0273-2289/03/109/0207/$20.00

Reductive Decolorization of a Textile Reactive Dyebath Under Methanogenic Conditions

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Abstract

The objective of the present study was to assess the biological decolorization of an industrial, spent reactive dyebath and its three dye components (Reactive Blue 19 [RB 19], Reactive Blue 21 [RB 21], and Reactive Red 198 [RR 198]) under methanogenic conditions. Using a mixed, methanogenic culture, batch assays were performed to evaluate the rate and extent of color removal as well as any potential toxic effects. Overall, a high rate and extent of color removal (>10 mg/[L·h] and 88%, respectively) were observed in cultures amended with either RB 19 (an anthraquinone dye) or spent dyebath at an initial dye concentration of 300 mg/L (expressed as RB 19 equivalent) and 30 g/L of NaCl. Inhibition of acidogenesis and, to a larger degree, of methanogenesis resulting in accumulation of volatile fatty acids was observed in both RB 19– and spent dyebath–amended cultures. RB 21 (a phthalocyanine dye) and RR 198 (an azo dye) tested at an initial concentration of 300 mg/L did not result in any significant inhibition of the mixed methanogenic culture. Based on results obtained with cultures amended with RB 19 with and without NaCl, as well as a control culture amended with 30 g/L of NaCl, salt was less inhibitory than either RB 19 or the dyebath. Therefore, the toxic effect of the spent dyebath is at least partially attributed to its major dye component RB 19 and NaCl. Further testing of the effect of RB 19 decolorization products on the methanogenic activity in the absence of NaCl demonstrated that these products are much less inhibitory than the parent dye. Although color removal occurred despite the severe culture inhibition, biological decolorization of full-strength reactive spent dyebaths using methanogenic cultures in a repetitive, closed-loop system is not deemed feasible. For this reason, a fermentative and halotolerant culture was

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developed and successfully used in our laboratory for the decolorization of industrial reactive dyebaths with 100 g/L of NaCl.

Index Entries: Anthraquinone dyes; decolorization; methanogenesis; phthalocyanine dyes; reactive textile dyes; salt; toxicity.

Introduction

Reactive textile dyes are now the dyes of choice for cotton and cotton blend fabrics because of their superior colorfastness, ease of application, brilliant colors, and wide variety of possible color shades *(1)*. In addition, the growing use of cotton worldwide has resulted in a steady increase in reactive dye usage. The worldwide annual use of reactive textile dyes increased from about 60,000 to 110,000 t between 1988 and 1992 and is projected to exceed 180,000 t by the year 2004 *(2)*. Reactive dyes are the only textile colorants designed to bond covalently with cellulosic fibers (mainly cotton). Under typical reactive dyeing conditions (pH ≥ 10.0, temperature of ≥60°C, 60–100 g/L of salt), as much as 50% of the initial dye mass remains in the spent dyebath in its unfixed, hydrolyzed form, which has no affinity for the fabric and thus results in colored process water that needs to be discharged *(1,3,4)*. It is estimated that about 1 to 2 million gallons of wastewater per day are generated by an average dyeing facility in the United States, with reactive and direct dyeing generating the most wastewater *(5)*. In addition to the color issue, the high salt concentration of the reactive dyebaths results in high salinity wastewaters, further exacerbating both their treatment and disposal. As a result, the textile industry is confronted with the challenge of both color removal (for aesthetic and toxicity reasons) and effluent salt content reduction. The management of spent reactive dyebaths is, therefore, the single most pressing environmental problem facing the textile industry. As more stringent regulations are adopted regarding the textile effluents, the need for more technically and economically efficient color and salt reduction from textile plant effluents grows more acute.

Because of their high water solubility, reactive dyes pass through conventional (aerobic) municipal treatment systems without any significant removal *(6–10)*. Although physicochemical methods for the treatment of textile wastewaters are technically feasible, they are costly. Anaerobic biological processes are more effective and hold promise in providing a low-cost and efficient means for the reductive decolorization of spent reactive dyebaths—which can then be reused as process water—and/or the treatment of textile effluents before their final disposal *(9,11–15)*. Commonly used classes of reactive dyes include azo, stilbene, carotenoid, thiazole, anthraquinone, and phthalocyanine *(1)*. Although considerable research has been conducted on the biological treatment of textile wastewaters bearing mainly azo dyes, investigation of the biotransformation of anthraquinone-based reactive dyes has been very limited, in spite of the fact that these reactive dyes are extensively used for green, blue, or violet hues *(4)*.

In addition, although trichromatic textile dye baths (i.e., combination of three different dyes) are usually used in various combinations to achieve a particular color shade, relatively few studies have investigated the biotransformation and toxicity of commercial, spent textile reactive dyebaths.

The objective of the work presented here was to assess the rate and extent of the reductive decolorization of an industrial-strength, spent textile reactive dyebath, as well as to quantify potential toxic effects on a mixed, methanogenic culture. In addition to the industrial, spent dyebath, a simulated textile dyebath and its individual dye components were also tested. It should be emphasized that relatively high dye concentrations were used in this study that are consistent with the overall scope of a research project which is centered on the development of a biologically based decolorization system for a closed-loop, in-plant process for the renovation of spent reactive dyebaths and reuse as process water in the dyeing operation.

Materials and Methods

Plant Dyebath and Dyes

A spent textile dyebath was collected at the Washington Manufacturing (formerly SCT Yarns) textile plant in Washington, GA, and stored under refrigeration in the laboratory. The industrial dyebath consisted of three dye components as follows: Remazol Brilliant Blue R-W (C.I. Reactive Blue 19, an anthraquinone dye) (RB 19); Remazol Turquoise R-P (C.I. Reactive Blue 21, a Cu-phthalocyanine dye) (RB 21); and Remazol Red RB (C.I. Reactive Red 198, an azo dye) (RB 198). The three dyes—RB 19, RB 21, and RR 198—were used in the dyeing operation at concentrations of 4.9, 1.2, and 0.32 g/L, respectively. To achieve the high salinity and alkaline pH conditions typical of reactive dyebaths, in addition to the three dyes, the industrial dyebath contained NaCl, Na_2CO_3, and NaOH at concentrations of 113, 3.0, and 0.95 g/L, respectively. Spectrophotometric scanning of dilute dyebath solutions was performed and absorbance maxima for the spent dyebath were identified. The dyebath pH, alkalinity, total organic carbon, chemical oxygen demand (COD), total solids (TS), and volatile solids (VS) were determined. For comparison purposes, a composite dyebath mixture was prepared by simulating the industrial dyebath conditions as follows: Dry aliquots of RB 19, RB 21, and RR 198 were added to a volumetric flask resulting in a final concentration of 4.9, 1.2, and 0.32 g/L, respectively. Then powder Na_2CO_3 and NaOH were added corresponding to a final concentration of 3.0 and 0.95 g/L, respectively. The solution was heated at 80°C for 1 h with continuous mixing and then diluted to 1 L. Reacted (i.e., hydrolyzed) solutions of the individual three dyes were prepared separately by dissolving powder dye, Na_2CO_3, and NaOH in deionized water corresponding to a final concentration of 5, 3, and 0.95 g/L, respectively, heated to 80°C for 1 h and then diluted to 1 L.

Methanogenic Seed Culture

A suspended-growth, mixed, methanogenic culture developed with inoculum obtained from a mesophilic, municipal anaerobic digester and fed with a dextrin/peptone solution and a medium containing salts and vitamins was used *(9,16,17)*. To avoid any abiotic dye reduction and color removal, the culture medium did not include any chemical reductant. The culture was maintained at 35°C with a hydraulic and solids retention time of 35 d. The steady-state gas-phase CH_4 and CO_2 concentration of this culture was 58.4 and 41.6%, respectively.

Batch Decolorization Assays

Batch assays were performed in 500-mL serum bottles (400-mL liquid volume), sealed with rubber stoppers and aluminum crimps and flushed with helium gas. An aliquot of 350 mL of the mixed, methanogenic seed culture was anaerobically transferred to each serum bottle, and the remaining 50 mL consisted of spent dyebath or specific dyes and organic feed solution. Dextrin and peptone at initial concentrations of 750 and 375 mg/L, respectively, were added to each bottle. The initial dyebath or dye concentration was 300 mg/L (expressed as RB 19 equivalents). A control culture was also set up with the same methanogenic seed culture and organic feed solution, without any dye. All incubations were carried out in the dark at 35°C and the bottles were agitated daily by hand. The following analyses were performed: initial and final pH and oxidation-reduction potential (ORP), volatile fatty acids (VFA) concentration, absorbance, total gas, and methane production. Further details on the experimental setup for individual decolorization assays are provided in Results and Discussion.

Batch Toxicity Assay of RB 19 Decolorization Products

A batch assay was conducted using supernatant from a methanogenic culture initially amended with RB 19 at 2000 mg/L and incubated for 32 d until complete decolorization was achieved. Then 26-mL serum bottles were set up with methanogenic seed culture, dextrin/peptone organic feed, and media. Aliquots of RB 19 decolorization products were anaerobically transferred from the stock-decolorizing culture to the serum bottles resulting in 0 (i.e., control), 50, 300, 500, 1000, and 1500 mg/L of RB 19 decolorization products, expressed as RB 19 equivalents. Incubation and measurements followed the same procedures used for the batch decolorization assays as discussed in the previous section.

Analytical Methods

ORP, pH, alkalinity, TS, VS, and COD were measured by following procedures outlined in ref. *18*. ORP measurements were performed using a Model 611 Orion Digital pH/millivolt meter (Fisher, Pittsburgh, PA) and a platinum electrode with an Ag/AgCl reference in a 3.5 *M* KCl gel (Sensorex, Stanton, CA). To obtain ORP values with reference to the stan-

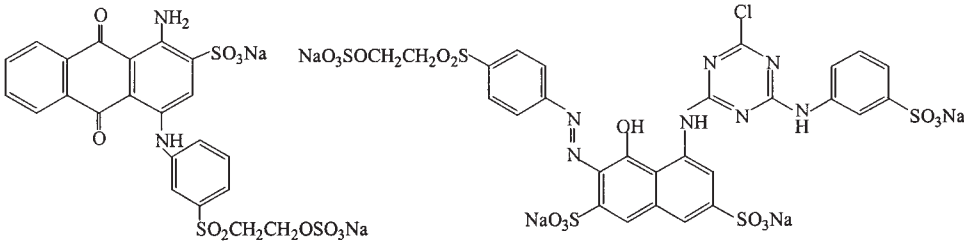
dard hydrogen electrode, a correction factor of +220 mV must be added to the reported measurements. Total gas production, CH_4, CO_2, VFA, and total carbon were measured following previously reported procedures *(9,16,17)*. Low levels of H_2 were measured using a gas chromatography unit equipped with a reduction gas analyzer (Trace Analytical, Sparks, MD). All spectrophotometric analyses were carried out using a UV/V_{IS} HP model 8453 spectrophotometer equipped with a diode array detector (Hewlett-Packard, Palo Alto, CA). Absorbance scans were carried out at a wavelength range of 300–800 nm, and the concentration of the dyes was quantified based on sample absorbance at the maximum wavelength (591, 669, and 516 nm for RB 19, RB 21, and RR 198, respectively) and previously prepared calibration curves. Dye concentration measurement of the dyebath was based on absorbance measurements at 591 nm and concentrations are reported as RB 19 equivalents. To assess the effect of sample exposure to air during sample preparation and absorbance measurements, liquid samples from the dye-amended cultures were centrifuged and tested with and without ascorbic acid (250 mg/L to prevent sample oxidation) after 1-h air bubbling. No significant difference in the absorbance between ascorbic acid–amended and unamended samples was observed, indicating that dye reoxidation under aerobic conditions was not significant. All spectrophotometric analyses reported here were conducted without the addition of ascorbic acid.

Results and Discussion

Dyebath and Dye Characterization

Figure 1 shows the chemical structures of the three dye components of the industrial dyebath: RB 19, an anthraquinone dye with a vinyl sulfonyl reactive group; RB 21, a phthalocyanine dye with a vinyl sulfonyl reactive group; and RR 198, a monoazo, bifunctional dye with both a vinyl sulfonyl and a monochlorotriazinyl reactive group. The spectra of the spent reactive dyebath and its three dye components are shown in Fig. 2. Above a wavelength of 400 nm, the spectra of the spent dyebath, simulated dyebath, and RB 19, all at the same matrix and at an equivalent dye concentration of 100 mg/L (expressed as RB 19), were very similar, further indicating that the characteristics of the spent dyebath are predominantly determined by RB 19, its major dye component. Absorbance scans of spent dyebath solutions at pH 5.0, 7.0, and 12.3 (original, unadjusted dyebath pH) resulted in almost identical absorbance readings, especially above a wavelength of 400 nm (data not shown). Table 1 shows the results of characterization of the plant spent dyebath, as well as its three dye components. As stated, RB 19 is the major dye component of the industrial dyebath. Based on the initial dye concentration used in the dyeing operation and that found in the resulting spent dyebath, a dye exhaustion of 84% was achieved in this dyeing process, which is typical of reactive dyeing *(1,3,4)*.

Reactive Blue 19 **Reactive Red 198**



Reactive Blue 21

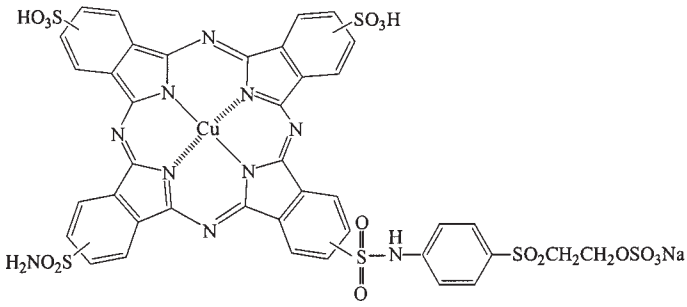


Fig. 1. Chemical structures of three dye components of industrial dyebath.

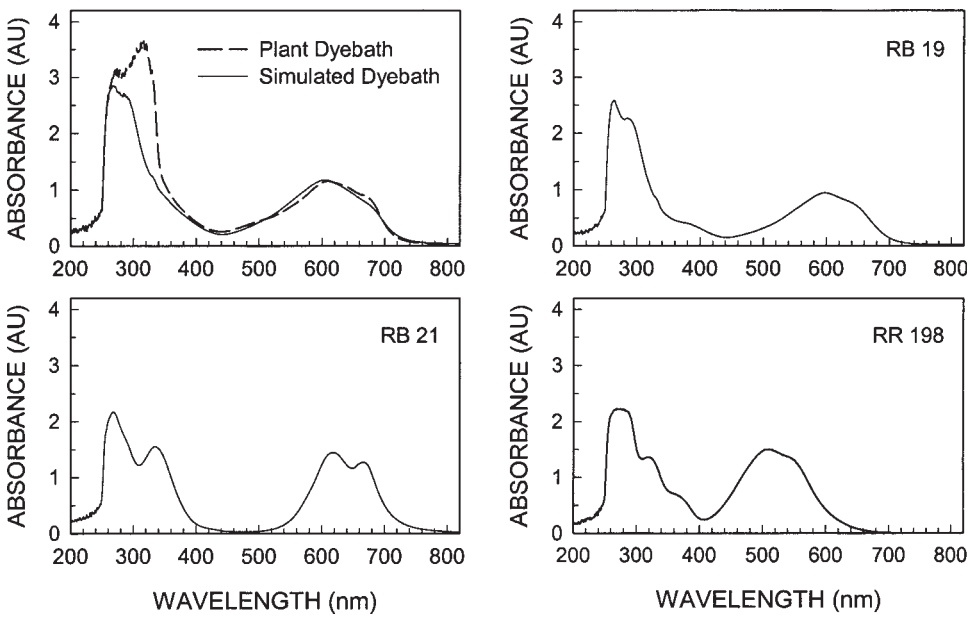


Fig. 2. Spectra of plant spent dyebath, simulated dyebath, and its three dye components: RB 19, RB 21, and RR 198 (all at 113, 3.0, and 0.95 g/L of NaCl, Na_2CO_3, and NaOH, respectively; 100 mg/L dye concentration; and pH 11.5).

Table 1
Results of Characterization of a Textile Spent Reactive Dyebath and Its Three Dye Components

Parameter	Dye-bath	RB 19	RB 21	RR 198
Dye type	NA[a]	Anthraquinone	Phthalocyanine	Azo
λ_{max} (nm)	591	591	621/669	516
Dye concentration (mg/L)	1032[b]	NA	NA	NA
pH	12.3	5.6[d]	6.3[d]	5.6[d]
Alkalinity (mg $CaCO_3$/L)	3173	NA	NA	NA
Carbon content	1186[c]	42.6[e]	19.3[e]	11.7[e]
COD (mg/L)	4670	1410[f]	696[f]	610[f]
TS (g/L)	108.4	NA	NA	NA
VS (g/L)	2.3	NA	NA	NA

[a]NA, not applicable.
[b]Based on absorbance measurement and expressed as RB 19 equivalent.
[c]Milligrams of C/L in plant dyebath.
[d]Unreacted, 50 mg/L dye solution in deionized water.
[e]Percent dry weight.
[f]COD value of a 1000 mg/L unreacted dye solution in deionized water.

Decolorization of Plant Spent Reactive Dyebath

In this assay, the rate and extent of decolorization, as well as the toxicity of the plant spent dyebath were compared to that of RB 19. Incubation lasted for about 32 d. The rate and extent of decolorization of the plant spent dyebath were very similar to those of RB 19 (Table 2, Fig. 3), as expected, because RB 19 is the major dye component of the industrial dyebath. It is noteworthy that during the initial stages of the decolorization assay, removal of RB 19 was facilitated by dye association with biomass, as evidenced by the blue pellet after centrifugation of culture samples. However, with prolonged incubation time, the biomass-associated dye was reductively transformed, leading to decolorization. Color removal by biomass association was less noticeable for Procion Blue MX-R (C.I. Reactive Blue 4 [RB 4]), another reactive anthraquinone dye tested in a similar study under methanogenic conditions *(17)*. The main difference between these two dyes is in the reactive group (dichlorotriazinyl for RB 4 and vinyl sulfonyl for RB 19). Fast color removal attributed to biomass association has also been observed in our laboratory for reactive phthalocyanine dyes with a vinyl sulfonyl reactive group, such as the RB 21, which is a secondary dye component of the industrial dyebath used in the present study. Similar results, related to biomass-associated color removal, have been reported by Panswad and Luangdilok *(19)* for two anthraquinone reactive dyes, RB 19 and Reactive Blue 5.

Reactive anthraquinone dyes with sulfonic acid groups are mainly used for green, blue, or violet hues. The color of anthraquinone dyes is partially associated with the anthraquinone nucleus and modified by the

Table 2
Results of Bioassay Comparing Decolorization and Toxicity of Plant Spent Dyebath and Reacted RB 19 Dye to Control Culture Under Methanogenic Conditions

	Methanogenic culture		
Parameter	Control	RB 19	Dyebath
pH[b]	7.4/7.3	8.8/8.5	7.4/7.2
ORP (mV)[b]	–300/–210	–338/–238	–244/–180
Methane (%)[c]	100.0	1.4	1.3
Extent of color removal (%)	NA[a]	88.8	88.7
Initial decolorization rate (mg/[L·h])	NA	10.8	10.6
VFA (m*M*)[d]			
Acetic	0.1	5.0	4.6
Propionic	ND[a]	2.7	2.1
iso-Butyric	ND	0.1	0.1
n-Butyric	ND	0.1	0.1
iso-Valeric	ND	0.2	0.2
Total	0.1	8.1	7.1

[a]NA, not applicable; ND, not detected.
[b]Initial/final values after 32 d of incubation.
[c]Expressed as a fraction of methane produced by the control (i.e., culture without dye amendment).
[d]After 32 d of incubation.

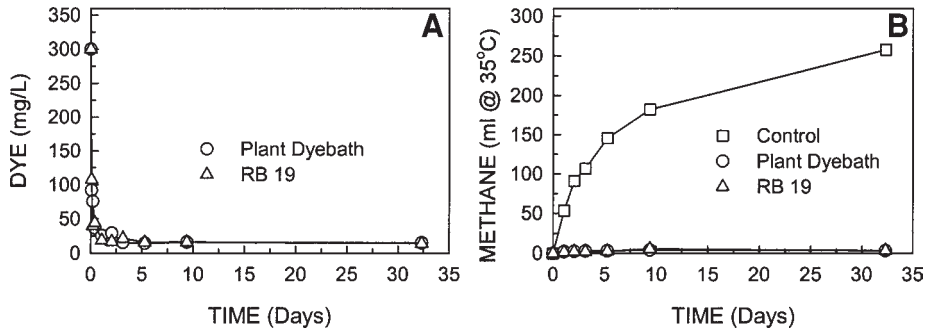


Fig. 3. **(A)** Dye concentration profiles and **(B)** methane in methanogenic cultures amended with plant spent dyebath and RB 19 (control: culture without any dye or dyebath).

type, number, and position of substituents *(4)*. Unsubstituted anthraquinone is pale yellow and has a weak band at about 405 nm, owing to an $n \rightarrow \pi^*$ transition. Electron-withdrawing substituents have little influence on the anthraquinone spectrum. By contrast, electron-donating substituents (e.g., OH^-, $–NH_2$), especially in the α-positions, have bathochromic effects owing to a charge transfer band involving the electron lone pair of hydroxyl or amino groups. The reduction of anthraquinone and its derivatives takes

place by the well-known mechanism of reversible quinone reduction to hydroquinone in a two-step reaction scheme as follows *(4)*:

$$\text{Benzoquinone} \xrightarrow{H^+ + e^-} \text{Semiquinone} \xrightarrow{H^+ + e^-} \text{Hydroquinone}$$

Likewise, in aprotic media, anthraquinone is reduced through the anthrasemiquinone radical anion ($AQ^{\bullet -}$) to the dihydroxyanthracene dianion (AQ^{-2}). In protic media (e.g., water), dihydroxyanthracene (AHQ) is formed *(20,21)*. Therefore, the decolorization of reactive anthraquinone-based dyes is achieved by the reductive transformation of the anthraquinone moiety to the stable AHQ. The rate and extent of decolorization of anthraquinone dyes by *Bacillus subtilis* cultures followed a descending series relative to their substituents: dihydroxy > aminohydroxy > aminomethyl > diamino *(22)*. In general, under anaerobic conditions, a lower rate and extent of decolorization of anthraquinone dyes have been observed compared with azo dyes *(19,23,24)*.

Very low methane production was observed in both the dyebath– and RB 19–amended cultures compared with the control culture (Table 2, Fig. 3). Acetic acid was the only VFA detected in the control culture. By contrast, much higher VFA concentrations, mainly acetic and propionic, accumulated in the dyebath– and RB 19–amended cultures, compared with the control culture (i.e., culture without any dye amendment) (see Table 2). At the end of the 32-d incubation period, the control culture had produced 258 mL of CH_4 (at 35°C and 1 atm) and only 0.1 m*M* acetate was detected. In contrast to the control culture, the dyebath– and RB 19–amended cultures were severely inhibited (i.e., methane production was less than 2% of the control), and VFA accumulated to 7.1 and 8.1 m*M*, respectively, mainly in the form of acetate and propionate, and traces of *iso*-butyric, *n*-butyric, and *iso*-valeric (Table 2). Based on electron equivalent calculations, the accumulation of VFA in the dyebath– and RB 19–amended cultures corresponds to about 35 and 41% of the methane produced by the control culture, respectively. Thus, RB 19 and the spent dyebath inhibited both the acidogenic and methanogenic populations in the mixed cultures, although the highest inhibitory effect was on the methanogens. As discussed later, NaCl at 30 g/L (equivalent to the diluted dyebath salt content tested) also inhibited the mixed methanogenic culture, but to a lesser degree than either the simulated dyebath or RB 19 at an initial dye concentration of 300 mg/L without any salt addition. Based on the similarity of the RB 19– and dyebath–amended cultures, it is concluded that the toxicity of the dyebath is at least partially owing to the RB 19 dye component. In a previous study conducted with the same mixed methanogenic culture, RB 19 and RB 4—another reactive anthraquinone, dichlorotriazinyl dye—inhibition of the acidogenic and, to a larger extent the methanogenic populations was observed at an initial dye concentration as low as 50 mg/L *(17)*.

Inhibition of methanogenic and other anaerobic cultures by anthraquinone dyes and anthraquinone derivatives has been reported by other

researchers. Malpei et al. *(24)* reported 78.9 and 59.6% inhibition of the specific methane yield and production via acetoclastic methanogenesis for a mixed methanogenic culture amended with 250–300 mg/L of Brilliant Red Resolin BLS, an anthraquinone dispersed dye. Panswad and Luangdilok *(19)* reported that addition of two anthraquinone dyes (RB 19 and Cibacron Blue CR, i.e., C.I. Reactive Blue 5) to an anaerobic/aerobic sequencing batch reactor at an initial concentration of 20 mg/L resulted in 53 and 41% removal of phosphorus, respectively. Two other reactive dyes, Remazol Black B (C.I. Reactive Black 5, an azo dye) and Procion Blue H-EGN (C.I. Reactive Blue 198, an oxazine dye) tested at an initial dye concentration of 20 mg/L resulted in 78 and 96% removal of phosphorus, respectively. The anthraquinone dyes had a negative effect on both phosphorus release and subsequent uptake. Cooling et al. *(25)* reported that anthraquinone and anthraquinone derivatives inhibited respiratory sulfate reduction in pure cultures of sulfate-reducing bacteria, in enrichment cultures developed from a large number of freshwater and salt marsh sediment samples, as well as in wastewater and sludge samples collected from two municipal wastewater treatment facilities. The I_{50} values (i.e., the concentration of compounds resulting in 50% inhibition of sulfate reduction) ranged from 0.5 to >18 μ*M*. An increased degree of substitution of the anthraquinone nucleus and addition of charged ring substituents resulted in an increased I_{50} value, i.e., a lower degree of inhibition. Uncoupling of adenosine triphosphatase synthesis from electron transfer reactions was suggested as the underlying mechanism of inhibition by anthraquinone and its derivatives. Inhibition of methanogenesis in mixed enrichment cultures derived from rumen or anaerobic digester samples by 1,8-dihydroxyanthraquinone and accumulation of H_2 was also reported *(25)*.

In spite of the high degree of inhibition by both the plant dyebath and RB 19 on the mixed methanogenic culture used in the present study, a high degree of color removal was achieved by both cultures. Continued decolorization in spite of the observed culture inhibition indicates that maintenance of reduced conditions was sufficient for color removal for only one dye addition. A relatively high (above 96%) extent of color removal was achieved by the same mixed methanogenic culture amended three consecutive times with an initial RB 19 concentration of 300 mg/L *(17)*.

Decolorization of a Simulated, Composite Reactive Dyebath and Its Dye Components

Another batch assay was conducted to assess the decolorization as well as potential toxic effects of a simulated, composite dyebath and its individual component dyes. The individual dyes and the composite dyebath were tested at an initial equivalent dye concentration of 300 mg/L. Incubation lasted for about 15 d. Data on this decolorization assay including the initial volumetric decolorization rate and extent of color removal are given in Table 3. Figure 4A shows the color profiles during the incubation period of the second decolorization assay. Both the RB 19 and the simulated

Table 3
Results of Bioassay Comparing Decolorization and Toxicity of Simulated, Composite Dyebath and Its Three-Component, Reacted Dyes to Control Culture Under Methanogenic Conditions

	Methanogenic culture[a]				
Parameter	Control	RB 19	RB 21	RR 198	Composite
pH[b]	7.4/7.3	7.8/7.6	7.8/7.6	7.8/7.7	7.5/7.5
ORP (mV)[b]	–273/–167	–232/–128	–237/–180	–250/–150	–224/–129
Methane (%)[c]	100.0	0.9	83.3	96.0	1.3
Extent of color removal (%)	NA[a]	92.5	79.9	95.0	93.0
Initial decolorization rate (mg/[L·h])	NA	12.6	8.6	11.6	13.2
VFA (m*M*)[d]					
Acetic	0.1	3.3	ND	ND	2.8
Propionic	ND[a]	2.6	ND	ND	2.5
n-Butyric	ND	0.2	0.2	0.3	0.4
iso-Valeric	ND	0.1	ND	ND	0.1
Total	0.1	6.2	0.2	0.3	5.8

[a]NA, not applicable; ND, not detected.
[b]Initial/final values after 15 d of incubation.
[c]Expressed as a fraction of methane produced by the control (i.e., culture without dye amendment).
[d]After 15 d of incubation.

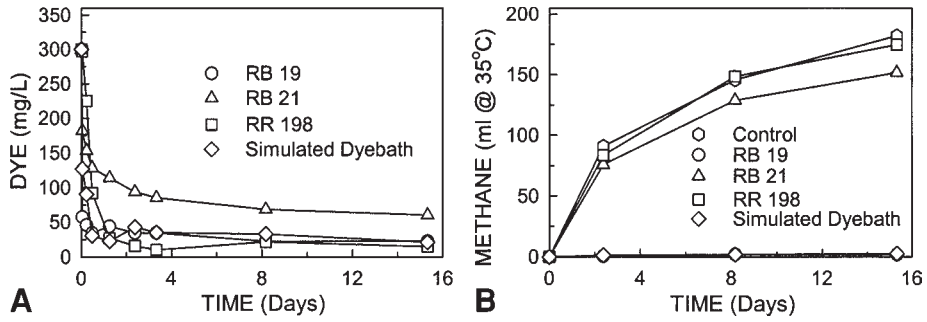


Fig. 4. **(A)** Dye concentration profiles and **(B)** methane in methanogenic cultures amended with a composite, simulated dyebath and its three component dyes (control: culture without any dye or dyebath).

dyebath had a relatively high initial rate and extent of decolorization, very similar to those observed during the first decolorization assay conducted with the plant dyebath at an initial RB 19 equivalent concentration of 300 mg/L (see Fig. 3A and Table 2). The azo dye (RR 198) had the greatest extent of color removal (95%), which is consistent with results obtained in our laboratory with the same and other reactive azo dyes *(9,12,26)*.

The Cu-phthalocyanine dye (RB 21) proved to be the most resistant to biological decolorization, resulting in about an 80% extent of decolorization, again corroborating previously reported results of biological decolorization of another reactive Cu-phthalocyanine dye, Cibacron Turquoise G-E (C.I. Reactive Blue 7 [RB 7]), using the same mixed methanogenic culture *(16)*. Phthalocyanines have a range of hues limited to shades of greenish blue. Metal complex formation does not alter the hue of phthalocyanine significantly. Because of the fluidity of the electrons and ring current around the phthalocyanine molecule, it is difficult to determine exactly which part of the phthalocyanine molecule conveys the color *(4)*. Relatively slow and partial bacterial decolorization of phthalocyanine dyes has been reported, owing to their chemical stability and resistance to biotransformation *(11,16,24,27,28)*. By contrast, studies conducted with fungi, mainly white-rot fungi, have achieved a relatively higher extent of decolorization of phthalocyanine dyes *(14,29–33)*. In addition, fungal cultures have achieved at least partial biotransformation of phthalocyanine dyes. Two sulfophthalimide isomers were identified as the major breakdown products of two reactive phthalocyanine dyes, C.I. Reactive Blue 15 and C.I. Reactive Blue 38 *(34)*, and were further transformed to 3- and 4-sulfophthalamic acid by chemical hydrolysis under aerobic conditions *(35)*. Further degradation and mineralization of 4-sulfophthalamic acid was achieved with a mixed, activated sludge culture, and the 3-sulfophthalamic acid remained stable over the 35-d incubation period. Although reduction of phthalocyanine dyes leads to at least partial decolorization, the exact mechanism is not well understood.

Both the simulated dyebath– and the RB 19-amended cultures exhibited significantly lower methane production, compared with the control culture. Similar to the results obtained in the previous batch decolorization assay, the anthraquinone dye (RB 19) was highly toxic to the methanogenic cultures. The culture amended with the azo dye (RR 198) did not affect the rate and extent of methane production (Table 3, Fig. 4B). The extent of methane production of the Cu-phthalocyanine-amended culture within the 15-d incubation period was about 83% of that produced by the control culture, and very low levels of VFA were detected. Therefore, the decreased methane production, relative to the control culture, is attributed to the inhibition of the hydrolytic/fermentative microbial populations as opposed to inhibition of the methanogenic population. Similar results were previously obtained with another Cu-phthalocyanine monochlorotriazinyl reactive dye (RB 7) tested at an initial dye concentration of 300 mg/L with the same mixed methanogenic culture *(16)*.

To delineate and separate the effect of the spent dyebath salt from that of the dye components on the activity of the mixed methanogenic culture, another batch assay was conducted following the previously described procedures for the decolorization assays. Three sets of cultures were prepared: one without any dye, one with a simulated dyebath, and one with RB 19. Each set included two cultures: one with 30 g/L of NaCl (equivalent to the diluted dyebath salt as tested in our study), and one without any salt. The initial dye concentration in the dye- and dyebath–amended cultures was about 300 mg/L (expressed as RB 19), similar to the initial dye concentration used in the previously presented decolorization assays. Incubation was carried out at 35°C, in the dark, for 43 d. Data on this decolorization assay are given in Table 4. The RB 19 and simulated dyebath decolorization data are shown in Fig. 5A. A very similar rate and extent of decolorization was observed, regardless of the presence or absence of NaCl for both the RB 19– and the dyebath–amended cultures. In addition, both the initial rate and the extent of decolorization were very similar for the RB 19– and the dyebath–amended cultures. Figure 6 shows the dye and dyebath spectra as a function of incubation time for the salt-amended, RB 19– and dyebath–amended cultures. Very similar spectra were obtained with the RB 19– and dyebath–amended cultures in the absence of salt (data not shown). The striking similarity of the spectra of the four dye- and dyebath–amended cultures demonstrates that the presence of NaCl did not affect the decolorization process. Furthermore, the dominance of RB 19 on the spectra of the composite dyebath is apparent.

Methane production was completely inhibited in all cultures amended with RB 19 or simulated dyebath, regardless of the presence or absence of NaCl (Fig. 5B). It is noteworthy that the NaCl-amended control culture produced about 49% of the methane observed in the NaCl-free control culture at 43 d of incubation. Although NaCl at 30 g/L resulted in a significant inhibition of both the acidogenic and methanogenic populations, the deleterious effect of RB 19 at an initial concentration of 300 mg/L is appar-

Table 4
Gas Composition and VFA in Control and in Cultures Amended with Simulated Dyebath and RB 19, with and without NaCl[a]

	Methanogenic culture					
	Control		Dyebath		RB 19	
Parameter	Without salt	With salt	Without salt	With salt	Without salt	With salt
Gas composition						
Methane (%)	53.9	34.9	0.0	0.0	0.0	0.0
Carbon dioxide (%)	21.0	22.1	24.4	26.3	25.4	34.0
Hydrogen (atm)	1.3×10^{-5}	2.2×10^{-5}	1.3×10^{-3}	3.9×10^{-3}	1.6×10^{-3}	4.9×10^{-3}
VFA (m*M*)						
Acetic	ND[b]	ND	6.61	6.22	6.67	6.25
Propionic	ND	3.15	4.18	2.32	4.60	2.56
iso-Butyric	ND	0.10	0.11	0.04	0.12	0.03
n-Butyric	ND	0.04	0.09	0.11	0.10	0.12
iso-Valeric	ND	0.16	0.17	0.09	0.19	0.07
n-Valeric	ND	0.03	0.02	ND	ND	ND
Total	ND	3.48	11.18	8.78	11.68	9.03
COD processed (%)[c]						
VFA	0	32	74	54	79	56
Methane	100	49	0	0	0	0
Total	100	81	74	54	79	56

[a]After 43 d of incubation.
[b]ND, not detected.
[c]Normalized to the total COD processed (i.e., VFA- and CH_4-COD measured at 43 d of incubation) by the control culture without salt addition.

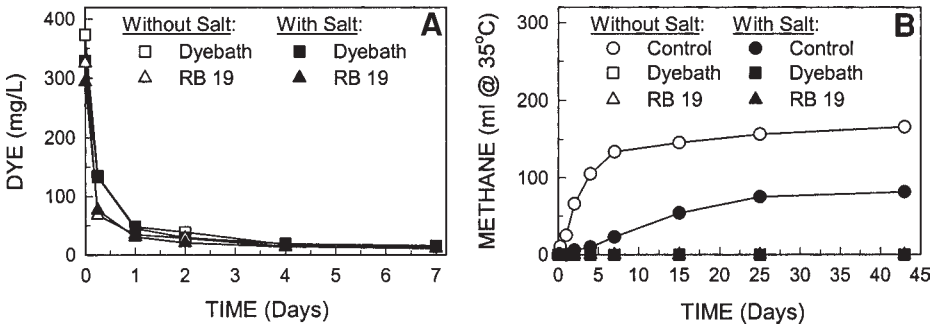


Fig. 5. **(A)** Dye concentration profiles and **(B)** methane in methanogenic cultures amended with a composite, simulated dyebath and RB 19 with and without NaCl (control: culture without any dye or dyebath).

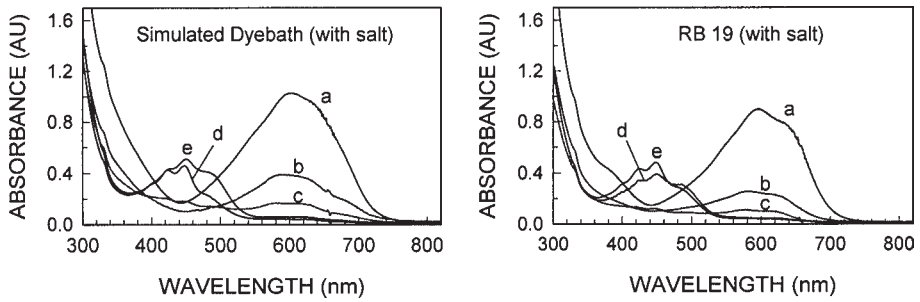


Fig. 6. Spectra of simulated dyebath and RB 19 in methanogenic cultures with NaCl addition as a function of incubation time (a, 0; b, 0.25; c, 1; d, 4; and e, 7 d) (Similar spectra were obtained for the simulated dyebath and RB 19 without NaCl addition.)

ent from these data (Fig. 5, Table 4). Furthermore, at the end of the 43-d incubation, the H_2 concentration in the two control cultures with and without salt addition was very low and similar. By contrast, the two dyebath– and two RB 19–amended cultures had an H_2 concentration two orders of magnitude higher compared with the two control cultures, regardless of salt addition (Table 4). Overall, these results confirm that, in addition to NaCl and regardless of its inhibitory effect, the toxicity of the industrial dyebath is at least partially associated with its RB 19 dye component. Earlier studies on the inhibitory effect of NaCl on mixed methanogenic consortia concluded that NaCl in the range of 27–30 g/L could be tolerated, and gradual adaptation to increasing salt concentrations led to an acclimated anaerobic population that could withstand NaCl concentrations of up to 65 g/L *(36,37)*. Based on these reports and the present study data, the inhibitory effect of the anthraquinone dyes is more severe than that of the dyebath salt.

Toxicity of RB 19 Decolorization Products

This assay lasted for about 7 d. The pH and ORP values in all cultures ranged from 7.3 to 7.4 and from –375 to –398 mV, respectively. Methane

Table 5
Methane and VFA Production by Control and by Cultures Amended with Different Levels of RB 19 Decolorization Products[a]

	Cultures amended with RB 19 decolorization products (mg/L)[b–d]					
Parameter	0[e]	50	300	500	1000	1500
Methane (%)[f]	100 (100)	95.8 (63.4)	95.8 (3.2)	87.5 (1.0)	16.7 (0.8)	14.7 (0.7)
VFA (m*M*)						
Acetic	0.1	ND	ND	0.8	4.5	5.8
Propionic	ND[c]	ND	ND	ND	3.8	4.8
iso-Butyric	ND	ND	ND	ND	0.1	0.2
iso-Valeric	ND	ND	ND	ND	0.3	0.3
Total	0.1	ND	ND	0.8	8.7	11.1

[a]After 7.3 d of incubation.
[b]Expressed as RB 19 equivalent.
[c]ND, not detected.
[d]Data in parentheses are for methanogenic cultures amended with RB 19.
[e]Control culture.
[f]Expressed as a fraction of methane produced by the control (i.e., culture without amendment of RB 19 decolorization products).

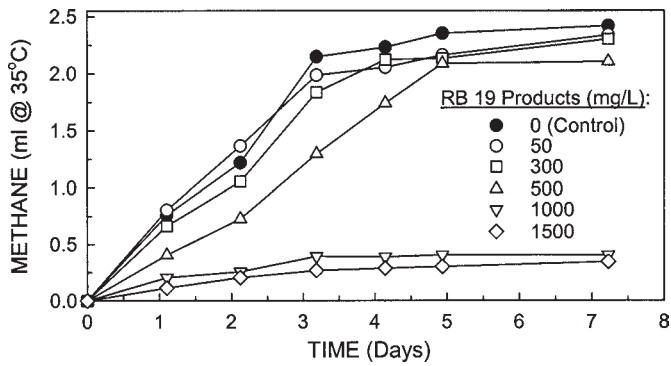


Fig. 7. Methane production in cultures amended with different concentrations of RB 19 decolorization products (expressed as RB 19 equivalent), as compared to control culture.

production and VFA concentration data after about 7 d of incubation are shown in Table 5. The methane production over the incubation period is shown in Fig. 7. RB 19 decolorization products up to an RB 19 equivalent concentration of 500 mg/L did not affect VFA and methane production significantly. At initial concentrations of RB 19 decolorization products equivalent to 1000 and 1500 mg/L of RB 19, accumulation of VFA occurred, but at levels much lower than those observed in cultures amended with equivalent RB 19 concentrations (Table 5). Therefore, the methane production data coupled with the decrease in VFA accumulation provide strong evidence that the RB 19 decolorization products are much less inhibitory to

the mixed methanogenic culture than the parent dye. Similar to these results, decolorization of two anthraquinone dyes, RB 19 and C.I. Acid Blue 225, under aerobic conditions by an immobilized laccase from *Trametes hirsuta* resulted in 84.4 and 78% detoxification, respectively, as determined based on the oxygen consumption rate of *Pseudomonas putida (38)*.

Conclusion

Microbial decolorization of a plant spent dyebath and its reactive dye components (anthraquinone RB 19; azo RR 198; and, to a lesser degree, Cu-phthalocyanine RB 21) was achieved by a mixed methanogenic culture at an initial dye concentration of 300 mg/L. Dye reduction and decolorization were not reversible under oxic conditions. The dyebath, as well as its predominant dye, RB 19, were inhibitory to the acidogenic and, to a larger degree, the methanogenic populations. A much lower degree of inhibition was observed in cultures amended with RB 21. By contrast, RR 198 did not inhibit the mixed methanogenic culture. NaCl at 30 g/L (equivalent to the diluted dyebath salt content tested) also inhibited the mixed methanogenic culture, but to a lesser degree than either RB 19 or the simulated dyebath at an initial dye concentration of 300 mg/L without any salt addition. Therefore, the observed toxicity of the dyebath is at least partially owing to its RB 19 dye component. RB 19 decolorization products up to an RB 19 equivalent concentration of 500 mg/L did not inhibit the acidogenic and methanogenic populations of the mixed culture. Therefore, the RB 19 decolorization products are much less inhibitory than the parent dye.

Fast initial dye removal attributed to dye association with the biomass, followed by dye reductive transformation, was observed in cultures amended with RB 19 and the dyebath. Color removal occurred despite the severe culture inhibition. Continued decolorization, in spite of the observed culture inhibition, indicates that maintenance of reduced conditions was sufficient for color removal. In view of the observed severe culture inhibition in the batch assays conducted in the present study, which is attributable to both the anthraquinone dye and NaCl, biological decolorization of full-strength, spent reactive dyebaths using methanogenic cultures in a repetitive, closed-loop system is not deemed feasible. For this reason, a fermentative and halotolerant culture has been developed and successfully used in our laboratory for the decolorization of industrial reactive dyebaths with 100 g/L of NaCl.

Acknowledgments

We thank D. K. Durden and A. B. Young, Washington Manufacturing (formerly SCT Yarns), Washington, GA, for providing the spent reactive dyebath and technical advise as the project's industrial partners. This work was initially supported by the US Department of Commerce/National Textile Center and the State of Georgia/Consortium on Competitiveness for the Apparel, Carpet, and Textile Industries. This study is based on work

supported in part by the US National Science Foundation under grant no. BES-0114169. Any opinions, findings, and conclusions or recommendations expressed in this text are those of the authors and do not necessarily reflect the views of the US National Science Foundation.

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