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Azo Dyes and Their Metabolites: Does the Discharge of the Azo Dye into Water Bodies Represent Human and Ecological Risks?

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1. Introduction

1.1 History of sintetic dyes

Colorants (dyes and pigments) are important industrial chemicals. According to the technological nomenclature, pigments are colorants which are insoluble in the medium to which they are added, whereas dyes are soluble in the medium. The world's first commercially successful synthetic dye, named mauveine, was discovered by accident in 1856 by William Henry Perkin. These synthetic compounds can be defined as colored matters that color fibers permanently, such that they will not lose this color when exposed to sweat, light, water and many chemical substances including oxidizing agents and also to microbial attack (Rai et al., 2005; Saratele et al., 2011). By the end of the 19th century, over ten thousand synthetic dyes had been developed and used for manufacturing purposes (Robinson et al., 2001a; Saratele et al., 2011), and an estimate was made in 1977 that approximately 800,000 tons of all recognized dyestuffs had been produced throughout the world (Anliker, 1977; Combes & Haveland-Smith, 1982). The expansion of worldwide textile industry has led to an equivalent expansion in the use of such synthetic dyestuffs, resulting in a rise in environmental pollution due to the contamination of wastewater with these dyestuffs (Pandey et al., 2007; Saratele et al., 2011).

The Ecological and Toxicological Association of the Dyestuffs Manufacturing Industry (ETAD) was inaugurated in 1974 with the goals of minimizing environmental damage, protecting users and consumers and cooperating with government and public concerns in relation to the toxicological impact of their products (Anliker, 1979; Robinson et al., 2001a). A survey carried out by ETAD showed that of a total of approximately 4,000 dyes that had

been tested, more than 90% showed LD₅₀ values above 2×10^3 mg/kg, the most toxic being in the group of basic and direct diazo dyes (Shore, 1996; Robinson et al., 2001a). Thus it appears that exposure to azo dyes does not cause acute toxicity, but with respect to systemic bioavailability, inhalation and contact with the skin by azo dyes is of concern, due to the possible generation of carcinogenic aromatic amines (Myslak & Bolt, 1988 and Bolt & Golka, 1993 as cited in Golka et al., 2004).

Of the approximately 10^9 kg of dyestuffs estimated to be manufactured annually throughout the World, the two most widely used in the textile industry are the azo and anthraquinone groups (Krizanec & Marechal, 2006; Forss, 2011). Thus, this chapter is a comprehensive review on the azo dyes and their effects on human and environmental health.

2. Azo dyes

Azo dyes are diazotized amines coupled to an amine or phenol, with one or more azo bonds ($-N=N-$). They are synthetic compounds and account for more than 50% of all the dyes produced annually, showing the largest spectrum of colors (Carliell et al., 1995; Bae & Freeman, 2007; Kusic et al., 2011). Nearly all the dyestuffs used by the textile industry are azo dyes, and they are also widely used in the printing, food, papermaking and cosmetic industries (Chung & Stevens, 1993; Chang et al., 2001a). An estimate was made in the 80's, that 280,000 t of textile dyes were annually discharged into industrial effluents worldwide (Jin et al., 2007; Saratale et al., 2011). Since the azo dyes represent about 70% by weight of the dyestuffs used (Zollinger, 1987), it follows that they are the most common group of synthetic colorants released into the environment (Chang et al., 2001b; Zhao & Hardin, 2007; Saratale et al., 2011).

One only needs very small amounts of dyes in the water (less than 1 ppm for some dyes) to cause a highly visible change in color (Banat et al., 1996), and colored wastewater not only affects the aesthetic and transparency aspects of the water being received, but also involves possible environmental concerns about the toxic, carcinogenic and mutagenic effects of some azo dyes (Spadaro et al., 1992; Modi et al., 2010; Lu et al., 2010). It can also affect the aquatic ecosystem, decreasing the passage of light penetration and gas dissolution in lakes, rivers and other bodies of water (Saranaik & Kanekar, 1995; Banat et al., 1996; Modi et al., 2010).

The more industrialized the society, the greater the use of azo dyes, and hence the greater the risk of their toxic effects affecting the society. It has already been noted that, as from the 70's, intestinal cancer has been more common in highly industrialized societies, and therefore there may be a connection between the increase in the number of cases of this disease and the use of azo dyes (Wolff & Oehme, 1974; Chung et al., 1978).

Bae and Freeman (2007) already demonstrated the biological toxicity of the direct azo dyes used in the textile industry. The results indicated that C.I. Direct Blue 218 was very toxic to daphnids, with a 48-h LC₅₀ between 1.0 and 10.0 mg/L. It must be remembered that toxicity to daphnids is sufficient to suggest potential damage to every receptor ecosystem, and emphasizes the need for the synthetic dye manufacturing industry to carry out toxicological studies (Bae & Freeman, 2007).

2.1 Azo dyes and their mutagenic effects

The azo dyes show good fiber-fixation properties as compared other synthetic dyes, showing up to 85% fixation, but nevertheless this explains why so much dye is released into

the environment, representing the other 10 to 15% of the amount used. Most of the synthetic dyestuffs found in this class are not degraded by the conventional treatments given to industrial effluents or to the raw water (Nam & Reganathan, 2000; Oliveira et al., 2007). Shaul et al. (1991) studied 18 azo dyes, and found that 11 passed practically unchanged through the activated sludge system, 4 were adsorbed by the activated sludge and only 3 were biodegraded, resulting in the release of these substances into bodies of water. Oliveira et al. (2007) showed that even after treatment, effluent from dyeing industries was mutagenic and contained various types of dye. Such data are of concern, especially when one considers that the effluent from the same industry was studied by Lima et al. (2007), who found an increase in the incidence of aberrant crypt in the colon of rats exposed to this sample, this being an early biomarker of carcinogenesis (Lima et al., 2007).

Azo dyes can also be absorbed after skin exposure, and such dermal exposure to azo dyes can occur as an occupational hazard or from the use of cosmetic products. It was postulated in the 80s that the percutaneous absorption of azo dyes from facial makeup could even be a risk factor in reproductive failures and chromosomal aberrations in a population of television announcers (Kučerová et al., 1987; Collier et al., 1993).

Various azo dyes have been shown to produce positive toxic results for different parameters. Tsuboy et al. (2007) analyzed the mutagenic, cytotoxic and genotoxic effects of the azo dye CI Disperse Blue 291, and the results clearly showed that this azo dye caused dose-dependent effects, inducing the formation of micronuclei (MNs), DNA fragmentation and increasing the apoptotic index in human hepatoma cells (HepG₂). A variety of azo dyes have shown mutagenic responses in *Salmonella* and mammalian assay systems, and it is apparent that their potencies depend on the nature and position of the aromatic rings and the amino nitrogen atom. For instance, 2-methoxy-4-aminoazobenzene is an extremely weak mutagen, whereas under similar conditions, 3-methoxy-4-aminoazobenzene is a potent hepatocarcinogen in rats and a strong mutagen in *Escherichia coli* and *Salmonella typhimurium* (Hashimoto et al., 1977; Esancy et al., 1990; Garg et al., 2002, Umbuzeiro et al., 2005a).

According to Chequer et al. (2009), the azo dyes Disperse Red 1 and Disperse Orange 1 increase the frequency of MNs in human lymphocytes and in HepG₂ cells in a dose-dependent manner. According to Ferraz et al. (2010), the azo dyes Disperse Red 1 and Disperse Red 13 showed mutagenic activity in the *Salmonella*/microsome assay with all the strains tested and in the absence of metabolic activation, except for Disperse Red 13, which was negative with respect to strain TA100. After adding the S9 mix, the mutagenicity of the two azo dyes decreased (or was eliminated), indicating that the P450-dependent metabolism probably generated more stable products, less likely to interact with DNA. It was also shown that the presence of a chlorine substituent in Disperse Red 13 decreased its mutagenicity by a factor of about 14 when compared with Disperse Red 1, which shows the same structure as Disperse Red 13, but without the chlorine substituent. The presence of this substituent did not cause cytotoxicity in HepG₂ cells, but toxicity to the water flea *Daphnia similis* increased in the presence of the chlorine substituent (Ferraz et al., 2010).

Chung and Cerneglia (1992) published a review of several azo dyes that had already been evaluated by the *Salmonella* / microsome assay. According to these authors, all the azo dyes evaluated that contained the nitro group showed mutagenic activity. The dyes Acid Alizarin Yellow R and Acid Alizarin GG showed this effect in the absence of metabolic activation (Brown et al., 1978). The dyes C.I. Basic Red 18 and Orasol Navy 2RB, which also contained

nitro groups, were shown to be mutagenic both in the presence and absence of metabolic activation (Venturini & Tamaro, 1979; Nestmann et al., 1981). This review also showed the results obtained in the Salmonella/microsomal test of azo dyes containing benzeneamines, and found that Chrysodin was mutagenic in the presence of a rat-liver preparation (Sole & Chipman, 1986; Chung & Cerniglia, 1992).

Another study applied the micronucleus assay in mouse bone marrow to the azo dye Direct Red 2 (DR2) and the results identified DR2 as a potent clastogen and concluded that excessive exposure to this chemical or to its metabolites could be a risk to human health (Rajaguru et al., 1999).

Al-Sabti (2000) studied the genotoxic effects of exposing the Prussian carp (*Carassius auratus gibelio*) to the textile dye Chlorotriazine Reactive Azo Red 120, and showed its mutagenic activity in inducing MNs in the erythrocytes. They also showed that the dye had clastogenic activity, a potent risk factor for the development of genetic, teratogenic or carcinogenic diseases in fish populations, which could have disastrous effects on the aquatic ecosystem since the fate of compounds found in effluents is to be discharged into water resources (Al-Sabti, 2000).

In addition to the effects caused by exposure to contaminated water and food, workers who deal with these dyes can be exposed to them in their place of work, and suffer dermal absorption. Similarly, if dye-containing effluents enter the water supply, possibly by contamination of the ground water, the general population may be exposed to the dyes via the oral route. This latter point could be of great importance in places where the existent waste treatment systems are inefficient or where there is poor statutory regulation concerning industrial waste disposal (Rajaguru et al., 1999).

2.2 Effects of the azo dyes metabolites

Sisley and Porscher carried out the earliest studies on the metabolism of azo compounds in mammals in 1911, and found sulphanilic acid in the urine of dogs fed with Orange I, demonstrating for the first time that azo compounds could be metabolized by reductive cleavage of the azo group (Sisley & Porscher, 1911 as cited in Walker, 1970).

The mutagenic, carcinogenic and toxic effects of the azo dyes can be a result of direct action by the compound itself, or the formation of free radicals and aryl amine derivatives generated during the reductive biotransformation of the azo bond (Chung et al., 1992; Collier et al., 1993; Rajaguru et al., 1999) or even caused by products obtained after oxidation via cytochrome P450 (Fujita & Peisach, 1978; Arlt et al., 2002; Umbuzeiro et al., 2005a).

One of the criteria used to classify a dye as harmful to humans is its ability to cleave reductively, and consequently generate aromatic amines when in contact with sweat, saliva or gastric juices (Pielesz et al., 1999, 2002). Some such aromatic amines are carcinogenic and can accumulate in food chains, for example the biphenylamines such as benzidine and 4-biphenylamine, which are present in the environment and constitute a threat to human health and to the ecosystems in general (Choudhary, 1996; Chung et al., 2000).

After an azo dye is orally ingested, it can be reduced to free aromatic amines by anaerobic intestinal microflora and possibly by mammalian azo reductase in the intestinal wall or the liver (Walker, 1970; Prival & Mitchel, 1982; Umbuzeiro et al., 2005a). Such biotransformations can occur in a wide variety of mammalian species, including both *Rhesus* monkeys and humans (Rinde & Troll, 1975; Watabe et al., 1980; Prival & Mitchel, 1982). As

previously mentioned, the main biotransformation products of azo dyes are aromatic amines, and thus a brief description of this class of compounds is shown below.

2.2.1 Aromatic amines

As early as the late nineteenth century, a doctor related the occurrence of urinary bladder cancer to the occupation of his patients, thus demonstrating concern about the exposure of humans to carcinogenic aromatic amines produced in the dye manufacturing industry, since his patients were employed in such an industry and were chronically exposed to large amounts of intermediate arylamines. Laboratory investigations subsequently showed that rats and mice exposed to specific azo dye arylamines or their derivatives developed cancer, mainly in the liver (Weisburger, 1997, 2002). Briefly, as mentioned above, in 1895, Rehn showed concern about the urinary bladder cancers observed in three workers from an 'aniline dye' factory in Germany. This led to the subsequent testing in animals of various chemicals to which these workers were exposed, and, as a result, the carcinogenic activity of the azo dye, 2,3-dimethyl-4-aminoazobenzene for the livers of rats and mice was discovered (Yoshida, 1933 as cited in Dipple et al., 1985). An isomeric compound, N,N-dimethyl-4-aminoazobenzene was also found to be a liver carcinogen (Kinosita, 1936 as cited in Dipple et al., 1985). Only in 1954 was the cause of the bladder tumors observed in the workers in the dye industry established to be 2-naphthylamine. This aromatic amine induced bladder cancer in dogs, but not in rats (Hueper et al., 1938 as cited in Dipple et al., 1985).

In addition, workers in textile dyeing, paper printing and leather finishing industries, exposed to benzidine based dyes such as Direct Black 38, showed a higher incidence of urinary bladder cancer (Meal et al., 1981; Cerniglia et al., 1986). Cerniglia et al. (1986) demonstrated that the initial reduction of benzidine-based azo dyes was the result of azoreductase activity by the intestinal flora, and the metabolites of Direct Black 38 were identified as benzidine, 4-aminobiphenyl, monoacetylbenzidine, and acetylaminobiphenyl (Manning et al., 1985; Cerniglia et al., 1986). Furthermore, these metabolites tested positive in the Salmonella/microsome mutagenicity assay in the presence of S9 (Cerniglia et al., 1986).

In the opinion of Ekici et al. (2001), although general considerations concerning the kinetics of azo dye metabolism indicate that an accumulation of intermediate amines is not very likely, this possibility cannot be excluded under all conditions. According to legislation passed in the European Community on 17th July 1994, the application of azo dyes in textiles is restricted to those colorants which cannot, under any circumstances, be converted to any of the following products: 4-Aminodiphenyl; 4-Amino-2',3'-dimethylazobenzene (*o*-aminoazo-toluene); 4-Aminophenylether (4,4'-oxydianiline); 4-Aminophenylthioether (4,4'-thiodianiline); Benzidine; Bis-(4-aminophenyl)-methane (4,4'-diaminodiphenylmethane); 4-chloroaniline (*p*-chloroaniline); 4-Chloro-2-methylaniline (4-chloro-*o*-toluidine); 2,4-Diaminotoluene (2,4-toluylenediamine); 3,3'-Dichlorobenzidine dihydrochloride; 3,3'-Dimethoxybenzidine (*o*-dianisidine); 3,3'-Dimethylbenzidine (*o*-toluidine); 3,3'-Dimethyl-4,4'-diamino-diphenyl methane; 2-Methoxy-5-methylaniline (*p*-kresidine); 4-Methoxy-1,3-phenylenediamine sulfate hydrate (2,4-diaminoanisole); 4,4'-Methylene-bis (2-chloroaniline); 2-Methyl-5-nitroaniline (2-amino-4-nitrotoluene); 2-Naphthylamine; *o*-Toluidine; 2,4,5-Trimethylaniline (Bundesgesetzblatt, 1994 and Directory of Environmental Standards, 1998 as cited in Ekici et al., 2001).

More recently, the scientific community has come to consider the possibility of manufactured azo dyes breaking down generating amines to be a health hazard. The International Agency for Research on Cancer only includes benzidine-based dyes in Group 2A and eight other dyes in Group 2B. Nevertheless, the possibility of azo bond reduction leading to the production of aromatic amines has been demonstrated under a variety of conditions, including those encountered in the digestive tract of mammals (Chung & Cerniglia, 1992; Pinheiro et al., 2004). Therefore, the majority of the attention concerning possible hazards arising from the use of azo dyes is now being directed at their reduction products (Pinheiro et al., 2004).

Nitroanilines are aromatic amines that are commonly generated during the biodegradation of azo dyes under anaerobic conditions, formed by reductive cleavage of the azo bonds ($-N=N-$) by the action of microorganisms present in the wastewaters (Pinheiro et al., 2004; Van der Zee & Villaverde, 2005; Khalid et al., 2009). Depending on the individual compounds, many aromatic amine metabolites are considered to be non-biodegradable or only very slowly degradable (Saupe, 1999), showing a wide range of toxic effects on aquatic life and higher organisms (Weisburger, 2002; Pinheiro et al., 2004; Khalid et al., 2009).

2.3 Metabolic pathways involved in the reduction and oxidation of azo dyes

Following oral or skin exposure to azo dyes, humans can subsequently be exposed to biotransformation products obtained by the action of intestinal microorganisms or that of others present on the skin, or due to reactions in the liver (Esancy et al., 1990; Chadwick et al., 1992; Chung et al., 1992; Stahlmann et al., 2006). Therefore it is extremely important to study the metabolic pathways of azo dyes that can contaminate the environment, in order to understand the overall spectrum of the toxic effects.

The metabolic pathways the azo dyes actually follow depend on several factors, such as, (a) the mode of administration; (b) the degree of absorption from the gastro-intestinal tract after oral ingestion; (c) the extent of biliary excretion, particularly after exposure to different routes other than the oral one; (d) genetic differences in the occurrence and activity of hepatic reducing-enzyme systems; (e) differences in the intestinal flora; and (f) the relative activity and specificity of the hepatic and intestinal systems, particularly those responsible for reducing the azo link, and all these factors are interrelated (Walker, 1970).

Azo dyes behave as xenobiotics, and hence after absorption, they are distributed throughout the body, where they either exert some kind of action themselves or are subjected to metabolism. Biotransformation may produce less harmful compounds, but it may also form bioactive xenobiotics, ie, compounds showing greater toxicity (Kleinow et al., 1987; Livingstone, 1998). The main routes involved in the biotransformation of dyes are oxidation, reduction, hydrolysis and conjugation, which are catalyzed by enzymes (Zollinger, 1991; Hunger, 1994), but in humans, biological reductions and oxidations of azo dyes are responsible for the possible presence of toxic amines in the organism (Pielesz et al., 2002).

Orange II can be reductively metabolized producing 1-amino-2-naphthol, a bladder carcinogen for rats (Bonser et al., 1963; Chung et al., 1992). This suggests that any toxicity induced by unchanged azo dye molecules should not be accepted as the only effect of these compounds, since the reductive cleavage products from these dyes can be mutagenic/carcinogenic (Field et al., 1977; Chung et al., 1992).

2.3.1 Oxidative metabolism

Highly lipid-soluble dyes such as azo dyes, with chemical structures containing amino groups, either alkylamino or acetylamino, but without sulfonated groups, are preferentially biotransformed by oxidative reactions (Hunger, 1994).

Oxidation processes are mainly catalyzed by a microsomal monooxygenase system represented by cytochrome P450 (Hunger, 1994), which belongs to a superfamily of heme proteins, present in all living organisms and involved in the metabolism of a wide variety of chemical compounds (Denisov et al. 2005; Mansuy, 2007).

The general mechanism of metabolic oxidation involves an electron transport chain, which first transfers an electron to the P-450-Fe³⁺ complex, which, on reduction, receives an oxygen atom and in the final steps, leads to the formation of an oxidation product in the organism (Furhmann, 1994 as cited in Hunger, 1994).

There are three different oxidation pathways of importance for azo dyes: I) C-Hydroxylation, ring hydroxylation in the case of azo dyes, probably via an epoxidation mechanism and subsequent rearrangement to a phenol. II) N-Hydroxylation at primary or secondary amino groups, or with acetyl amino groups in the liver. This reaction is followed by esterification with glucuronate or sulfate. The activated esters, which are water-soluble, can be excreted, or the ester group can split off with the formation of a nitrenium compound -NH⁺, which can covalently bind to a nucleophilic group of the DNA. III) Demethylation, which is the stepwise oxidation of the methyl groups of dialkylamino compounds, and the N-hydroxy derivative so formed can be further demethylated or react to form a nitrenium compound (Hunger, 1994).

Studies on the metabolism and carcinogenicity of N,N-dimethylaminoazobenzene (Butter Yellow), a classical hepatocarcinogen in rats, have shown that N-methylaminoazobenzenes are mainly metabolized by N-demethylation. In this way, Butter Yellow was first reversibly demethylated to the mono-N-methyl compound, which, in turn, was irreversibly demethylated to form p-aminoazobenzene. These changes were shown to precede reduction of the azo link, by isolating N-methyl-p-amino azobenzene and p-aminoazobenzene from the animal tissues (Miller et al., 1945; Walker, 1970). Radiotracer studies have shown (Miller et al., 1952) that demethylation occurs via the formation of a hydroxymethyl compound, followed by elimination of the methyl group in the form of formaldehyde (Mueller & Miller, 1953; Walker, 1970).

Hydroxylation of the aromatic ring can occur before reductive fission of the azo group, and also on the amines produced by such a reduction, and this pathway appears to be very important in compounds which contain an unsulphonated phenyl moiety (Walker, 1970).

2.3.2 Reductive metabolism

Trypan Blue has been shown to have carcinogenic and teratogenic properties (Field et al., 1977). Although original Trypan Blue is not mutagenic, it was reduced by the cell-free extract of an intestinal anaerobe, *Fusobacterium* sp.2, to a mutagenic product, O-toluidine (3,3'-dimethylbenzidine) (Hartman et al., 1978; Chung et al., 1992). In addition to Trypan Blue, Benzopurpurine 4B and Chlorazol Violet N were also shown to be Ames-positive frame-shift mutagens, but only in the presence of metabolizing systems capable of effecting azo reduction. The activity of these dyes may therefore be attributed to the benzidine metabolite, O-toluidine, which is generated because these amines are themselves indirect frame-shift agents (Hartman, et al., 1978; Matsushima et al., 1978). As mentioned above, the

benzidine produced after the reduction of some dyes can induce bladder cancer in humans and tumors in some experimental animals (Combes & Haveland-Smith, 1982; Chung, 1983). Some azo dyes, such as Brown FK, have been shown to be directly mutagenic in bacterial tests (Haveland-Smith & Combes, 1980a, b; Rafii et al., 1997). However many other azo dyes, such as Congo Red and Direct Black 38, only give a positive result for mutagenicity after chemical reduction or incubation with the contents of the human intestinal tract (Haveland-Smith & Combes, 1980 a,b; Reid et al., 1983; Cerniglia et al., 1986; Chung and Cerniglia, 1992; Rafii et al., 1997).

Reductive cleavage of the azo linkages is probably the most toxicologically important metabolic reaction of azo compounds. This reaction can be catalyzed by mammalian enzymes, especially in the liver (Walker, 1970; Kennelly et al., 1982) or by intestinal (Chung et al., 1978; Hartman et al., 1978) or skin bacteria such as *Staphylococcus aureus* (De France, 1986; Platzek et al., 1999; Golka et al., 2004). Azo compounds can reach the intestine directly after oral ingestion or via the bile after parenteral administration. They are reduced by azo reductases produced by intestinal bacteria, and to a lesser extent by enzymes from the cytosolic and microsomal fractions of the liver. The first catabolic step in the reduction of azo dyes is the cleavage of the azo bond, producing aromatic amines (Cerniglia et al., 1986), accompanied by a loss of color of the dye, and bacterial azoreductases show much greater activity than hepatic azoreductases (Watabe et al., 1980; Collier et al., 1993; Raffi et al., 1997). This reduction process may produce compounds that are more or less toxic than the original molecule (Collier et al., 1993; Rafii et al., 1997), depending on the chemical structure of the metabolite generated. Although its occurrence in the liver has been regarded as the result of a detoxification reaction, azo reduction may be the first step in azo dye carcinogenesis (Chung et al., 1992).

In addition, Nam & Reganathan (2000) demonstrated that both nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) are capable of reducing azo dyes in the absence of any enzyme, under mildly acidic conditions. The reduced forms of NADH and NADPH are ubiquitous sources of electrons in biological systems, and these function as cofactors in many reductive enzyme reactions. This suggests that the introduction of methyl and methoxy substituents at the 2-,2,3-,2,6-, or 2,3,6-positions of the aromatic ring, accelerates the reduction of phenolic azo dyes by NADH, as compared to that of unsubstituted dyes (Nam & Reganathan, 2000).

It is possible that both the mutagenicity and carcinogenicity of azo dyes are in fact frequently due to the generation of aromatic amines, with subsequent N- and ring hydroxylation and N-acetylation of the aromatic amine (Chung & Cerniglia, 1992). If the azo dyes contain nitro groups, they can also be metabolized by the nitroreductases produced by microorganisms (Chadwick et al., 1992; Umbuzeiro et al., 2005a). Mammalian enzymes in the liver and in other organs can also catalyze the reductive cleavage of the azo bond and the nitroreduction of the nitro group. However, it has been shown that the intestinal microbial azoreductase and nitroreductase play a more important role in this type of metabolism. In both cases, the formation of N-hydroxylamines can cause DNA damage, and if the dyes are completely reduced to aromatic amines, they can then be oxidized to N-hydroxyderivates by P450 enzymes. In addition, N-hydroxy radicals can be acetylated by enzymes such as O-acetyltransferase, generating nitrenium electrophilic ions which are able to react with DNA forming adducts (Chung et al., 1992; Arlt et al. 2002; Umbuzeiro et al., 2005a). Research carried out by Zbaida (1989) showed that the hydroxylation of non-reactive

azo dyes such as azobenzene, increased their binding to microsomal cytochrome P-450 and consequently their rate of reduction (Zbaida, 1989).

Studies with mono-azo dyes have indicated that low electron densities close to the azo bond favor reduction (Walker & Ryan, 1971; Combes & Haveland-Smith, 1982), and this may occur due to hydrogen bonding of an azo N atom together with a proximal naphthol group, producing a keto-hydrazone configuration. It is possible that such a structure, which is present in many food colors, may generate dyes which are more resistant to hepatic and microbial reduction (Parke, 1968 cited as in Combes & Haveland-Smith, 1982).

Kennelly et al. (1984) showed that Direct Black 38, Direct Brown 95, Direct Blue 6, Congo Red, Trypan Blue and Chicago Sky Blue were easily reduced by the intestinal microflora when orally administered to rats by gavage, however when administered via the hepatic portal vein, only Direct Black 38, Direct Brown 95 and Direct Blue 6 were reduced, all of which are potent liver carcinogens (Robens et al., 1980; Chung et al., 1992).

Sweeney et al. (1994) tested azo dyes for genotoxicity following bacterial reduction of the dye. They found that both reduced amaranth and reduced sunset yellow induced cytotoxicity when incubated with a repair deficient *E. coli* strain in the absence of hepatic enzymes, indicating DNA damage. On the other hand they failed to mutate the *S. typhimurium* strains TA98 and TA100, but in contrast, strain TA102, which detects oxidative mutagens (De Flora et al., 1984), was mutated by reduced amaranth and reduced sunset yellow (Sweeney et al., 1994).

Reduction can also modify the type of activity observed. The direct mutagenicities of Alizarin Yellow GG and Acid Alizarin Yellow R were eliminated by reduction, but in the presence of the exogenous metabolic system (S9), the resulting products were mutagenic and exhibited frame-shift activity (Brown et al., 1978; Combes & Haveland-Smith, 1982).

The Sudan dyes I, II, III and IV are oil-soluble azo dyes (1-amino-2-naphthol-based azo dyes), widely used in coloring plastics, leather, fabrics, printing inks, waxes and floor polishes (An et al., 2007; Xu et al., 2010). Sudan I is a liver and urinary bladder carcinogen in mammals and is also considered as a possible human mutagen, since it can produce the benzenediazonium ion during metabolism catalyzed by cytochrome P450, which could be the mechanism by which Sudan I is activated leading to a carcinogenic final product (Stiborová et al., 2002, 2005; Xu et al., 2010). An et al. (2007) found a dose-dependent increase in DNA migration in the comet assay, and in the frequency of micronuclei with all the concentrations of Sudan I tested (25–100 μ M). These data suggest that Sudan I caused breaks in DNA strands and chromosomes. Sudan II causes mutations in *Salmonella Typhimurium* TA 1538 in the presence of a rat liver preparation (Garner & Nutman, 1977; Xu et al., 2010). Concern about the safety of Sudan III, which is used in cosmetics, has arisen from its potential metabolic cleavage by skin bacteria producing 4-aminoazobenzene and aniline (Pielesz et al., 2002), and Sudan IV has been shown to require reduction and microsomal activation in order to be mutagenic (Brown et al., 1978). These are important mechanisms, since, with the exception of Sudan II, Xu et al. (2010) showed that the bacteria found in the human colon are frequently able to reduce Sudan dyes (Xu et al., 2010).

Almost all the azo dyes are reduced *in vivo*, but the reduction of the ingested dose is frequently incomplete, and thus a certain amount of the dye can be excreted in the unchanged or conjugated form. For instance, in the case of orally dosing rats with Sudan III, none of the expected reduction products was excreted, although p-aminophenol could be detected in the urine after i.p. injection (IARC, 1975; Combes & Haveland-Smith, 1982). This

may have been due to the formation of hydrazones at one of the azo bridges of this diazo dye, which might make it resistant to intestinal reduction (Combes & Haveland-Smith, 1982).

Stahlmann et al. (2006) reported investigations made to evaluate the sensitizing and allergenic potentials of two metabolites expected to be formed by the metabolic activity of skin bacteria and/or by metabolism in the skin. Two metabolites (4-aminoacetanilide and 2-amino-*p*-cresol) of Disperse Yellow 3, an azo dye widely used in the textile industry, were tested using modified local lymph node assay protocols in NMRI mice. The metabolite 2-amino-*p*-cresol gave a clearly positive response in the sensitisation protocol, showing marked increases in lymph node weight and cell proliferation, accompanied by a relative decrease in T-cells and relative increases in B-cells and 1A⁺ cells. Hence, 2-amino-*p*-cresol can be considered to be a stronger allergen in this model. In contrast, 4-aminoacetanilide only led to an increase in lymph node weight and cellularity at the higher concentration of 30%, with no consistent changes in the phenotypic analysis, indicating that this metabolite alone was a weak sensitizer (Stahlmann et al., 2006).

2.4 Dying processing plants effluents and their treatments

The textile industry accounts for two-thirds of the total dyestuff market (Fang et al., 2004; Elisangela et al., 2009). As mentioned before, part of dye used in the textile dyeing process does not attach to the fibers, remaining in the dye baths and eventually being discharged in the wastewater (Fang et al., 2004). The resulting wastewater is usually treated with activated sludge, and the liquid effluent is released to adjacent surface waters (Umbuzeiro et al., 2005 b).

Many dyes do not degrade easily due to their complex structure and textile dye effluent does not decolorize even if the effluent is treated by the municipal wastewater treatment systems (Shaul et al., 1991; Robinson et al., 2002; Forgacs et al., 2004). A study carried out in 1989 showed that the commercial aminoazobenzene dye, C.I. Disperse Blue 79, was not degraded by a conventionally operated activated sludge process and that 85% of the dye remained in the system. Of this 85%, 3% was retained by the primary sludge, 62% by the activated sludge and 20% was found in the final liquid effluent released into the environment (US EPA, 1989; Umbuzeiro et al., 2005 b). The use of an anaerobic system before the activated sludge treatment can result in cleavage of the azo bonds and the release of the corresponding aromatic amines. However, the colourless aromatic amines produced by these anaerobic microorganisms can be highly toxic and carcinogenic (Hu, 1994; Banat et al., 1996; Robinson et al., 2002).

Ekici et al. (2001) tested the stability of selected azo dye metabolites in both activated sludge and water and concluded that they were relatively stable in the aquatic environment and could not be efficiently degraded in wastewater plant systems. With respect to their mutagenicity, Fracasso et al. (1992) showed that dye factory effluents from primary and secondary biological treatments increased their levels of mutagenic activity as compared to the raw (untreated) effluent. The use of activated carbon filtration was beneficial but did not completely remove the mutagenic activity of the final effluent (Fracasso et al., 1992; Umbuzeiro et al., 2005 b).

Azo dyes are usually designed to resist biodegradation under aerobic conditions, the recalcitrance of these compounds being attributed to the presence of sulfonate groups and azo bonds. On the other hand, the vulnerability of reducing the azo bonds by different

mechanisms (e.g. biotreatment in anaerobic conditions) could result in the generation of aromatic amines, which are somewhat toxic and carcinogenic (Öztürk & Abdullah, 2006; Bae & Freeman, 2007; Kusic et al., 2011). It should also be mentioned that azo dyes are associated with various health risks to humans, and therefore colored wastewaters should be efficiently treated prior to discharge into the natural water bodies (Kusic et al., 2011).

Several methods are used to decolorize textile effluents including physicochemical methods such as filtration and coagulation, activated carbon and chemical flocculation (Gogate & Pandit, 2004). These methods involve the formation of a concentrated sludge, which, in turn creates a secondary disposal problem (Maier et al., 2004; Elisangela et al., 2009), since these methods merely transfer the pollution from one phase to another, which still requires secondary treatment (Gogate & Pandit, 2004; Kusic et al., 2011). Recently, new biological processes have been developed for dye degradation and wastewater reuse, including the use of aerobic and anaerobic bacteria and fungi (Elisangela et al., 2009).

The decolorizing of azo dyes using a fungal peroxidase system is another promising method (Hu, 1994). The ligninolytics are the most widely researched fungi for dye degradation (Elisangela et al., 2009) and of these, the white-rot fungi have been shown to be the most efficient organisms for the degrading of various types of dye such as azo, heterocyclic, reactive and polymeric dyes (Novotný et al., 2004). These fungi produce lignin peroxidase, manganese-peroxidase and laccase, which degrade many aromatic compounds due to their nonspecific systems (Forgacs et al., 2004; Revankar & Lele, 2007; Madhavi et al., 2007). However, all these processes for the mineralization of azo dyes need to be carried out in a separate process, since the dye compounds cannot be incorporated into the medium, and this would be impractical due to the great volume of wastewater requiring treatment (Hu, 1994). In addition, the long growth cycle and complexity of the textile effluents, which are extremely variable in their compositions, limit the performance of these fungi. Although the stable operation of continuous fungal bioreactors for the treatment of synthetic dye solutions has been achieved, the application of white-rot fungi for the removal of dyes from textile wastewaters still confronts many problems due to the large volumes produced, the nature of the synthetic dyes and the biomass control (Nigam et al., 2000; Mielgo et al., 2001; Robinson et al., 2001b; Elisangela et al., 2009).

Of the chemical methods under development, advanced oxidation processes (AOPs) seem to be a promising option for the treatment of toxic and non-biodegradable organic compounds in various types of wastewater, including the colored ones (Forgacs et al., 2004; Gogate & Pandit, 2004; Kusic et al., 2011). AOPs have received considerable attention due to their potential to completely oxidize the majority of the organic compounds present in the water. AOPs could serve as oxidative pretreatment method to convert non or low-biodegradable organic pollutants into readily biodegradable contaminants (Mantzavinos & Psillakis, 2004; Kusic et al., 2011). The electron beam (EB) treatment is also included in the class of AOPs, and laboratory investigations, pilot-plant experiments and industrially established technology have shown the efficiency of the EB treatment in destroying textile dyes in aqueous solutions (Han et al., 2002; Pálfi et al., 2011).

Chlorine has been extensively used as a complementary treatment to remove or reduce the color of industrial effluents containing dyes, and also to disinfect the water in drinking water treatment plants (Sarasa et al., 1998; Oliveira et al., 2010). The discoloration process using sodium hypochlorite (NaOCl) or chlorine gas, is based on the electrophilic attack of the amino group, and subsequent cleavage of the chromophore group (responsible for the

dye color) (Slokar & Marechal, 1998). However, the treatment of textile effluents using the conventional activated sludge method followed by a chlorination step, is not usually an effective method to remove azo dyes, and can generate products which are more mutagenic than the original untreated dyes, such as PBTAs (chlorinated 2-phenylbenzotriazoles). It has been reported that conventional chlorination should be used with caution in the treatment of aqueous samples contaminated with azo dyes (Umbuzeiro et al., 2005b; Oliveira et al., 2010).

Another alternative could be the use of photoelectrocatalysis on titanium supported nanocrystalline titanium dioxide thin film electrodes, where active chlorine is produced promoting the rapid degradation of reactive dyes (Carneiro et al., 2004; Osugi et al., 2009). Osugi et al. (2009) investigated the decolorizing of the mutagenic azo dyes Disperse Red 1, Disperse Red 13 and Disperse Orange 1 by chemical chlorination and photoelectrochemical oxidation on Ti/TiO₂ thin-film electrodes using NaCl and Na₂SO₄ media. After 1 h of treatment, 100% decolorizing was achieved with all the methods tested. After 1 h of photoelectrocatalytic oxidation, all the dye solutions showed complete reduction of the mutagenic activity using the strains TA98 of *Salmonella* in the absence or presence of the S9 mix, suggesting that this process could be a good option for the removal of disperse azo dyes from aqueous media. The results involving conventional chlorination showed that this method did not remove the mutagenic response from the dyes, and in fact promoted an increase in mutagenic activity in the presence of metabolic activity for Disperse Red 13 (Osugi et al., 2009).

3. Conclusions

The discharge of azo dyes into water bodies presents human and ecological risks, since both the original dyes and their biotransformation products can show toxic effects, mainly causing DNA damage. Azo dyes are widely used by different industries, and part of the dyes used for coloring purposes is discharged into the environment. The azo dyes constitute an important class of environmental mutagens, and hence the development of non-genotoxic dyes and investment in research to find effective treatments for effluents and drinking water is required, in order to avoid environmental and human exposure to these compounds and prevent the deleterious effects they can have on humans and aquatic organisms.

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5. References

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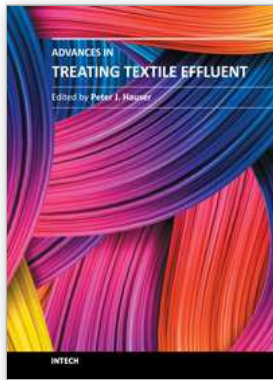
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