

The wastewater used for the decolourisation assays was a dark acid polyamide dye wastewater from a textile factory located near Gent, Belgium. It consisted of a mix of streams from the dyebath, fixation and rinsing steps. A co-substrate concentration of 2 g COD l^{-1} (glucose:VFA mixture at a COD ratio of 1:3) ensured the supply of primary electron donors. To evaluate the electron shuttling effect of a redox mediator to anthraquinone dyes, AQS (0.5 mM) was added to some bottles. Both mesophilic and thermophilic acclimated sludges were incubated with the wastewater at a concentration of 1.3 ± 0.1 g VSS l^{-1}. Abiotic colour removal was assessed with both autoclaved sludge and sludge-free controls.

2.3.2. Decolourisation assays and standardized activity tests with model compounds

The anthraquinone dyes RB5 and RB19 were selected for decolourisation assays and added in varying concentrations in standardized activity tests under mesophilic and thermophilic conditions. A VFA mixture at a concentration of 2 g COD l^{-1} was used as co-substrate. To evaluate the electron shuttling effect of a redox mediator to anthraquinone dyes, AQS (0.012 mM) was added to some bottles. Abiotic colour removal was assessed with both autoclaved sludge and sludge-free controls.

2.4. Analysis

For anthraquinone model compounds colour removal was determined photometrically (Spectronics 60, Milton-Roy Analytical Products Division, Belgium), reading the absorbance at the maximum absorbance wavelength, i.e. RB5 at 602 nm and RB19 at 593 nm. The extinction coefficients used (AU cm^{-1} mM^{-1}) were 8.2 and 9.5 for RB5 and RB19, respectively. Wastewater decolourisation was determined in a 1 cm quartz cuvette by scanning the UV/VIS spectra (Perkin-Elmer UV/VIS Lambda 12, Rodgau-Jügesheim, Germany) and comparing the wavelength of two absorbance peaks. Samples were centrifuged for 3 min at 10,000 rpm prior to analysis. Methane production was determined on a gas chromatograph model 438/S (Packard-Becker, Delft, The Netherlands), as previously described (Cervantes et al., 2000). VFA, methanol and ethanol were measured on a Hewlett Packard 5890 gas chromatograph (Palo Alto, USA), as previously described (Cervantes et al., 2000). 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. (1997). Volatile suspended solids (VSS) were analysed ac-

cording to APHA standard methods (1998). The pH was determined using a Schott Gerate N32A double electrode (Hofheim, Germany) connected to a Knick 511 pH meter (Berlin, Germany).

3. Results

3.1. Decolourisation assays with anthraquinone dye-containing wastewater

The capacity of anaerobic granular sludge to decolourise an anthraquinone dye-containing wastewater was tested. The decolourisation assays demonstrate that colour removal rates were enhanced at 55 °C as compared with 30 °C (Fig. 2A and B). In the presence of the redox mediator, AQS, and assuming a first-order reaction, the first-order rate constant "*k*" was 0.19 day^{-1} at 55 °C and 0.08 day^{-1} at 30 °C, a 2.6-fold enhancement in decolourisation rates. In the absence of AQS the impact of temperature was even greater (6.7-fold increasing), as expressed in the *k*-value 0.20 day^{-1} at 55 °C and 0.03 day^{-1} at 30 °C. Metabolic activity of the inoculum was very likely the main mechanism of colour removal, as a negligible decolourisation was observed in the sludge-free controls, and higher rates of colour removal were observed in the bottles supplemented with co-substrate. As predicted, the redox mediator AQS at a concentration of 0.5 mM had no significant impact on colour removal rates under thermophilic conditions, although mesophilic decolourisation was increased 2.6-fold by AQS addition. Nevertheless, both rates at 30 °C were significantly lower than those obtained at 55 °C (Fig. 2A and B).

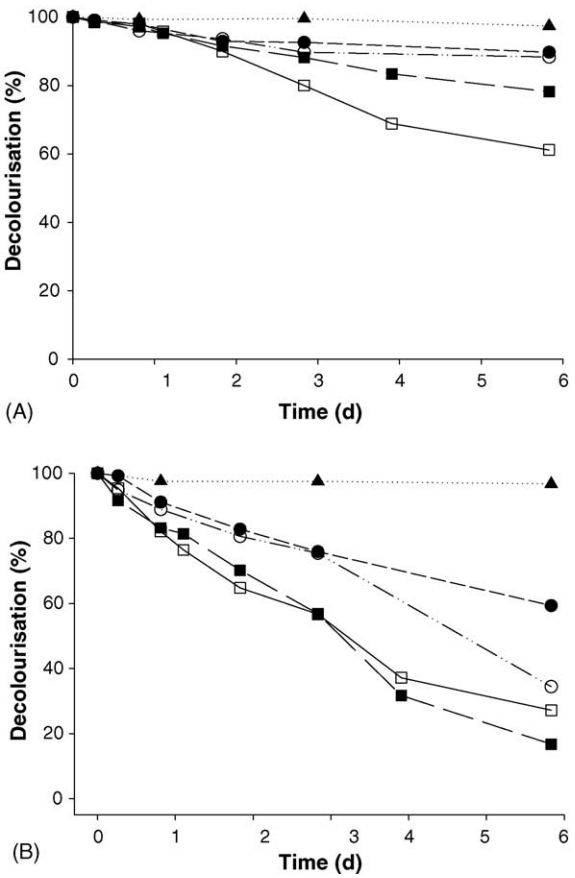

Fig. 2. Decolourisation of the textile wastewater at 585 nm by mesophilic (A) and thermophilic (B) anaerobic granular sludge. Incubations contained: wastewater (●), wastewater/AQS (○), wastewater/co-substrate (■), wastewater/co-substrate/AQS (□). Sludge-free bottles were used as control for the abiotic colour removal (▲). AQS (0.5 mM) and 2 g COD l^{-1} co-substrate were added to some bottles. The results are means of duplicate bottles. The standard deviation was lower than 5% in all cases.

3.2. Decolourisation assays and standardized activity tests with model compounds

3.2.1. Decolourisation assays with Reactive Blue 5 (RB5)

Decolourisation assays with RB5 clearly confirm that a complete colour removal was only possible at 55 °C (Fig. 3). Assuming a first-order reaction in the presence of AQS, the *k*-values were 0.33 day^{-1} at 55 °C and 0.03 day^{-1} at 30 °C, a 11-fold enhancement due to the temperature increase (Fig. 3). In the absence of AQS, the *k*-values were 0.18 day^{-1} at 55 °C and 0.03 day^{-1} at 30 °C, a six-fold difference in decolourisation rates (Fig. 3). The complete decolourisation at 55 °C was likely irreversible as no colour resurgence was seen after exposing the sample to oxygen (data not shown). Contrary to the experiments with textile wastewater, AQS at a concentration of 0.012 mM supplemented with 23.2 mg l^{-1} of RB5 enhanced 1.8-fold the decolourisation rate at 55 °C (Fig. 3). However, at high concentrations of RB5, e.g. 232 mg l^{-1}, the same concentration of AQS did not have any effect on colour removal (data not shown). In mesophilic bottles (30 °C) supplemented

with 23.2 mg l^{-1} of RB5, the decolourisation rates were 0.035 day^{-1} and 0.044 day^{-1} for the AQS-free and AQS-supplemented incubations, respectively (Fig. 3). Therefore, AQS had a very slight impact on colour removal. Negligible (<3%) colour removal occurred in sludge-free controls in the presence of AQS (results not shown) at 30 and 55 °C.

For the VFA mixture added as co-substrate, the conversion of acetate and butyrate to methane was almost complete at 30 and 55 °C (Table 1). Such a conversion corresponds to an excess of reducing equivalents required to completely reduce the dye. For instance, if just the conversion of acetate to methane is considered, consumption of less than 1% of the acetate present is required to reduce all dye molecule, if all reducing equivalents from acetate conversion are preferentially channelled to the anthraquinone group. Therefore, the difference between the potential for colour removal at 30 and 55 °C was not due to a lack of reducing equivalents, but may be rather associated to differences in the rate of electrons transfer. Table 1 shows that the oxidation of propionate is affected by the presence of AQS (0.012 mM) at both temperatures. Moreover, propionate conversion at 55 °C ceased for concentrations of RB5 above 23.2 mg l^{-1}.

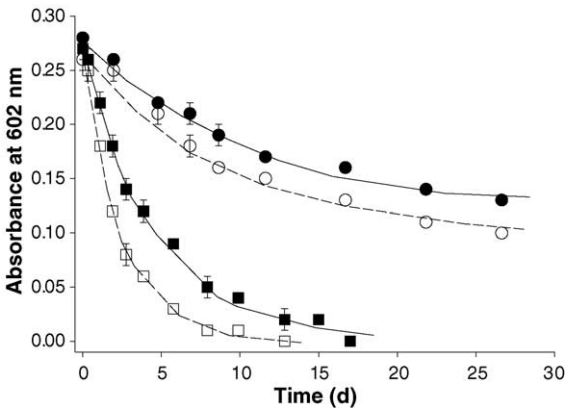

Fig. 3. Colour removal of the anthraquinone dye Reactive Blue 5 (23.2 mg l^{-1}) by mesophilic (30 °C) and thermophilic (55 °C) anaerobic granular sludge. The redox mediator AQS (0.012 mM) was added to certain bottles. Symbols for: AQS-free (●) and AQS-supplemented (○) at 30 °C; AQS-free (■) and AQS-supplemented (□) at 55 °C. A VFA mixture (2 g COD l^{-1}) was used as co-substrate. Sludge-free controls were used to assess the stability of the basal medium and contained RB5, AQS and co-substrate. The results are means of duplicate bottles and the bars indicate the standard deviation.

In the absence of granular sludge, no colour removal was observed at 55 °C either in the presence or absence of AQS during a pure chemical reduction of RB5 (232 mg l^{-1}) with sulphide as potential reducing agent (4.5 mM, pH 7.2) (data not shown). These results indicate that the biogenically produced sulphide does not significantly contribute to the decolourisation of this type of dye. Furthermore, this experiment suggests that although AQS may be reduced by sulphide, the expected reduced form of AQS (AH_2QS) does not have the capacity of transferring the electrons to the dye, being the rate-limiting step in the process. On the other hand, a complete decolourisation of the azo dye Reactive Red 2 (RR2, 0.3 mM) was observed both in the presence and absence of AQS with the same sulphide concentration, the presence of AQS increasing the decolourisation rates 1.7-fold (data not shown).

3.2.2. Decolourisation assays with Reactive Blue 19

Although a complete colour removal of RB19 was achieved at 30 and 55 °C (data not shown), the experiments revealed that decolourisation was mainly due to abiotic mechanisms. Both a considerable adsorption

Table 1
Results of the standardized activity test for different concentrations of the anthraquinone dye Reactive Blue 5 during mesophilic (30°) and thermophilic (55°) incubations with anaerobic granular sludge

RB 5 (mg l^{-1})	COD balance (mg l^{-1})				
	Acetate	Propionate	Butyrate	Methane	Total
Mesophilic					
Control	24	–	–	1900	1924
23.2	24	–	–	2280	2304
23.2 + AQS	27	595	–	1568	2190
69.6	23	–	–	1840	1862
116.1	25	–	–	1857	1882
Thermophilic					
Control	45	46	–	1897	1987
23.2	24	115	–	2135	2273
23.2 + AQS	76	682	–	1508	2265
69.6	75	668	–	1310	2052
116.1	59	696	–	1157	1911

A concentration of 2 g COD l^{-1} of a VFA mixture (1:1:1 at a COD basis) was used as co-substrate. Anthraquinone-2-sulfonate (0.012 mM) was added to some of the bottles. Mean of duplicate incubations, with the standard deviation lower than 10% in all cases. The molecular weigh of RB5 is 774.2 g mol^{-1}.

on the granules and formation of precipitates in the medium were observed.

A very strong toxic effect on VFA degradation and methane production was also observed for both inocula, even when low concentrations of RB19 (37.5 mg l^{-1}) were added. The apparent inhibitory concentrations for RB19 exerting 50% reduction in methanogenic activity (IC_{50}-value) were about 55 mg l^{-1} at 30 °C and 45 mg l^{-1} at 55 °C. If the occurring precipitation of RB19 is taken into account, the actual IC_{50} is even lower than the mentioned apparent values. Despite the more complex molecular structure of RB5 compared with the RB19 structure (Fig. 1), the toxicity of RB19 in the inoculum was more pronounced than for RB5 (Table 1).

Experiments were also conducted at 30 and 55 °C to verify the reversibility of the toxicity induced by RB19 (93.9 mg l^{-1}). The medium was replaced by a new medium free of RB19 and a fresh VFA mixture was added afterwards. The methane formation was compared with the control, i.e., free of RB19 for the whole period. As shown in Fig. 4 for batch assays at 30 °C, the toxicity was indeed irreversible as no methane was detected after replacing the medium containing RB19. In experiments conducted at 55 °C the same toxic effect on methanogenesis was observed.

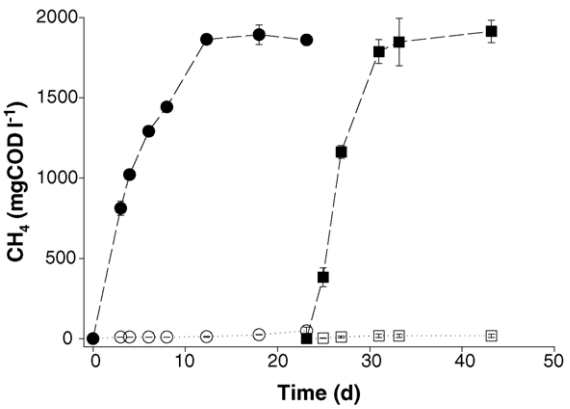

Fig. 4. Evaluation of the permanent toxicity of Reactive Blue 19 by mesophilic anaerobic granular sludge relative to the control (dye-free). Symbols for: dye-free control (●) and 93.9 mg l^{-1} of RB19 (◯); control after re-feeding (■) and RB19 after re-feeding (□). AQS (0.012 mM). A VFA mixture (2 g COD l^{-1}) was used as co-substrate. The results are means of duplicate bottles and the bars indicate the standard deviation.

Further experiments focused on the evaluation of which group of microorganisms was mainly affected by the toxicity of RB19. Glucose (1.5 g COD l^{-1}) was selected as co-substrate and was tested in standardized activity tests at 30 °C either in the presence or absence of the dye RB19 (93.9 mg l^{-1}) and AQS (0.012 mM). Results revealed that the glucose oxidizers, including acetogens, propionate-forming, butyrate-forming and ethanol-forming microorganisms, were not affected by RB19, as all glucose was consumed after 1 day of incubation in all bottles. In the absence of RB19 the produced acetate (Fig. 5A) was immediately converted into methane (Fig. 5B). However, in the RB19-containing bottles acetate accumulated

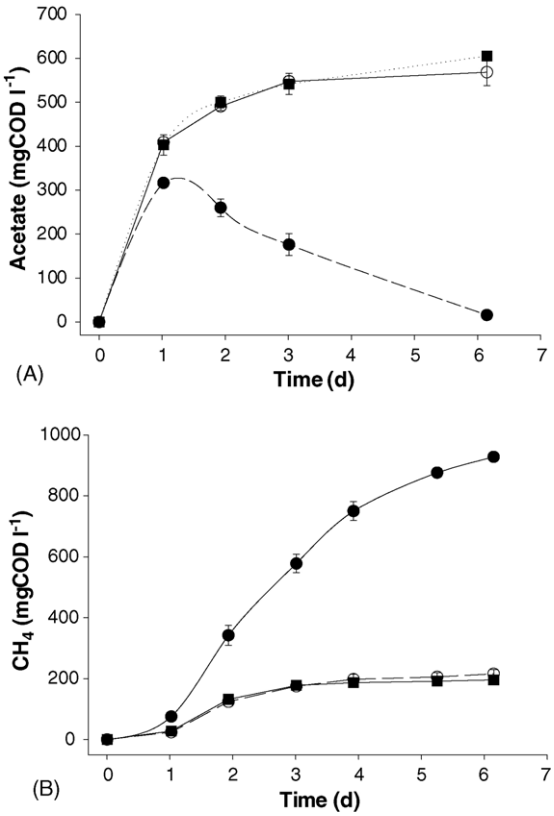

Fig. 5. Acetate concentration (A) and CH_4 formation (B) during investigations with mesophilic (30 °C) anaerobic granular sludge with glucose (1.5 g COD l^{-1}) as co-substrate. RB19 (93.9 mg l^{-1}) and AQS (0.012 mM) were added to some bottles. Symbols for: dye-free control (●), RB19 AQS-free (◯), and RB19 AQS-supplemented (■). The results are means of triplicate bottles and the bars indicate the standard deviation.

Table 2
Investigations on anaerobic colour removal and toxicity of anthraquinone dyes reported in the literature

Dye	Decolour (%)	Additional information	Reference
Reactive Blue 4	78	After 27.7 days. CH_4 production comparable to the control	Fontenot et al. (2002)
Reactive Blue 5	68	After 16 days. C = 20 mg l^{-1}	Luangdilok and Paswad (2000)
	37	After 16 days. C = 100 mg l^{-1}	
Reactive Blue 19	83	After 27.7 days. CH_4 production severely inhibited	Fontenot et al. (2002)
	57, 32	After 16 days. C = 20 mg l^{-1}	Luangdilok and Paswad (2000)
	30–100	Average: 70%	Brown and Laboureur (1983)
Reactive Blue 49	7–10	After 2 h	Carliell et al. (1994)
Acid Blue 80	4–16	Average: 7%	Brown and Laboureur (1983)
Acid Blue 25	36–100	Average: 67% insoluble blue pigment formed	Brown and Laboureur (1983)
	100	After 21 days. 1:1 formation of 1-amino-4-phenyl-aminoanthraquinone (blue solid)	Brown and Hamburger (1987)
Basic Blue 22	46–86	Average: 62%	Brown and Laboureur (1983)
Disperse Red 159	0	After 3 days. C = range 300–2400 mg l^{-1}. Rate inhibited by 60% at 300 mg l^{-1} and by >90% at 2400 mg l^{-1}	Malpei et al. (1998)
Disperse Blue 56	0	C = 30 mg l^{-1} dye made UASB reactor collapse	Delee et al. (1998)

(Fig. 5A), and methanogenesis ceased after 3 days of incubation (Fig. 5B). This indicated that acetate-utilizing methanogens was the most important group affected by RB19, which was also verified in experiments at 55 °C.

4. Discussion

Results from batch experiments reveal that compared with mesophilic treatment at 30 °C, thermophilic treatment at 55 °C presents distinctly higher decolourisation rates with both textile wastewater and with the model compound RB5. A complete colour removal of the anthraquinone model compound RB5 was only possible at 55 °C. Additionally as hypothesized, the redox mediator AQS did not significantly increase the decolourisation rates of anthraquinone dyes.

The few publications on anaerobic anthraquinone dye decolourisation under mesophilic conditions are not very consistent (Table 2). Our results under mesophilic conditions are in accordance with Carliell et al. (1994) who reported that compared with azo dyes, other dyes such as anthraquinone and phtalocyanine, are less susceptible to reduction and present very low colour removal capacity. Moreover, Fontenot et al. (2002) achieved an incomplete decolourisation, i.e. 78% colour removal of the anthraquinone dye Reactive Blue 4, in both amended and un-amended cultures at 35 °C. In our experiments with the industrial textile wastewater, the temperature increase from 30 to 55 °C distinctly enhanced the decolourisation rates. This was probably due to an improvement of the colour removal capacity not only for the anthraquinone dyes, but also for the azo dyes present in the wastewater. For instance, compared with anaerobic incubations at 30 °C, the decolourisation of the azo dye Reactive Red 2 at 55 °C was enhanced six-fold in the absence of the external mediator anthraquinone-2,6-disulfonate (AQDS) (Dos Santos et al., 2004a, 2004b).

Our observation that the decolourisation of RB5 by a pure chemical reaction with sulphide was not possible confirms that the anthraquinone link at neutral pH is indeed electronically very stable, making the nucleophilic attack by sulphide inefficient. Particularly in the case of RB5, the hydrogen bond formed between the carbonyl group of the quinone-substituent and the amino-substituent, likely precludes the RB5 molecule from receiving an additional electron. On the other

hand, the complete decolourisation of the azo dye RR2 at neutral pH by sulphide evidences that the azo link is electronically less stable and therefore susceptible for reductive cleavage. As sulphate is a common pollutant in textile wastewaters, a significant quantity of sulphide might be produced via sulphate reduction. Thus, in the application of anaerobic treatment, biogenic sulphide may play an important role as a reductant and contribute to the decolourising processes (Yoo, 2002; Van der Zee et al., 2003). However, for dyebaths composed of a mix of dyes, e.g., anthraquinone and azo dyes, the purely chemical decolourisation by the biogenic sulphide will occur mostly with azo dyes, as anthraquinone dyes are shown extremely stable.

Generally, the low decolourisation rate of anthraquinone dyes is attributed to toxic effects, which display different inhibition levels on anaerobic inocula (Table 2). It was verified that less than 100 mg l^{-1} of RB19 was enough to completely inhibit the methane production of both mesophilic and thermophilic inocula with a VFA co-substrate. Contrarily, it was observed that glucose oxidizers were not affected by RB19 toxicity, as no trace of glucose (1.5 g COD l^{-1}) was detected after one day of incubation when this sugar was supplemented as co-substrate. Anthraquinone dyes are also toxic to sulphate reducers (Lie et al., 1996), but the effects on methanogens seem to be less predictable (Cooling III et al., 1996). An explanation for the toxicity is that the active site of enzymes can be occupied by anthraquinone dye molecules, thus blocking the binding of substrate and cofactors (Prestera et al., 1992; Denizli and Piskin, 2001). Cooling III et al. (1996) proposed another inhibition mechanism, which was the uncoupling of electron transfer from ATP synthesis via an anthraquinone-mediated electron transfer reaction. Nevertheless, in the current experiment acetate was one of the most important glucose oxidation products that accumulated in the bottles when RB19 was present (Fig. 5A). This was an indication that the acetyl-CoA pathway used by almost all acetate forming microorganisms was not blocked by the dye (Diekert, 1991). On the other hand, methane production ceased in the RB19-supplemented bottles (Fig. 5B), suggesting the blockage of methanogenesis pathway by the dye.

It was found that the toxicity was permanent in terms of methane production, and that just the removal of RB19 from the medium was not enough to re-establish the enzyme functionality. This is in accordance with the above-mentioned observation of Denizli and Piskin (2001) that the dye-enzyme bond, i.e., when the active site of enzymes is occupied by the anthraquinone dye, could not easily be broken. In the latter study the use of an elutant to break the enzyme bond did reverse the process. Delee et al. (1998) reported that the dye Disperse Blue 56 severely inhibited the inoculum of a lab scale UASB reactor, causing a process collapse, although it had been decolourising other dyes successfully.

With Reactive Blue 19 (RB19), a complete decolourisation at 30 and 55 °C was observed, which was mainly attributed to abiotic mechanisms. This conclusion is initially based on the observation that the reducing equivalents required to remove the colour were not formed, due to the inhibitory effect of RB19 on VFA oxidation and CH_4 formation. Therefore, the sole maintenance of reducing conditions was not enough to remove the colour. This contradicts the conclusions of Fontenot et al. (2002) who attributed the decolourisation of RB19 to the reduced conditions in the medium despite a severe inoculum inhibition. The abiotic mechanism in the current experiment consisted of a considerable adsorption of RB19 on the granules and the formation of precipitates. This is in accordance with the results of Luangdilok and Paswad (2000) who reported that the decolourisation of Reactive Blue 19 was only due to biomass adsorption, and that no biotransformation of the dye could be observed. Brown and Hamburger (1987) also found a blue precipitate while investigating the reduction of Acid Blue 25 under anaerobic conditions. The formation of precipitates is a result of the pure chemical release of the reactive species of reactive anthraquinone dyes, e.g. the release of the free amine from the protonated group of anthraquinone dye incubated under neutral to alkaline solutions (Mccallum et al., 2000). As the pH of the current RB19 incubations was neutral, our results are in agreement with the latter.

In summary, anthraquinone dyes present in textile wastewaters represent a potential problem to the applicability of cost-effective anaerobic treatment technologies or even chemical treatment based on a nucleophilic attack. Therefore, a better insight in both the transformation and the toxicity mechanisms of anthraquinone dyes is required for closing process water cycles of all dye-containing wastewaters to avoid their discharge in the environment.

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References

APHA, 1998. Standard methods for the examination of water and wastewater, twentieth ed. American Public Health Association, Washington, DC.

Baughman, G.L., Weber, E.J., 1994. Transformation of dyes and related compounds in anoxic sediment: kinetics and products. Environ. Sci. Technol. 28, 267–276.

Brown, D., Hamburger, B., 1987. The degradation of dyestuffs: part III—investigations of their ultimate degradability. Chemosphere 16, 1539–1553.

Brown, D., Laboureur, P., 1983. The degradation of dyestuffs: part I—primary biodegradation under anaerobic conditions. Chemosphere 12, 397–404.

Carliell, C.M., Barclay, S.J., Naidoo, N., Buckley, C.A., Mulholland, D.A., Senior, E., 1994. Anaerobic decolorisation of reactive dyes in conventional sewage treatment processes. Water SA 20, 341–344.

Cervantes, F.J., van der Velde, S., Lettinga, G., Field, J.A., 2000. Competition between methanogenesis and quinone respiration for ecologically important substrates in anaerobic consortia. FEMS Microbiol. Ecol. 34, 161–171.

Cervantes, F.J., Van der Zee, F.P., Lettinga, G., Field, J.A., 2001. Enhanced decolourisation of Acid Orange 7 in a continuous UASB reactor with quinones as redox mediators. Water Sci. Technol. 44, 123–128.

Christie, R., 2001. Colour Chemistry. The Royal Society of Chemistry, Cambridge, UK.

Cooling III, F.B., Maloney, C.L., Tabinowski, J., Odom, J.M., 1996. Inhibition of sulfate respiration by 1,8-dihydroxyanthraquinone and other anthraquinone derivatives. Appl. Environ. Microbiol. 62, 2999–3004.

Delee, W., O'Neill, C., Hawkes, F.R., Pinheiro, H.M., 1998. Anaerobic treatment of textile effluents: a review. J. Chem. Technol. Biotechnol. 73, 323–335.

Denizli, A., Piskin, E., 2001. Dye-ligand affinity systems. J. Biochem. Biophys. Methods 49, 391–416.

Diekert, G., 1991. The acetogenic bacteria. In: The Prokaryotes. Springer-Verlag, New York, USA.

Dos Santos, A.B., Cervantes, F.J., Yaya-Beas, R.E., Van Lier, J.B., 2003. Effect of redox mediator, AQDS, on the decolourisation of a reactive azo dye containing triazine group in a thermophilic anaerobic EGSB reactor. Enzyme Microbiol. Technol. 33, 942–951.

Dos Santos, A.B., Cervantes, F.J., Van Lier, J.B., 2004a. Azo dye reduction by thermophilic anaerobic granular sludge, and the impact of the redox mediator AQDS on the reductive biochemical transformation. Appl. Microbiol. Biotechnol. 64, 62–69.

Dos Santos, A.B., Traverse, J., Cervantes, F.J., Van Lier, J.B., 2004. Enhancing the electron transfer capacity and subsequent colour removal in bioreactors by applying thermophilic anaerobic treatment and redox mediators. Biotechnol. Bioeng, in press.

Fontenot, E.J., Beydilli, M.I., Lee, Y.H., Pavlostathis, S.G., 2002. Kinetics and inhibition during the decolorization of reactive anthraquinone dyes under methanogenic conditions. Water Sci. Technol. 45, 105–111.

Hao, O.J., Kim, H., Chang, P.C., 2000. Decolorization of wastewater. Crit. Rev. Environ. Sci. Tec. 30, 449–505.

Lie, T.J., Godchaux, W., Leadbetter, E.R., 1996. Sulfonates as terminal electron acceptors for growth of sulfite-reducing bacteria (Desulfitobacterium spp.) and sulfate-reducing bacteria: effects of inhibitors of sulfidogenesis. Appl. Environ. Microbiol. 65, 4611–4617.

Luangdilok, W., Paswad, T., 2000. Effect of chemical structures of reactive dyes on color removal by an anaerobic–aerobic process. Water Sci. Technol. 42, 377–382.

Malpei, F., Andreoni, V., Daffonchio, D., Rozzi, A., 1998. Anaerobic digestion of print pastes: a preliminary screening of inhibition by dyes and biodegradability of thickeners. Biores. Technol. 63, 49–56.

Mccallum, J.E.B., Madison, S.A., Alkan, S., Depinto, R.L., Wahl, R.U.R., 2000. Analytical studies on the oxidative degradation of the reactive textile dye Uniblue A. Environ. Sci. Technol. 34, 5157–5164.

Prestera, T., Prochaska, J., Talalay, P., 1992. Inhibition of NAD(P)H: (Quinone-acceptor) oxireductase by Cibracon Blue and related anthraquinone dyes: a structure-activity study. Biochemistry 31, 824–833.

Van der Zee, F.P., Bisschops, I.A.E., Blanchard, V.G., Bouwman, R.H.M., Lettinga, G., Field, J.A., 2003. The contribution of biotic and abiotic processes during azo dye reduction in anaerobic sludge. Water Res. 37, 3098–3109.

Van Lier, J.B., Rebac, S., Lens, P., Van, B.F., Oude, E.S., Stams, A.J.M., Lettinga, G., 1997. Anaerobic treatment of partly acidified wastewater in a two-stage expanded granular sludge bed (EGSB) system at 8 °C. Water Sci. Technol. 36, 317–324.

Yoo, E.S., 2002. Kinetics of chemical decolorization of the azo dye C.I. Reactive Orange 96 by sulfide. Chemosphere 47, 925–931.