Microbial conversion of selected azo dyes and their breakdown products

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Abstract Four selected azo dyes (acid orange 6, acid orange 7, methyl orange and methyl red) were completely decolourised in the presence of anaerobic granular sludge, while only methyl red was degraded in aerobic conditions using a conventional activated sludge. Additional experiments with culture broth devoid of cells showed that anaerobic decolourisation of azo dyes was performed by extracellular reducing agents produced by anaerobic bacteria. This was further confirmed by abiotic experiments with sulphide and NADH. The presence of redox mediators such as riboflavin led to dramatic acceleration of the anaerobic biodecolourisation process. The azo dye reduction products were found to be sulphanilic acid and 4-aminoresorcinol for acid orange 6; sulphanilic acid and 1-amino-2-naphthol for acid orange 7; N,N-dimethyl-1,4-phenylenediamine and sulphanilic acid for methyl orange; and N,N-dimethyl-1, 4-phenylenediamine and anthranilic acid for methyl red. Anaerobic toxicity assays showed that the azo dyes were more toxic than their breakdown products (aromatic amines), except 1-amino-2-naphthol. In the presence of activated sludge, only anthranilic acid was completely mineralised while sulphanilic acid was persistent. 4-aminoresorcinol, 1-amino-2-naphthol and N,N-dimethyl-1,4-phenylenediamine underwent autooxidation in aerobic conditions yielding coloured polymeric products. On the contrary, in the presence of granular methanogenic sludge, 4-aminoresorcinol, 1-amino-2-naphthol and anthranilic acid were quantitatively methanised, sulphanilic acid was partially (70%) mineralised while N,N-dimethyl-1,4-phenylenediamine was only demethylated producing 1,4-phenylenediamine as an end product.
Keywords Aerobic; anaerobic; azo dye

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Introduction

Azo dyes – aromatics substituted with azo groups (-N=N-) – represent a major class of all colourants used worldwide. The presence of such compounds in industrial wastewater may create serious environmental problems (Hildenbrand *et al.*, 1999). Biological methods of organic pollution removal are commonly considered to be the most economically effective and environmentally sound. In spite of the fact that recalcitrance and toxicity of azo dyes may make biological treatment (especially aerobic ones) difficult (Razo-Flores *et al.*, 1997), a number of researches demonstrated azo dyes' reduction to colourless aromatic amines by anaerobic microbial consortia (Kalyuzhnyi and Sklyar, 2000; O'Neill *et al.*, 2000; Rajaguru *et al.*, 2000; Tan, 2001; van der Zee *et al.*, 2001; Yemashova *et al.*, 2004). Meantime, a wide range of azo dye structures decolourised in various anaerobic environments allows the assumption that the decomposition of azo dyes by splitting their azo bonds is a common property of any anaerobic sludge. At the present time, there is no generic opinion concerning the mechanism of azo dye decolourisation under anaerobic conditions. Generally speaking, the reduction of azo dye may imply different mechanisms, such as enzymatic, mediated, pure chemical, intracellular and extracellular (Stolz, 2001; van der Zee *et al.*, 2001, 2003). For example, it was demonstrated that ubiquitous sources of electrons, such as reduced forms of nicotinamide adenine dinucleotide (phosphate) (NAD(P)H), were able to reduce azo dyes in the absence of any enzymes (Nam and Renganathan, 2000). Another extracellular reducing

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agent – sulphide – produced via respiration by sulphate reducing bacteria also chemically decolourises azo dyes (Yoo, 2002; Yemashova *et al.*, 2004). Azo dye reduction was also greatly accelerated by the addition of redox mediators such as anthraquinone-sulphonate (dos Santos *et al.*, 2004). On the other hand, aromatic amines released from azo dye decomposition are known, or suspected, to be carcinogens for humans (Hildenbrand *et al.*, 1999). Meanwhile, aromatic amines can be easily biodegraded aerobically through hydroxylation and ring opening of the aromatic moiety. Consequently, it is suggested to combine the anaerobic cleavage of the azo dyes with the aerobic biodegradation for the amines formed (Kalyuzhnyi and Sklyar, 2000; O'Neill *et al.*, 2000; Rajaguru *et al.*, 2000; Tan, 2001). However, such an approach has serious drawbacks. Many of the aminoaromatics formed during the anaerobic decolourisation of azo dyes (especially with hydroxyl groups) are unstable under aerobic conditions and are extremely prone to autooxidation yielding recalcitrant polymeric products (Stolz, 2001). To avoid this, special attention should be given to the possibility of degradation of such compounds by anaerobic microorganisms. Some researches showed that aromatic amines substituted with hydroxyl or carboxyl groups could be degraded under anaerobic conditions (Battersby and Wilson, 1989; Razo-Flores *et al.*, 1997; Kalyuzhnyi *et al.*, 2000). The objective of this paper was to obtain a deeper insight into mechanisms of anaerobic decolourisation of four selected azo dyes (acid orange 6, acid orange 7, methyl orange and methyl red), as well as on anaerobic biodegradation of their breakdown products (aromatic amines) and compare the results obtained with the corresponding aerobic biodegradation data.

Materials and methods

Biomass and basal medium

Methanogenic granular sludge (16 g VSS/L, aceticlastic activity – 0.19 g CH_4-COD/g/VSS/L) from the EGSB-reactor treating brewery wastewater (Efes-Moscow) and activated sludge (6 g VSS/L) from Kur'yanovskaya municipal aeration station (Moscow) were used for experiments. The basal medium in all batch experiments contained (mg L^{-1}): $NaHCO_3$ (5,000), NH_4Cl (280), $CaCl_2{\cdot}2H_2O$ (10), K_2HPO_4 (250), $MgSO_4{\cdot}7H_2O$ (100), yeast extract (100), H_3BO_3 (0.05), $FeCl_3{\cdot}4H_2O$ (2), $ZnCl_2$ (0.05), $MnCl_2{\cdot}4H_2O$ (0.05), $CuCl_2{\cdot}2H_2O$ (0.03), $(NH_4)_2SeO_3{\cdot}5H_2O$ (0.05), $AlCl_3{\cdot}6H_2O$ (2), $NiCl_2{\cdot}6H_2O$ (0.05), $Na_2SeO_3{\cdot}5H_2O$ (0.1), 36% HCl to adjust the pH value to 7.

Assays

The toxicity of the azo dyes at concentrations of 150–1,200 mg/L and aromatic amines at concentrations of 100–300 mg/L was determined using aceticlastic methanogenic activity assay (Razo-Flores *et al.*, 1997). These assays were performed in 120 mL closed glass serum bottles containing 50 mL of basal medium. After flushing with argon, the aqueous solution of investigated dyes and granular sludge (1.0 g VSS L^{-1}) were added. Then, 0.5 mL of acetate was added from a solution containing 2.85 M sodium acetate. The assay bottles were incubated at 30 °C for several days. The concentration of CH_4 and pressure in the headspace of the serum flasks were recorded throughout the incubation. Specific aceticlastic activities of the sludge were calculated from a linear segment of kinetic curves of methane production. The aceticlastic activity of the untreated sludge with a toxicant was taken as control. From the concentration dependence of the specific activities, 50% of the inhibiting concentration (IC_{50}) values for the azo dyes and aromatic amines were estimated. The assays were performed in duplicate.

The anaerobic biodegradation assays with azo dyes and aromatic amines were performed in 120 mL closed serum bottles filled with 50 mL of basal medium, granular sludge (10 vol. %) and 0.2–1 mM corresponding substrate. Since some biodegradable

organics were introduced with the sludge, no other primary electron donors were added. Moreover, in the preliminary experiments, it was shown that their addition (e.g. ethanol) did not enhance biodegradation rates (data not shown). The headspace of the bottles was flushed with argon. The assays were performed in duplicate at 30 °C. The concentrations of azo dyes, aromatic amines, methane, carbon dioxide, sulphide and ammonium were measured. To take into account a production of methane from the cells' autolysis, control experiments were carried out without aromatic substrates. Since the concentration of azo dyes may decrease due to adsorption onto the sludge particles, the experiments with autoclaved sludge (killed cells) were performed for checking this effect. In experiments with riboflavin, the latter was added to the standard biodegradation assays at concentrations of 5–15 μM (sludge concentration – 5 vol.%).

The aerobic biodegradation assays with azo dyes and aromatic amines were carried out at 27 °C in 100 mL shake flasks filled with 40 mL of basal medium, activated sludge (10 vol.%) and 0.2–0.9 mM corresponding substrate. The assays were performed for 30 d using a thermostatic rotary shaker (New Brunswick Scientific, USA) with 180 rpm. The concentrations of azo dyes and aromatic amines were monitored.

The chemical azo dyes reduction assays were performed identically to the anaerobic biological assays with the exception that sulphide was added to the final concentrations of 5, 0.5 or 0.05 mM instead of granular sludge (sterile conditions). Concentrations of dyes were 0.5, 0.25 or 0.025 mM, respectively. In the assays with reduction by NADH, concentration of azo dyes was 0.25 mM and concentration of NADH was 0.5 mM.

For elucidation of azo dyes' decolourisation mechanisms, the assay with culture broth devoid of microorganisms was performed. After 10 days of anaerobic biodegradation tests, the culture broth was passed through a 0.20 μm syringe microfilter (Corning Inc, USA) used for microbial broth sterilisation, and was then placed into the sterile closed 15 mL flasks. In this way, anaerobiosis was not disturbed. The flasks were flushed with argon, cultivation was performed at 30 °C for 40 d and the concentrations of azo dyes tested were 0.17–0.4 mM.

Chemicals

All azo dyes and 1-amino-2-naphthol were purchased from Acrus (USA), 4-aminoresorcinol, N,N-dimethyl-1,4-phenylenediamine, anthranilic acid, sulphanilic acid were purchased from Aldrich (USA). All chemicals were of the analytical grade and were not further purified. All aromatic substrates used are presented in Figure 1.

Analysis

The methane and carbon dioxide contents in the headspace of the serum flasks were determined on a gas chromatograph LHM-8MD (Yagat, Russia) equipped with a steel column (2 m) packed with Porapak QS. The temperature of the column, injector port and conductivity detector was 50 °C. Argon was used as a carrier gas with a flow rate of $30\,mL \cdot min^{-1}$. The volume of gas sample was 200 μL. The azo dyes' concentrations were measured spectrophotometrically with a UV-1202 spectrophotometer (Shimadzu, Japan) at a maximum absorbance wavelength (pH 7.0) of 428, 431, 465 and 490 nm for acid orange 6, methyl red, methyl orange and acid orange 7, respectively. Samples were centrifuged and aliquots of 100 μL were diluted in a 2 mL 0.1 M phosphate buffer (pH 7.0) solution and measured in a 1.0 cm quartz cuvette. The aromatic amines formed were analysed with high performance liquid chromatography (HPLC). The reverse phase column was packed with a Diasorb 130-C_{16}T and had a film thickness of 6 μm. The chromatograph (Gilson, France) was equipped with a UV detector operated at 225 nm. Methanol with 1% acetic acid was used as a liquid phase with a flow rate of

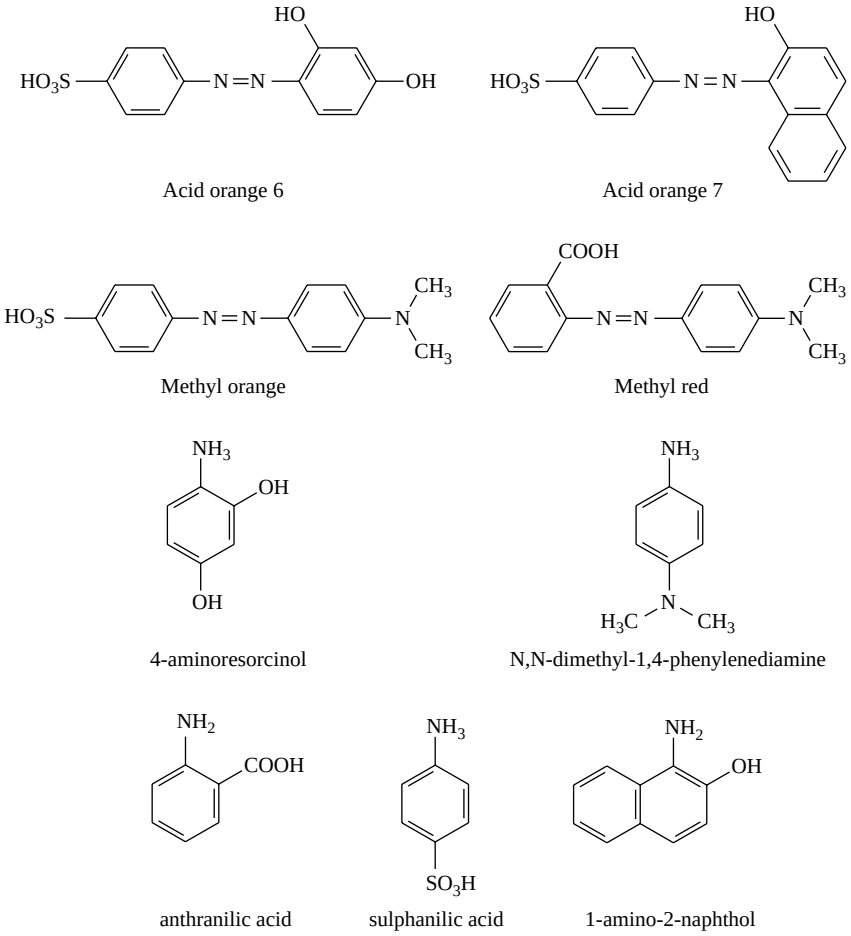

Figure 1 Structural formulas of N-substituted aromatic substrates used

$1\,mL \cdot min^{-1}$. The pressure was 14 MPa and the volume of aqueous sample was 10 μL. Ammonium and sulphide were measured spectrophotometrically as described by Fawcett and Scott (1960) and Truper and Schlegel (1964), respectively.

Results and discussion

Biodecolourisation of azo dyes

In the presence of activated sludge, only methyl red became colourless, whereas the other azo dyes were persistent during 25 days of aerobic incubation (Figure 2a). This fact may be explained by reduced electron density of the aromatic ring caused by electron-withdrawing azo group making difficult electrophilic oxygenase attack – characteristic for aerobic microorganisms. On the contrary, in the presence of granular methanogenic sludge, all the azo dyes tested were completely decolourised under anaerobic conditions (Figure 2b). Azo dyes become colourless as a result of reductive splitting of azo group with formation of aromatic amines:

$$Ar^1{-}N{=}N{-}Ar^2 + 4[H] \rightarrow Ar^1NH_2 + H_2N - Ar^2. \quad (1)$$

The bioreduction of azo dyes proceeded without significant lag periods and followed the first-order kinetics (Yemashova *et al.*, 2004) with respect to dye concentration (Figure 2b). The corresponding constants were calculated from the data of Figure 2b and their

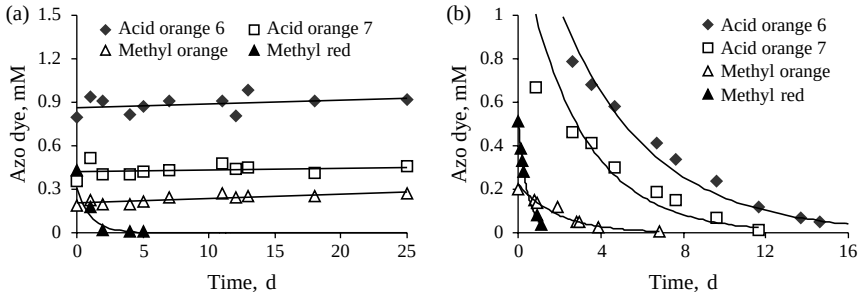

Figure 2 Aerobic (a) and anaerobic (b) biodegradation of selected azo dyes

values are presented in Table 1. It is seen that methyl red and acid orange 6 were the most prone and persistent dyes for anaerobic reduction, respectively. The addition of ethanol as a primary electron donor for reaction (1) did not enhance azo dye decolourisation rate (data not shown). It should also be noted that incubation of azo dyes with activated sludge in anaerobic conditions also led to their decolourisation with the first order constants comparable to those observed in the presence of methanogenic sludge (Table 1), witnessing that this ability is not an exclusive property of only anaerobic sludges. Azo dye reduction products identified by HPLC were found to be sulphanilic acid and 4-aminoresorcinol for acid orange 6; sulphanilic acid and 1-amino-2-naphthol for acid orange 7; N,N-dimethyl-1,4-phenylenediamine and sulphanilic acid for methyl orange; and N,N-dimethyl-1,4-phenylenediamine and anthranilic acid for methyl red (Table 2).

Mechanism of anaerobic azo dye reduction

For degradation of azo dyes, the vital functions of microorganisms are necessary because no azo dye reduction was found in the presence of autoclaved anaerobic sludge (data not shown). On the contrary, the culture broth obtained by anaerobic sludge liquor filtration through 0.2 μm microfilter (i.e. devoid of cells but containing the products of their vital functions) performed a complete decolourisation of all the azo dyes tested (Figure 3a). Thus, splitting of azo dye appears to be a result of action of reducing agents presented in anaerobic culture broth and followed the pseudo first-order kinetics without any lag period (Figure 3a). However, the observed constants (Table 1) were lower compared to those in the presence of anaerobic biomass, which is thus beneficial for maintaining the pools of the reducing agents. Since sulphide and NADH are usually encountered in anaerobic environments, azo dyes' decolourisation was studied without any sludge but in the presence of both compounds. It was found that these reducing agents completely decomposed all the azo dyes studied (data for acid orange 6 are shown in Figure 3b as an example). This abiotic azo dye's reduction also followed pseudo first-order kinetics with respect to the dye concentration though some lag-periods were observed (Figure 3b). The corresponding constants are presented in Table 1. For sulphide, it was found that the rate of reduction increased with an increase of its concentration (Table 1). However, the sulphide concentrations measured in the anaerobic culture broth were always below 0.05 mM. This implies that the observed rates of azo dyes' reduction by anaerobic sludge (Table 1) cannot be explained by the action of sulphide only; some other reducing agents such as NADH and other redox mediators can also contribute to this process.

The importance of the presence of redox mediators in anaerobic media for bioreduction of azo dyes was demonstrated by the addition of riboflavin to the standard anaerobic biodegradability assay (Figure 4). It is seen (data for acid orange 6 are shown in Figure 4

Table 1 The 1st order constants (day^{-1}) of azo dye reduction by sludges, broth, sulphide and NADH

Azo dye	Methanogenic sludge	Broth*	Activated sludge#	Sulphide, 5 mM	Sulphide, 0.5 mM	Sulphide, 0.05 mM	NADH, 0.5 mM
Acid orange 6	0.20 ± 0.02	0.09 ± 0.01	0.14 ± 0.01	1.07 ± 0.06	0.10 ± 0.01	0.07 ± 0.01	0.10 ± 0.01
Acid orange 7	0.30 ± 0.03	0.13 ± 0.02	0.32 ± 0.05	1.44 ± 0.09	0.40 ± 0.08	0.29 ± 0.04	0.62 ± 0.06
Methyl orange	0.49 ± 0.03	0.36 ± 0.03	0.60 ± 0.06	20 ± 1	0.88 ± 0.04	0.20 ± 0.02	0.13 ± 0.01
Methyl red	2.0 ± 0.2	0.18 ± 0.01	0.48 ± 0.04	3.9 ± 0.2	0.41 ± 0.05	0.26 ± 0.04	0.11 ± 0.01

*Anaerobic culture broth devoid of cells
#Anaerobic conditions

Table 2 Anaerobic toxicity of azo dyes and corresponding aromatic amines

Azo dye	IC_{50} of azo dye, mM	IC_{50} of corresponding aromatic amines, mM	
Acid orange 6	2.50	sulphanilic acid non toxic*	4-aminoresorcinol non toxic*
Acid orange 7	0.90	sulphanilic acid non toxic*	1-amino-2-naphthol 0.54
Methyl orange	0.25	sulphanilic acid non toxic*	N,N-dimethyl-1,4-phenylenediamine 1.26
Methyl red	0.99	anthranilic acid non toxic*	N,N-dimethyl-1,4-phenylenediamine 1.26

*Non toxic until 2 mM concentration

as an example) that even such a low riboflavin concentration as 5 μM led to the dramatic acceleration of azo dye reduction.

Anaerobic toxicity of azo dyes and corresponding aromatic amines

Analysis of the data presented in Table 2 showed that in all cases, except 1-amino-2-naphthol, the azo dyes were more toxic than their breakdown products. This fact is in agreement with literature data when aromatic amines are generally regarded as being less toxic compounds for anaerobic microorganisms compared to azo dyes (Razo-Flores *et al.*, 1997).

Biodegradation of aromatic amines

In the presence of activated sludge, only anthranilic acid was completely mineralised while sulphanilic acid was persistent (Figure 5). The latter fact is in contradiction to literature data (Tan, 2001) where aerobic biodegradation of sulphanilic acid was observed. Probably, the activated sludge used by us required an acclimation to this substrate. Meanwhile, 4-aminoresorcinol, 1-amino-2-naphthol and N,N-dimethyl-1,4-phenylenediamine, after exposure to aerobic conditions, underwent autoxidation and polymerisation yielding coloured products which was evident from a largely altered UV-VIS spectrum. Thus, aerobic treatment is not a solution for removal of these amines. On the contrary, in the presence of granular methanogenic sludge, 4-aminoresorcinol (Figure 6a), 1-amino-2-naphthol (Figure 6b) and anthranilic acid (data not shown) were quantitatively mineralised to methane, carbon dioxide and ammonium according to the following equations:

$$C_6H_7O_2N + 4H_2O \rightarrow 3CH_4 + 3CO_2 + NH_3 \quad \text{for 4-aminoresorcinol,}$$

$$C_{10}H_9ON + 8H_2O \rightarrow 5.5CH_4 + 4.5CO_2 + NH_3 \quad \text{for 1-amino-2-naphthol}$$

$$C_7H_7O_2N + 5H_2O \rightarrow 3.5CH_4 + 3.5CO_2 + NH_3 \quad \text{for anthranilic acid.}$$

Sulphanilic acid was also slowly degraded in anaerobic conditions producing methane, ammonium and sulphide; however, the initial substrate was not fully mineralised

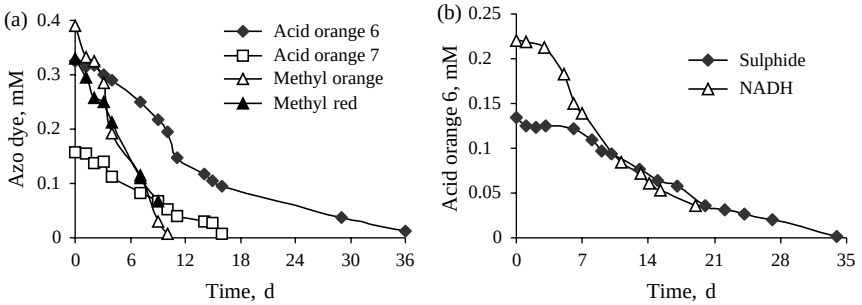

Figure 3 Decolourisation of azo dyes by anaerobic culture broth devoid of cells (a) as well as sulphide and NADH (initial concentration of both – 0.5 mM) (b)

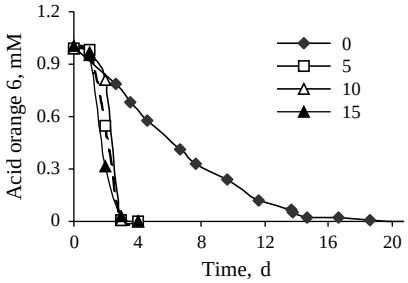

Figure 4 Influence of riboflavin concentrations (numbers on graph in μM) on acid orange 6 and anaerobic biodecolourisation (5 vol.% sludge)

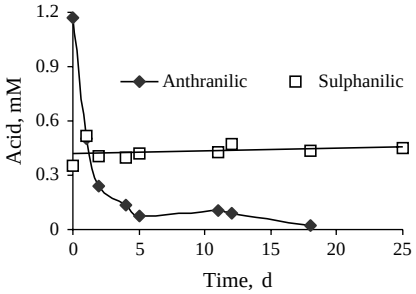

Figure 5 Aerobic biodegradation of anthranilic and sulphanilic acids

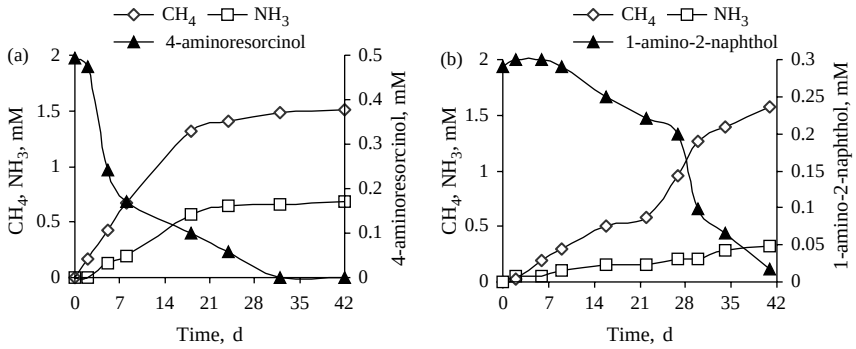

Figure 6 Anaerobic biodegradation of 4-aminoresorcinol (a) and 1-amino-2-naphthol (b)

(30% left) after 100 d of anaerobic incubation (data not shown). N,N-dimethyl-1,4-phenylenediamine was demethylated but phenylenediamine formed was not further degraded in anaerobic conditions during 100 d of incubation (data not shown). According to our knowledge, this is the first report demonstrating anaerobic biodegradation of 4-aminoresorcinol, 1-amino-2-naphthol and sulphanilic acid. Two latter findings are in contradiction to some researchers (Bras *et al.*, 2001; Tan, 2001) who reported on the nonbiodegradability of these substances in anaerobic conditions.

Conclusions

1. All four selected azo dyes were completely decolourised by an anaerobic granular sludge, while only methyl red was aerobically biodegraded.

2. The anaerobic decolourisation of azo dyes proceeds due to the action of extracellular reducing agents (sulphide, NADH etc) produced by anaerobic bacteria. The presence of redox mediators such as riboflavin led to a dramatic acceleration of this process.
3. Anaerobic toxicity assays showed that the azo dyes were more toxic than their breakdown products (aromatic amines), except 1-amino-2-naphthol.
4. Under aerobic conditions, only anthranilic acid was completely mineralised while sulphanilic acid was persistent. 4-aminoresorcinol, 1-amino-2-naphthol and N,N-dimethyl-1,4-phenylenediamine underwent autoxidation in these conditions yielding coloured products.
5. Under anaerobic conditions, 4-aminoresorcinol, 1-amino-2-naphthol and anthranilic acid were quantitatively (sulphanilic acid partially) methanised, while N,N-dimethyl-1,4-phenylenediamine was only demethylated producing 1,4-phenylenediamine as an end product.

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