Microbial decolourisation of a reactive azo dye under anaerobic conditions

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Abstract

Water-soluble azo dyes are used extensively in the textile industry and are known to be problematic with respect to the removal of colour from textile waste waters. Under anaerobic conditions azo dyes can be utilised as terminal electron acceptors in microbial respiration, and are reduced and decolourised concurrently with re-oxidation of reduced flavin nucleotides. The microbial decolourisation of an azo dye (C.I. Reactive Red 141) was investigated with respect to the kinetic order of azo reduction and rate-controlling factors of the reaction. Decolourisation of C.I. Reactive Red 141 was found to be first order with respect to dye concentration, although increasing the initial dye concentration in the serum bottles resulted in decreasing k values of -0.441/h (100 mg/ℓ of C.I. Reactive Red 141), -0.316/h (150 mg/ℓ) and -0.252/h (200 mg/ℓ). The presence of labile carbon in the anaerobic system was found to be essential in order to obtain an acceptable rate of decolourisation. The k value obtained for decolourisation of the azo dye without a supplemental carbon source (glucose) was -0.012/h, in comparison to a k value of -0.441/h when supplemented with glucose (1 g/ℓ). The presence of nitrate in the anaerobic system was found to inhibit decolourisation, while the presence of sulphate was found to have no discernible effect on the rate of decolourisation. A low redox potential (-450 to -500 mV) was found to be conducive to rapid decolourisation of C.I. Reactive Red 141. A C.I. Reactive Red 141 degradation product was positively identified as 2-aminonaphthalene-1,5-disulphonic acid, confirming that azo reduction was responsible for decolourisation of the azo dye. A toxicity assay was performed which showed that C.I. Reactive Red 141 was inhibitory to the anaerobic microbial community at concentrations >100 mg/ℓ, but that prior exposure of the biomass to the dye increased the resistance to previously inhibitory dye concentrations.

Introduction

Azo dyes account for 60 to 70% of all textile dyestuffs produced and are the most common chromophore of reactive textile dyes. Colouration of textile effluents (in particular red hues) can usually be linked to the presence of water-soluble (reactive) azo dyes in the waste water. It is generally accepted that the aerobic biological processes at conventional treatment works do not substantially decrease the colouration of these effluents, usually resulting in colour contamination of the receiving water body. It is also well known that substantial decolourisation of azo dyes occurs by reduction of the azo bonds and subsequent destruction of the dye chromophore/s, a process that was linked to the activity of anaerobic micro-organisms as early as 1911 (Meyer, 1981). Thus, although aerobic processes are traditionally used for the treatment of high volume liquid effluents, the potential of anaerobic applications should be explored in the field of textile effluent treatment.

A review of the literature shows that research into anaerobic microbial azo reduction was initiated by concern over metabolic products resulting from the reduction of azo food dyes in the mammalian intestine. For this reason, much of the early research focused on the mechanism of azo reduction by intestinal micro-organisms, whereas later studies tended towards environmental applications. One of the first papers to contain a comprehensive investigation into the mechanism of microbial azo reduction was published by Gingell and Walker (1971), using *Streptococcus faecalis* and the azo dye, Red 2G. The researchers proposed that reduced soluble flavins act as electron shuttles to ferry electrons from the flavoproteins of the microbial electron transport chain to the acceptor azo compound. That is, the azo dye acts as an oxidising agent for the reduced flavin nucleotides of the electron transport chain and is reduced and decolourised concurrently with re-oxidation of the reduced flavin nucleotides. Dubin and Wright (1975), investigating the reduction of various azo food colourants by *Proteus vulgaris*, expanded this theory to include a rate-controlling step which involved a redox equilibrium between the dye and an extracellular reducing agent, with the site of reduction being extracellular. That is, the specific reduction potential of the dye was proposed to be the principal rate-limiting factor in azo reduction. Subsequent research by Yatome et al. (1991) showed that the rate of azo reduction was not limited by the specific dye reduction potentials but by the degree of sulphonation of the dyes. These researchers concluded that azo reduction must occur intracellularly, with the rate of permeation of the dye through the cell membrane being the principal rate-limiting factor. Additional evidence of cell permeability as a primary rate-limiting factor in microbial azo reduction was reported by Mechsner and Wuhrmann (1982), who managed to substantially increase the reduction rates of azo compounds by permeabilising bacterial cells prior to azo reduction. Anaerobic decolourisation of azo dyes has also been reported using micro-organisms such as *Bacillus subtilis* (Horitsu et al., 1977), *Bacillus cereus* (Wuhr-mann et al., 1980), *Pseudomonas cepacia* (Ogawa et al., 1986; and Ogawa and Yatome, 1990), *Pseudomonas stutzeri* (Yatome et al., 1990) and *Aeromonas hydrophilia* (Idaka and Ogawa, 1978; Yatome et al., 1987).

Although the mechanism of microbial azo reduction has been investigated and reported in the literature, many questions arise about the application of this process for the decolourisation of textile effluent. Some salient concerns are the fate and effect of the

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dye metabolites on an anaerobic treatment system, and the physical and nutritional requisites of such a treatment system. A study of microbial azo reduction was undertaken using a mixed microbial population and a commercial reactive azo dye, C.I. Reactive Red 141. Decolourisation of the azo dye was investigated under anaerobic conditions and the results of the study are reported in this paper. Initial stages of the investigation focused on determining the kinetic order of azo reduction. From this point rate-limiting factors were identified that would be significant to a waste-water treatment system. Studies were undertaken to identify the degradation products of azo reduction (decolourisation) and to determine whether increasing concentrations of the dye or dye degradation products would have an adverse effect on the anaerobic microbial population.

Materials and methods

A series of experiments were undertaken to define the nature of anaerobic decolourisation. The first experiment aimed to determine the order of decolourisation for C.I. Reactive Red 141 with respect to dye concentration. Subsequently, probable rate-controlling factors were investigated with respect to their effect on decolourisation. In particular, the availability of labile carbon in the treatment system and the presence of additional (possibly competitive) electron acceptors in the system were considered pertinent to the operation of a treatment works. The role of redox potential in azo reduction was investigated when C.I. Reactive Red 141 was present as a sole electron acceptor and when additional electron acceptors (nitrate and sulphate) were added to the system. The inhibitory effects (if any) of C.I. Reactive Red 141 and/or C.I. Reactive Red 141 degradation products on an anaerobic microbial population were investigated using an anaerobic toxicity assay method based on that developed by Owen et al. (1979)

A set of experimental conditions (termed standard assay conditions) were developed for this investigation. These have been presented in detail in Carliell (1993) and are described briefly below. Some amendments were made to the standard assay conditions depending on the requirements of the particular experiment and these are detailed in the relevant sections. All experiments were performed in triplicate unless otherwise stated.

Standard assay conditions

Experimental procedure

Experiments were performed in serum bottles (120 mℓ, Aldrich Chemical Company).

A 30 % (v/v) inoculum was used per serum bottle. The inoculum was obtained from a laboratory anaerobic digester (Digester A) which was initially inoculated with anaerobic biomass from a sewage sludge digester. Digester A was operated for four months at 32°C (unmixed), during which time no additional substrate was added in order to exhaust the sludge of residual organic substrate. Low concentrations of C.I. Reactive Red 141 were added to Digester A during this period. The biomass obtained from Digester A is referred to as previously exposed biomass (PEB), in comparison with biomass that has not been exposed to the dye prior to the measured experiment (non-exposed biomass, NEB).

A mineral salts medium was prepared according to Owen et al. (1979) with the exception of the sodium sulphide solution. Glucose was added to the mineral salts medium to give a concentration of 1 g/ℓ. A volume of 100 mℓ (mineral salts medium plus glucose) was added to each serum bottle. Serum bottles were over-gassed with oxygen-free nitrogen at a flow rate of 0.5 mℓ/min for 15 min and sealed with butyl rubber stoppers and aluminium crimp seals. The serum bottles were pre-incubated for 18 h in a waterbath at 32°C (without shaking). Upon completion of pre-incubation 2 mℓ of C.I. Reactive Red 141 stock solution (5 000 mg/ℓ) was added to each assay bottle by means of a hypodermic needle and glass syringe to give an initial dye concentration of 100 mg/ℓ. The commercially available preparation of C.I. Reactive Red 141 (Zeneca) was used in these experiments.

Analytical procedure

Samples (2 mℓ) were withdrawn from the serum bottles with a hypodermic needle and syringe, centrifuged at 6 500 r/min for 25 min, and analysed for absorbance at the maximum wavelength of absorbance for C.I. Reactive Red 141 (520 nm). Quartz microcuvettes (vol = 1.5 mℓ; path length = 10 mm, Zeiss) and an LKB Biochrom Ultraspec (model 4050) were used. The absorbance figures were converted to the equivalent dye concentrations using a calibration curve of absorbance (520 nm) vs. C.I. Reactive Red 141 concentration (mg/ℓ).

The kinetic order of decolourisation of C.I. Reactive Red 141 with respect to dye concentration

Standard assay conditions were followed for these experiments in which the rate of decolourisation was measured using three concentrations of C.I. Reactive Red 141 (100, 150 and 200 mg/ℓ).

Rate of decolourisation of C.I. Reactive Red 141 when present as a sole carbon source

The rate of decolourisation of C.I. Reactive Red 141 was measured in the presence of an additional carbon source (glucose) as per standard assay conditions and compared with decolourisation rates in serum bottles with no additional carbon source.

Decolourisation of C.I. Reactive Red 141 in the presence of additional electron acceptors

Two oxidising agents (nitrate and sulphate) were added to the assay bottles to assess the effect of additional electron accepting compounds on the reduction of C.I. Reactive Red 141. Experimental bottles were prepared and pre-incubated according to standard assay conditions. The appropriate stock solutions (1 mℓ) were added by syringe to the pre-incubated serum bottles to give initial nitrate concentrations of 1, 5, or 10 mM and initial sulphate concentrations of 5 and 10 mM. Control bottles contained no nitrate or sulphate. C.I. Reactive Red 141 was added to the serum bottles as per standard assay conditions.

Redox potential trends observed during decolourisation of C.I. Reactive Red 141

These experiments required continuous monitoring of the redox potential of an anaerobic system during decolourisation of C.I. Reactive Red 141. As the dimensions of the serum bottles (used in the standard assay) precluded the insertion of an electrode, the following anaerobic system (designated Digester B) was

devised for these experiments. The conditions of Digester B followed those of the standard assay as closely as possible. Digester B consisted of a cylindrical reactor (1 ℓ) and flange (Quickfit) with ground glass ports for insertion of electrodes and collection of samples. A magnetic stirrer was used to mix the contents of the digester. A PHM82 standard Radiometer pH meter, fitted with a combined platinum-calomel redox electrode (PK 1401) was used for continuous redox potential measurements. The electrode was inserted into the digester through a ground glass port which was subsequently sealed. Redox potential readings (mV) were relayed to a PC-based data collection programme. The redox electrode was introduced into the digester after the 18 h pre-incubation period and allowed to stabilise for 30 min, after which one of three experimental routes was followed :

- C.I. Reactive Red 141 solution was added to Digester B to give an initial concentration of 100 mg/ℓ;
- sodium nitrate solution was added to Digester B to give an initial concentration of 20 mM nitrate, together with C.I. Reactive Red 141;
- sodium sulphate solution was added to Digester B to give an initial concentration of 5 mM sulphate, together with C.I. Reactive Red 141.

Digester B was incubated at 32°C with continuous measurement of redox potential (mV). Samples were withdrawn from the digester at regular intervals and the concentration (mg/ℓ) of C.I. Reactive Red 141 was determined.

Identification of the C.I. Reactive Red 141 degradation products remaining after decolourisation

The anaerobic metabolites of C.I. Reactive Red 141 were separated and identified using column chromatography, thin layer chromatography (TLC) and proton nuclear magnetic resonance spectroscopy (NMR). The samples were taken from Digester B when C.I. Reactive Red 141 was present as a sole electron acceptor.

Column chromatography : Liquid samples from Digester B were evaporated to dryness and the residue Soxhlet-extracted into methanol. The extract was concentrated by rotary evaporation and loaded onto a column packed with Merck 9385 silica gel (230 to 400 Å). A solvent system of methanol-methylene chloride with an increasing ratio from 10 to 90% (v/v) was used. Fractions were collected, concentrated by evaporation and analysed by TLC.

TLC : Merck 5554 silica gel aluminium backed F_{254} (20 x 20 mm) precoated (0.2 mm) TLC plates were used. A solvent system of 30% (v/v) methanol-methylene chloride was used. The fractions were spotted on the plate and like fractions identified. These fractions were redissolved in methanol, combined and evaporated to dryness for NMR analysis.

Proton NMR : A Varian Gemini-300 spectrophotometer was used for the analysis. The samples (10 to 20 mg) were dissolved in deuterium oxide (0.75 mℓ) and placed in a sample tube. The tube was suspended in the instrument and spun at 20 r/s. Ambient operating temperatures were used.

Inhibitory effects of C.I. Reactive Red 141 on the anaerobic microbial community

Experimental procedure : Five concentrations of C.I. Reactive Red 141 were chosen for the toxicity assay, namely, 20, 50, 100, 200 and 500 mg/ℓ. Both NEB and PEB were used in this study. The anaerobic toxicity assay was performed in serum bottles with a 30% inoculum, made up to a working volume of 100 mℓ with mineral salts medium (Owen et al., 1979) containing the appropriate dye concentration. Control bottles contained no dye. The bottles were overgassed at a flow rate of 0.5 ℓ/min for 15 min with high purity nitrogen (Fedgas), sealed and 4 mℓ of acetate-propionate solution added by hypodermic needle and syringe to give 75 mg acetate and 26.5 mg propionate per bottle. After 1 h of incubation the pressures inside the serum bottles were equilibrated to atmospheric by insertion of a hypodermic needle through the butyl rubber septa. Gas production resulting from the degradation of acetate and propionate was found to be limited and, therefore, glucose was added to the serum bottles to give an initial concentration of 1 g/ℓ.

Gas production in the serum bottles was monitored daily by insertion of a hypodermic needle and syringe through the butyl rubber septum of each serum bottle. Volume determinations were made by allowing the syringe plunger to move freely and equilibrate between the bottle and atmospheric pressure. Gas was exhausted after each measurement.

Total gas production data were employed to determine the relative rates of metabolism of the feed source among the samples. The maximum rate of gas production was computed for each sample over the same time period and the data normalised by computing ratios between respective rates for samples and the average of the controls. This ratio is designated the maximum rate ratio (MRR), with a value of less than 0.95 suggesting possible inhibition and a value less than 0.9 indicating significant inhibition (Owen et al., 1979).

Results

The kinetic order of decolourisation of CI Reactive Red 141 with respect to dye concentration

The exponential regressions of C.I. Reactive Red 141 (mg/ℓ) vs. time (h), for initial dye concentrations of 100, 150 and 200 mg/ℓ, are plotted in Fig. 1. The exponential curves suggest a first-order

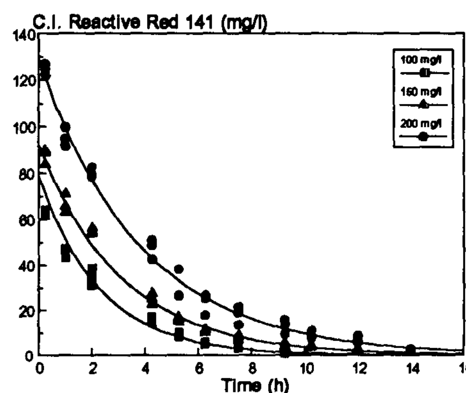

Figure 1

Exponential regression of C.I. Reactive Red 141 (mg/ℓ) with time (h) showing possible first-order reaction with respect to dye concentration. Decolourisation is shown with initial dye concentrations of 100, 150 and 200 mg/ℓ

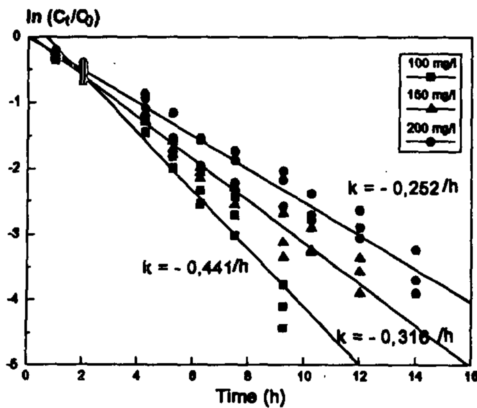

Figure 2
ln (C_t/C_o) versus time (h) is plotted for initial dye concentrations of 100, 150 and 200 mg/ℓ, confirming that C.I. Reactive Red 141 decolourisation is first-order with respect to dye concentration

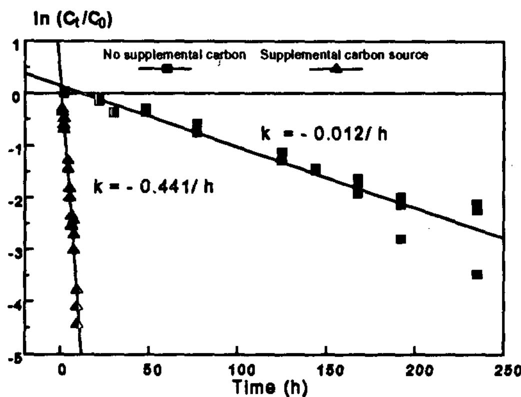

Figure 3
Decolourisation of C.I. Reactive Red 141 (mg/ℓ) in the presence and absence of a supplemental carbon source (glucose, 1 g/ℓ)

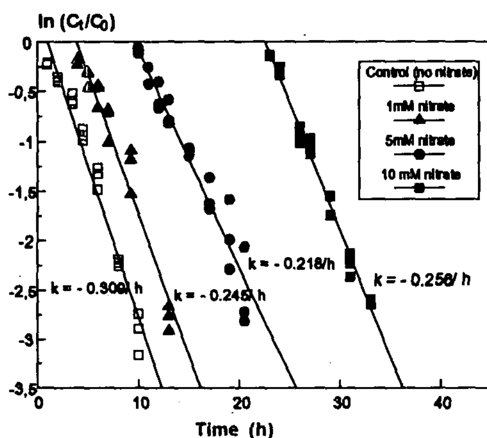

Figure 4
ln (C_t/C_o) versus time (h) is plotted for decolourisation of C.I. Reactive Red 141 in the presence of 0, 1, 5 and 10 mM nitrate. Time zero was taken as the sampling time recorded directly before the onset of decolourisation

relationship of CI Reactive Red 141 decolourisation vs. time with respect to (soluble) dye concentration. When ln (C_t/C_o) (where C_o is the concentration of dye at time zero and C_t the dye concentration at time t) is plotted against time (h), linear relationships are obtained, confirming a first-order relationship of dye decolourisation versus time (Fig. 2). It should be noted, however, that increasing the initial dye concentration in the anaerobic test system resulted in decreasing reaction rates for decolourisation of C.I. Reactive Red 141.

Rate of decolourisation of C.I. Reactive Red 141 when present as a sole carbon source

Figure 3 shows that decolourisation of C.I. Reactive Red 141 could occur in the anaerobic system when the dye was present as the sole carbon source and that this reaction could be related to first-order kinetics. However, this reaction proceeded at a very low rate when compared with the rate of decolourisation in assay bottles containing glucose at 1 g/ℓ. The rate constant (k) measured without glucose was only 2.72 % of that measured in the presence of glucose. No gas production was measured during the time period required for decolourisation (or for 5 days after decolourisation was complete) although gas bubbles were noted in the sludge.

Decolourisation of C.I. Reactive Red 141 in the presence of additional electron acceptors

Decolourisation of C.I. Reactive Red 141 was measured when incubated with an additional electron acceptor in the anaerobic system. Two oxidising agent, viz. nitrate and sulphate, were chosen for this experiment.

Figure 4 shows how the presence of nitrate in the standard assay system inhibits decolourisation of C.I. Reactive Red 141 for a period of time which will be termed the *lag phase*. The duration of these *lag phases* is directly related to the concentration of nitrate in the system, which is well illustrated by comparing the duration of the *lag phase* in the presence of 5 mM nitrate (approximately 12 h) to that in the presence of 10 mM nitrate (approximately 25 h). Thus, a twofold increase in nitrate concentration resulted in a doubling of the *lag phase*, suggesting that nitrate reduction occurs preferentially to reduction of C.I. Reactive Red 141. Once decolourisation of C.I. Reactive Red 141 commences, an exponential relationship of dye concentration vs. time is observed. The highest rate of decolourisation is observed in the control systems, i.e. those containing no nitrate, while the rate constants for the nitrate-containing samples were similar, irrespective of the initial nitrate concentration.

The addition of sulphate to the serum bottles containing C.I. Reactive Red 141 had no marked effect on the rate of decolourisation. The data in Fig. 5 demonstrates that there is no significant difference in reaction rates for the control bottles (no sulphate) and those containing 5 or 10 mM sulphate.

Redox potential trends observed during decolourisation of C.I. Reactive Red 141

The redox potential of an anaerobic digester was monitored during decolourisation of C.I. Reactive Red 141 (100 mg/ℓ) when C.I. Reactive Red 141 was present as the sole electron acceptor, and in the presence of nitrate (20 mM) and sulphate (5 mM). Figure 6 shows the decolourisation of C.I. Reactive Red 141 when the dye was present as a sole electron acceptor. The redox

potential of the anaerobic system was monitored continuously during decolourisation and for approximately 20 h subsequent to decolourisation. The results show that the redox potential of the system decreases from approximately -375 mV (addition of C.I. Reactive Red 141) to approximately -475 mV by the end of the 5 h decolourisation period. A redox potential reading of approximately -475 mV is maintained until 12.5 h of incubation, at which point the redox potential of the system can be seen to increase fairly rapidly to approximately -450 mV. Thereafter the reading remains fairly steady at -450 mV.

Figure 7 shows the redox potentials monitored in Digester B, when C.I. Reactive Red 141 and nitrate were added to this anaerobic system. The redox potential of the system (prior to addition of dye and nitrate) was approximately -475 mV. In the first 5 h subsequent to addition of dye and nitrate to the anaerobic system the redox potential remains below -400 mV, and some decolourisation of the dye is detected. Once the redox potential increases to approximately -375 mV decolourisation appears to be inhibited. The redox potential continues to rise to -225 mV, at which point the readings remain steady (within a range of -225 to -200 mV) for approximately 40 h of incubation. During this time no decolourisation is recorded and it may be speculated that nitrate reduction coincides with this redox potential plateau. After 40 h of incubation the redox potential of the system decreases sharply to reach -450 mV within 3 h. Once the redox potential decreases below -450 mV decolourisation of C.I. Reactive Red 141 is initiated. Decolourisation is achieved in approximately 5 h during which time the redox potential decreases to -500 mV.

Figure 8 shows the change in residual dye concentration and redox potential for an anaerobic system containing C.I. Reactive Red 141 (100 mg/ℓ) and sulphate (5 mM). The redox potential of the system is fairly high (-300 mV) before addition of the dye. Subsequent to addition of C.I. Reactive Red 141 and sulphate the redox potential decreases rapidly to -450 mV. Decolourisation of the dye is completed in approximately 6 h and the redox potential remains steady at -450 mV until 15 h of incubation at which point a sharp decrease in potential is recorded (below -500 mV). An increase in redox potential is recorded after approximately 20 h of incubation and the redox potential of the system remains steady at approximately -475 mV for the next 50 h.

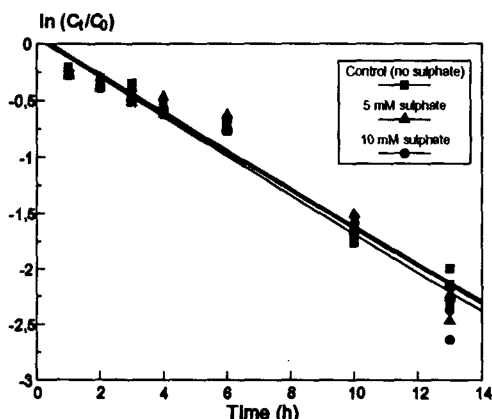

Figure 5
ln (C_t/C_o) versus time (h) is plotted for decolourisation of C.I. Reactive Red 141 in the presence of 0, 5 and 10 mM sulphate

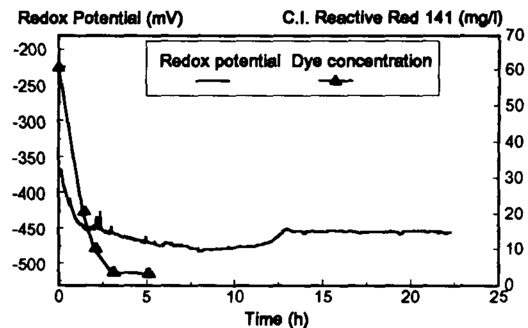

Figure 6
Redox potential (mV) measured in an anaerobic digester during decolourisation of C.I. Reactive Red 141 (approximately 5 h) and for 20 h subsequent to the completion of decolourisation.

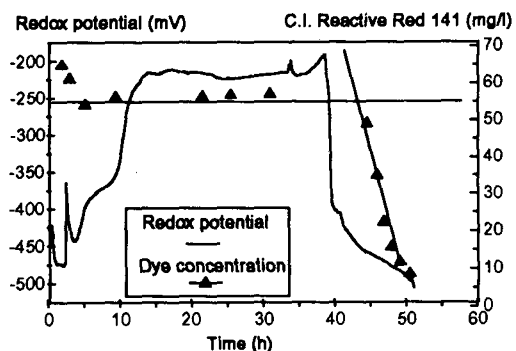

Figure 7
Redox potential (mV) measured in an anaerobic digester containing nitrate (20 mM) and C.I. Reactive Red 141 (100 mg/ℓ)

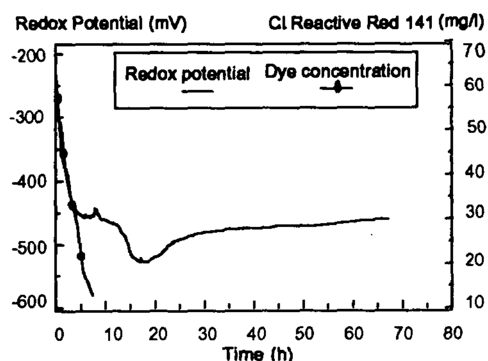

Figure 8
Redox potential (mV) measured in an anaerobic digester containing sulphate (5 mM) and C.I. Reactive Red 141 (100 mg/ℓ)

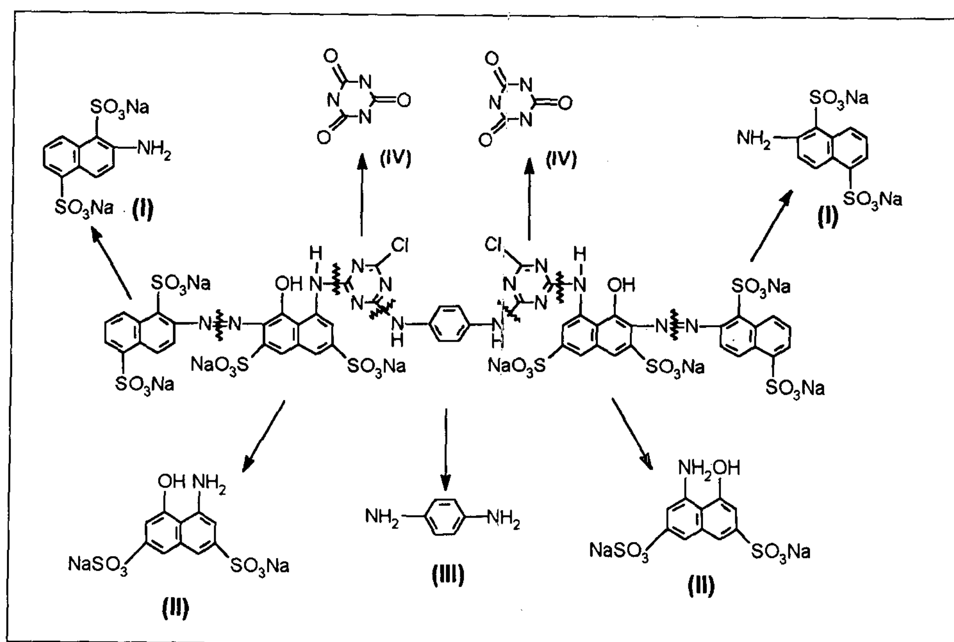

Figure 9
Proposed degradation of C.I. Reactive Red 141 in an anaerobic system

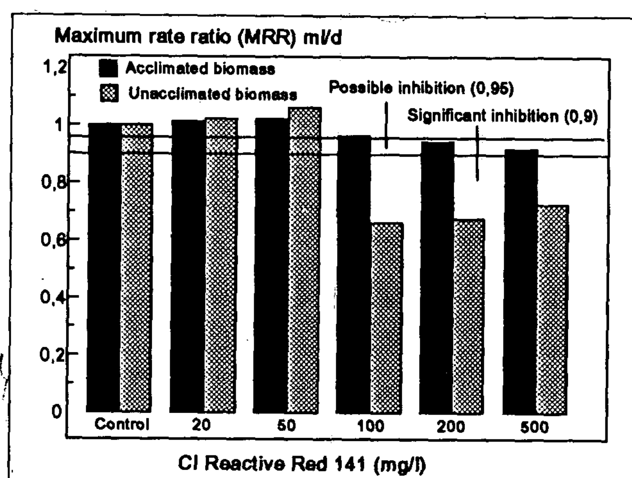

Figure 10
Maximum rate ratios for the anaerobic toxicity assay with C.I. Reactive Red 141

Identification of the C.I. Reactive Red 141 degradation products remaining after decolourisation

Figure 9 shows the structure of C.I. Reactive Red 141 and proposed degradation products resulting from the decolourisation of the dye. These compounds were separated by colomn chromatography and identified using NMR analysis. The identification of fragment (I) as 2-aminonaphthalene-1,5-disulphonic acid confirmed that decolourisation of C.I. Reactive Red 141 does occur as a result of reduction and cleavage of the azo chromophore. A further 2 fragments were identified (1,7-diamino-8-naphtho-3,6-disulphonic acid (II) and *p*-diamino-benzene (III)); however, further investigation is required to establish whether these compounds are degradation products or merely contaminants present in the commercial dye powder. Fragment (IV) was not identified using NMR due to the absence of any hydrogen atoms, but will probably be present as cyanuric acid if compounds (II) and (III) do result from degradation of C.I. Reactive Red 141.

The effect of increasing concentrations of C.I. Reactive Red 141 on the anaerobic microbial community

An anaerobic toxicity assay was performed to determine whether increasing concentrations of C.I. Reactive Red 141 would be inhibitory to anaerobic biomass. Two sources of biomass were used to assess this, PEB and NEB. Figure 10 presents the MRRs calculated for PEB and NEB when incubated with increasing concentrations of C.I. Reactive Red 141.

Concentrations of 20, 50 and 100 mg/ℓ of C.I. Reactive Red

141 are not shown to be inhibitory to the PEB, although higher concentrations of the dye (200 and 500 mg/ℓ) result in MRRs of 0.944 and 0.92 respectively, which indicate possible inhibition of the anaerobic biomass (Owen et al., 1979). However, for serum bottles containing NEB, significant inhibition is noted at concentrations of C.I. Reactive Red 141 above and including 100 mg/ℓ. No inhibititory effects are recorded at the lower dye concentrations. These results, therefore, suggest that prior exposure of the biomass to C.I. Reactive Red 141 increases the resistance of the microbial population to inhibitory concentrations of dye.

Discussion

Microbial decolourisation of C.I. Reactive Red 141 under anaerobic conditions is first-order with respect to dye concentration. These results are in agreement with the literature, with first-order reactions for the degradation of azo dyes by anaerobic micro-organisms being reported by Larsen et al. (1976), Wuhrmann et al. (1980), Mechsner and Wuhrmann (1982) and Kremer (1989). However, the measured rates of decolourisation are inversely proportional to the initial dye concentrations in the anaerobic system indicating that additional factors, possibly microbial inhibition, must be taken into account. In view of the results obtained in the toxicity assay, it is suggested that this was caused by increasingly inhibitive concentrations of C.I. Reactive Red 141 and/or C.I. Reactive Red 141 degradation products in the system, resulting in decreasing decolourisation rates with increasing initial dye concentrations.

The inhibitive effect of textile dyes was reported by Ogawa et al. (1988; 1989). Microbial inhibition was thought to be caused by intercalation of dye compounds between DNA base pairs, so preventing enzymatic activity and cell replication (Ogawa et al., 1988). However, this requires that the dye pass through the cell membranes of the micro-organisms and become inserted between the base pairs of DNA. C.I. Reactive Red 141 is a large, highly sulphonated compound (MW = 1 634 g/mol); therefore, permeation of the dye through the microbial cell membranes and subsequent intercalation of the dye between DNA base pairs is thought to be unlikely as a mechanism of inhibition. Although it is possible that an alternative mechanism is responsible for the inhibitive effects observed with C.I. Reactive Red 141, the potential toxicity of the dye degradation products must not be overlooked. These degradation products are smaller than the dye compound and may be capable of penetrating the cell and inhibiting the micro-organisms either by intercalation or some other intracellular mechanism. The microbial toxicity of the aromatic degradation products of dyes has been reported by Chung (1983) and Ganesh et al. (1992). The latter noted inhibition of biomass in a waste-water system treating reactive dye waste water and suggested that this was caused by the products of dye degradation rather than the dye itself. Wuhrmann et al. (1980) also noted that, with particular dyes, the rate of decolourisation decreased more rapidly than predicted by a first-order reaction, when a large percentage of the dye had already been reduced. This was attributed to accumulation of toxic metabolic products in the medium. Although the decreasing rate of degradation of C.I. Reactive Red 141 with increasing dye concentration does not directly correlate with the observation of Wuhrmann et al. (1980), it is probable that accumulating metabolic products were also responsible for this phenomenon.

Although the PEB demonstrate possible inhibition at the upper concentrations of C.I. Reactive Red 141 in the toxicity assay, prior exposure of the anaerobic biomass to the dye noticeably increases the tolerance of the micro-organisms. This is in accordance with the findings of Ogawa et al. (1988; 1989), who reported that acclimation of bacteria to growth in the presence of inhibitory dyes reduced the toxic effect of the dyes and partially restored the physiological activity of the cells. Therefore, it is possible that a microbial population in a dedicated treatment system could become adapted to withstand and decolourise increasingly concentrated waste waters. Moreover, the high gas production rates recorded for bottles supplemented with low levels of dye suggests that, at low concentrations, the presence of C.I. Reactive Red 141 in the anaerobic system may even be beneficial to the anaerobic micro-organisms, possibly due to its role as a terminal electron acceptor. Rahmen (1991) also reported that the addition of the reactive dye, Red B, to a biological culture capable of reducing the dye, appeared to enhance growth in the culture and proposed that the electron accepting nature of the dye was responsible for this.

Although it is probable that micro-organisms could be adapted to tolerate the dye degradation products in a treatment system, the ultimate fate of these compounds must be considered. Generally, research into the biodegradability of textile dye degradation products has shown that aerobic conditions are more amenable to mineralisation, with little or no degradation of these compounds occurring under the anaerobic conditions that give rise to decolourisation. The Ecological and Toxicological Association of Dyes and Organic Pigments Manufacturers (ETAD) reported that little degradation of dye metabolites occurred in the anaerobic systems tested and concluded that aerobic treatment was the more feasible option for mineralisation of the dye degradation products (Brown and Hamburger, 1987). Haug et al. (1991) also concluded that a two-phase anaerobic-aerobic system was neccessary for mineralisation of the sulphonated azo dye, Mordant Yellow 3. Research by Carliell (1993) indicated that no *ready* degradation of C.I. Reactive Red 141 metabolites occurred in the anaerobic system.

The principal factor limiting the degradation potential of dye metabolites in biological systems is the highly sulphonated nature of these compounds. Sulphonation tends to increase the recalcitrance of compounds by decreasing their ability to permeate through the microbial cell walls (Mechsner and Wuhrmann, 1982; Haug et al., 1991). Microbial degradation of sulphonated naphthalene compounds has been reported by Brilon et al. (1988 a, b) but desulphonation was found to be essential before these compounds could be utilised as sources of carbon and energy. As molecular oxygen is essential for oxygenolytic cleavage of the sulphonate bonds in order to liberate the sulphonate groups as sulphite, it does not seem likely that mineralisation of sulphonated dyes will occur under the strict anaerobic conditions required for decolourisation. However, it is possible that an anaerobic treatment system, in addition to decolourising textile effluent, will render the effluent more amenable to subsequent aerobic treatment.

The requirements of such an anaerobic treatment system were addressed in this research project with respect to the provision of additional labile carbon to the system and the effect of additional oxidising agents (nitrate and sulphate) on the decolourising performance of such a system.

Decolourisation of C.I. Reactive Red 141 as a sole carbon source is found to occur at a reduced rate when compared with the rate measured in the presence of a supplemental labile carbon source (glucose). Enhancement of azo reduction through addition of glucose was also noted by researchers such as Haug et al. (1991) and Wuhrmann et al. (1980). It is thought that the presence of glucose enhances the reduction rate of the azo compound by increasing the rate of formation of reduction equivalents (reduced flavin nucleotides) which are re-oxidised concurrently with

reduction of the azo compound. Gingell and Walker (1971) proposed that the rate of formation of these reduced flavins was the principal rate-limiting step in the microbial reduction of an azo dye, Red-2G. It is, therefore, not surprising that azo reduction rates are sensitive to the amount of available respiration substrate in an anaerobic system, since catabolism of these substrates is ultimately responsible for the production of reduced flavins. Thus, a treatment system designed to anaerobically decolourise textile dyes must take into account the requirement of this system for supplemental labile carbon in order to maintain the metabolic activity of the micro-organisms and, consequently, the formation of reduced flavin nucleotides to reduce and decolourise the dyes. This conclusion is substantiated by results published by Harmer and Bishop (1992) in which the rate of decolourisation of an azo dye (Acid Orange 7) was found to be closely related to the metabolic activity of the waste-water bacteria, with increasing concentrations of easily assimilable COD in the treatment system resulting in increased decolourisation of the azo dye.

The presence of nitrate in the standard assay system inhibits decolourisation for a period of time proportional to the concentration of nitrate added. This suggests that nitrate (as the more thermodynamically favourable electron accceptor) is reduced in preference to C.I. Reactive Red 141 and that only after all the nitrate (and possibly nitrite) has been reduced does decolourisation of C.I. Reactive Red 141 commence. Inhibition of azo reduction by nitrate or nitrite was also reported by Wuhrmann et al. (1980), although in this case the principal rate-limiting factor of azo reduction was thought to be permeation of the dyes through the microbial cell walls.

The presence of sulphate as an additional electron acceptor has no discernible effect on the rate of decolourisation of C.I. Reactive Red 141 in the standard assay system. It is probable that the dye was reduced preferentially to the sulphate compounds, suggesting that C.I. Reactive Red 141 is the more favourable electron acceptor. It is, therefore, proposed that the order of reduction of these compounds would be nitrate > C.I. Reactive Red 141 > sulphate.

Nitrate, sulphate, chloride and carbonate salts are often used in reactive dyeing and are, therefore, frequently present in textile effluents. Thus, the effect of additional oxidising agents on the reduction of azo dyes is important when designing a waste-water treatment system for decolourisation of textile effluent. It was reported by Wuhrmann et al. (1980) that in a nitrifying sewage works supplemented with an anaerobic treatment step for denitrification, azo compounds were not decolourised until all nitrite was denitrified. Therefore, it is not possible to combine denitrification and dye reduction in a simultaneous process unless the retention time in the anaerobic step corresponds to the sum of the retention times of the two reactions involved. Although sulphate has not been shown to interfere with the reduction of the azo dye C.I. Reactive Red 141 in a batch system, anaerobic digestion of sulphate-containing wastewaters is complicated by substrate competition between sulphate-reducing bacteria (SRB) and methane-producing bacteria (MPB) which can lead to the inhibition of methane production and the production of hydrogen sulphide.

The redox potential of the anaerobic digester, measured with C.I. Reactive Red 141 alone, or in combination with nitrate or sulphate shows that reduction of the dye is dependant on the redox potential of the anaerobic system. It is probable that decolourisation of C.I. Reactive Red 141 will occur within a range of redox potentials but that the rate will be influenced by whether the reduction potential of the system is at the upper or lower level of this range. This was also reported by Gingell and Walker (1971), when using cell-free extracts of *Streptococcus faecalis,* who stated that the azo dye (Red 2-G) was reduced at a rate depending on the redox potential of the system. Although the precise redox potential for optimum decolourisation is not known, it can be concluded that strictly anaerobic conditions are conducive to decolourisation of C.I. Reactive Red 141.

Conclusions

Microbial decolourisation of C.I. Reactive Red 141 in an anaerobic environment occurs as a result of reduction and cleavage of the azo bonds. This gives rise to the liberation of degradation products such as 2-aminonaphthalene-1,5-disulphonic acid. No *ready* biodegradation of these sulphonated metabolites is expected in the anaerobic system that gives rise to decolourisation. Decolourisation of C.I. Reactive Red 141 in the anaerobic system conforms to a first-order relationship, with respect to dye concentration. However, k values decrease with increasing initial dye concentration in the anaerobic serum bottles, suggesting inhibition of the micro-organisms. Prior exposure of the anaerobic biomass to C.I. Reactive Red 141 increases the resistance to the micro-organisms to previously inhibitory concentrations of the dye.

The metabolic state of the anaerobic microbial community is a rate-limiting factor in the decolourisation of C.I. Reactive Red 141, requiring the addition of labile carbon to the system to maintain the rate of azo reduction. The presence of competitive electron acceptors in the anaerobic environment is also a rate-controlling factor, with denitrification occurring preferentially to reduction of C.I. Reactive Red 141. The redox potential of the anaerobic system is thought to play a role in the rate of decolourisation, with strictly anaerobic conditions being conducive to azo reduction.

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