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Azo dyes in clothing textiles can be cleaved into a series of mutagenic aromatic amines which are not regulated yet

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ABSTRACT

Azo dyes represent the by far most important class of textile dyes. Their biotransformation by various skin bacteria may release aromatic amines (AAs) which might be dermally absorbed to a major extent. Certain AAs are well known to have genotoxic and/or carcinogenic properties. Correspondingly, azo dyes releasing one of the 22 known carcinogenic AAs are banned from clothing textiles in the European Union. In the present study, we investigated the mutagenicity of 397 non-regulated AAs potentially released from the 470 known textile azo dyes. We identified 36 mutagenic AAs via publicly available databases. After predicting their mutagenicity potential using the method by Bentzien, we accordingly allocated them into different priority groups. Ames tests on 18 AAs of high priority showed that 4 substances (22%) (CASRN 84-67-3, 615-47-4, 3282-99-3, 15791-87-4) are mutagenic in the strain TA98 and/or TA100 with and/or without rat S9 mix. Overall, combining the information from the Ames tests and the publicly available data, we identified 40 mutagenic AAs being potential cleavage products of approximately 180 different parent azo dyes comprising 38% of the azo dyes in our database. The outcome of this study indicates that mutagenic AAs in textile azo dyes are of much higher concern than previously expected, which entails implications on the product design and possibly on the regulation of azo dyes in the future.

1. Introduction

Azo dyes represent the most commonly used group of dyes in textile industry (Lacasse and Baumann, 2004; FriedliPartner, 2009a, 2009b; Brüschweiler et al., 2014). They were reported to constitute 60–70% of all dyestuff concerning textile production (Rawat et al., 2016). Dermal, systemic and bacterial biotransformation of azo dyes can release aromatic amines (AAs) (BGFA, 2009; Platzek et al., 1999; Stingley et al., 2010). AAs on the skin might be dermally absorbed to a major extent (Korinth et al., 2013).

AAs are used as intermediates in the synthesis of azo dyes (Weglarz-Tomczak and Gorecki, 2012; Freeman, 2013). As recently reviewed by Platzek (2010), AAs exposures from consumer products bear risks for human health, particularly associated to mutagenic and/or carcinogenic properties of certain AAs. Toxicity of AAs depends on the metabolic activation of the amino group, which can generate the reactive intermediate hydroxylamine known to damage DNA and proteins (Neumann, 2010).

Azo dyes which may release one of the 22 as yet regulated carcinogenic AAs are banned from clothing textiles in the European Union (Annex XVII of the REACH regulation; No, 1907/2006) (EC, 2009) and in national regulations, e.g. in Switzerland in the Ordinance about objects with human contact (SR 817.023.41) (FDHA, 2005). Regulation of these 22 AAs was based on their classification as carcinogens, whereupon 14 were assigned to category I and II according to the previous EU system (today 1A and 1B) and 8 to the previous carcinogenic class A1 and As by the German Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area (MAK) (LGC, 1998; CSTEE, 1999).

From the 896 azo dyes with known chemical structure in our textile dyes database (FriedliPartner, 2009b; Brüschweiler et al., 2014), 426 azo dyes (48%) are potential parent compounds of one or more of the 22 regulated AAs, while the other 470 azo dyes (52%) are exclusively metabolized to non-regulated[1] AAs.

Biotransformation of these 470 azo dyes can release 397

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[1] non-regulated only in the context of clothing textiles and not in the context of other regulations (e.g. CLP).

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different AAs from which Brüschweiler et al. (2014) could compile a priority list with 15 non-regulated AAs suspected mostly to be genotoxic and/or in some cases carcinogenic based on available literature. Following reductive cleavage according to EN 14362-1 (DIN, 2012) performed on 153 samples of clothing textiles that were purchased from clothing retail outlets in Switzerland, four potentially genotoxic and/or carcinogenic substances, 2,2'-dimethylbenzidine, 4-aminophenol, 4-ethoxyaniline and aniline, were detected. Within these analyses amounts up to 588 mg/kg aniline and 134 mg/kg 4-ethoxyaniline were measured in textiles (Brüschweiler et al., 2014; KL BE, 2014).

This study aims at the investigation of the mutagenicity of AAs representing cleavage products of azo dyes used in clothing textiles, since a systematic evaluation is missing so far. In a first step, we applied a modified *in silico* method by Bentzien et al. (2010) to predict Ames activities of primary AAs by calculating the stability of the metabolically intermediate nitrenium ions. Based on the outcome of this analysis and other criteria, we assigned the AAs into different priority groups. We selected 23 AAs and performed experimental Ames tests in the strains TA98 and TA100 with and without metabolic activation to clarify their mutagenicity. Additionally, we queried publicly available relevant databases and literature for relevant experimental Ames test data.

2. Materials and methods

2.1. Dataset

This study refers to an inventory of textile dyes using available data sources from dye producers, industrial associations, textile labels, seals of quality, official authorities and scientific institutions (FriedliPartner, 2009a, 2009b). The database contains 470 azo dyes that can be cleaved into 397 unique, non-regulated[1] AAs (Brüschweiler et al., 2014; this study).

2.2. Sources for mutagenicity data

The available experimental mutagenicity data were taken from the mutagenicity dataset by Kazius et al. (2005), the Chemical Carcinogenesis Research Information System (CCRIS), and the Carcinogenicity Potency Database (CPDB). The CCRIS database contains chemical records with mutagenicity, carcinogenicity, tumor promotion, and tumor inhibition test results. It was developed by the National Cancer Institute (NCI). Data are derived from studies cited in primary journals, current awareness tools, NCI reports, and other sources. Test results have been reviewed by experts in carcinogenesis and mutagenesis. Further important data sources were the informations on chemicals by ECHA and Jung et al. (1992).

2.3. Ames test

The bacterial reverse-mutation screening assay (Ames test) with the *S. typhimurium* strains TA98 and TA100 was designed to be compatible with the procedure indicated in the OECD test guideline No. 471, with and without metabolic activation (S9 mix) (OECD, 1997). The work was conducted by Envigo CRS GmbH (Rossdorf, Germany) following good laboratory practices and adhering to the applicable standard operation procedures. These strains were chosen as they are used in reduced versions of the Ames test because TA98 is capable of detecting frameshift mutations, while TA100 detects base pair substitutions. Harding et al., 2015 could demonstrate the significance of TA98 and TA100 for the detection of AA mutagenicity.

In a first experiment, a plate incorporation assay was performed with all test substances. In the case a borderline result was obtained, a second experiment in form of a pre-incubation assay was performed. The *S. typhimurium* strains TA98 and TA100 were obtained from Trinova Biochem GmbH (Giessen, Germany). All test substances were dissolved in DMSO. As positive control substance without metabolic activation, sodium azide (NaN_3) was used in the strain TA100 and 4-nitro-o-phenylene-diamine (4-NOPD) in the strain TA98. With metabolic activation, 2-aminoanthracene (2-AA) was used a positive control. Concurrent untreated and solvent controls were also performed. For the preparation of the S9-mix, phenobarbital/β-naphthoflavone-induced rat liver S9 was used as the metabolic activation system.

The assay was considered acceptable, if the following criteria were met: i) negative and solvent control show a regular background growth ii) spontaneous reversion rates in the negative and solvent control are in the range of the laboratory's historical control data, iii) positive control substances at least produce a twofold increase compared to the colony count of the corresponding solvent control, and iv) a minimum of five evaluable dose levels were present with at least three dose levels showing no signs of toxic effects, evident as a reduction in the number of revertants below the indication factor of 0.5. A substance was considered mutagenic, if the number of revertants shows a biologically relevant increase exceeding the threshold of twice the colony count of the corresponding solvent control. A dose dependent increase was considered biologically relevant, if the threshold is exceeded at more than one dose level. An increase exceeding the threshold at only one dose level was considered biologically relevant, if it was reproducible in an independent second experiment. A dose dependent increase in the number of revertant colonies below the threshold was regarded as an indication of a mutagenic potential, if it was reproducable in an independent second experiment. However, whenever the colony counts remained within the historical range of negative and solvent controls such an increase was not considered biologically relevant.

2.4. Test chemicals

2,5-Dichlorosulfanilic acid (CAS Registry Number (CASRN) 88-50-6, 98% purity), aniline-2,5-disulfonic acid (98-44-2, 95%), 1,2,4-triaminobenzene dihydrochloride (615-47-4, 96%), 3,3'-dihydroxybenzidine (2373-98-0, 95%), 4-amino-3-methylphenol (2835-99-6, 98%), 1,1-bis(4-aminophenyl)cyclohexane (3282-99-3, 98%), 3,5-dimethyl-1H-pyrazol-4-amine (5272-86-6, 95%), 4,6-diaminoresorcinol dihydrochloride (15791-87-4, 98%), 4-amino-3-hydroxy-N-(2-methoxyphenyl)-2-naphthamide (23342-49-6, 95%), N4-ethyl-N4-(2-hydroxyethyl)-2-methyl-1,4-phenylenediamine sulfate (25646-77-9, 98%), (4-amino-2-methylphenyl)dimethylamine (27746-11-8, 95%), 2-amino-1-naphthol hydrochloride (41772-23-0, 98%), 3-methyl-1-phenyl-1H-pyrazole-4,5-diamine (52943-88-1, 95%), and 4-(4-aminophenyl)thiomorpholine 1,1-dioxide (105297-10-7, 98%) were purchased from abcr GmbH (Karlsruhe, Germany). 2,2′-Dimethylbenzidine (84-67-3, 95%), 2-(4-amino(ethyl)anilino) ethanol (92-65-9, 98%), 3-amino-4-chlorobenzenesulfonic acid (98-36-2, 97%), 2-amino-1,5-naphthalenedisulfonic acid (117-62-4, purity not determined), 4-amino-1,3-benzenedisulfonic acid (137-51-9, 97%), N4,N4-diethyl-2-methyl-1,4-benzendiamine hydrochloride (2051-79-8, 97%), N-(4-Amino-3-methylphenyl)-N-ethylbenzamide (5856-00-8, 95%), 4-(1-(4-amino-3-methylphenyl)cyclohexyl)-2-methylphenylamine (6442-08-6, 95%), were obtained from Sigma-Aldrich Schweiz AG (Buchs SG, Switzerland). 1,2,4,5-Benzenetetraamine tetrahydrochloride (4506-66-5, 97%) was purchased from ChemPur (Karlsruhe, Germany).

3. Calculations

3.1. Validation dataset for the in silico predictions

Kazius et al. (2005) compiled a database with Ames results for 4337 substances (Organon Ames dataset). Bentzien et al. (2010) selected a subset of primary AAs out of the Kazius database that (i) contain no electric charge in the formula, (ii) have molecular weight below 500 Da, (iii) have no more than one stereocenter, (iv) have less than 10 rotatable bonds, (v) have only one AA functionality, (vi) do not contain aromatic nitro groups as they could exhibit Ames toxicity because of their nitro-moiety. This subset was used to validate our own implementation. Primary AAs CASRNs were downloaded from the supporting information in Bentzien et al. (2010). The complete Kazius database was retrieved as an SD file from www.cheminformatics.org and primary AAs were extracted based on the CASRN.

3.2. In silico preparation of the chemical structures

Cleavage products were downloaded as SMILES string from Brüschweiler et al. (2014) supplementary database and converted to an SD file. Structures were salt-stripped and neutralized, except those with a fixed formal charge (e.g. quaternary ammonium). In particular, carboxylic acids and basic nitrogen were drawn in their neutral forms. The most abundant protomer was selected on the basis of the primary AA structure. No reassessment of the major protomer form was performed on the nitrenium ions. The 3D geometry of structures was optimized using our own implementation of the MMFF94s force field (Halgren, 1996a, 1996b, 1996c, 1996d, 1999a, 1999b; Halgren and Nachbar, 1996) and the lowest energy conformation of the primary AA was used in further quantum mechanics calculations.

3.3. Quantum mechanics calculations

Energy calculations are based on the procedure proposed by Bentzien et al. (2010) and include some modifications proposed by McCarren et al. (2011). The Bentzien method requires only the calculation of $ArNH_2$ and ArNH + energies, but we also calculated the energy of ArNH- as proposed by Shamovsky et al. (2012). $ArNH^+$ and $ArNH^-$ forms were generated by removing hydrogen bond to the AA nitrogen. When several primary AAs were present in the molecule, each one was processed independently. This yielded $2n$ geometries for each species, where n is the number of primary AAs in the molecule. $ArNH^+$ and $ArNH^-$ geometries were not optimized after removing the hydrogen. A single point energy was calculated with GAMESS (Schmidt et al., 1993; Gordon and Schmidt, 2005), using B3LYP5 hybrid functional with a 6-31 + G* basis set for all atoms. Larger basis sets were tested, though no significant change in ranking was noticed, as already described by McCarren et al. (2011). The $ArNH^+$ formation energy (ΔE_{ArNH+}) is equal to the energy of the amine (E_{ArNH2}) subtracted from the energy of the lowest energy nitrenium ion (E_{ArNH+}) plus hydride anion. $ArNH^+$ formation energies are then expressed relatively to the formation energy of aniline ($PhNH_2$), and are therefore denoted $\Delta\Delta E_{ArNH+}$.

$$\Delta\Delta E_{ArNH+} = \Delta E_{ArNH+} - \Delta E_{PhNH+} = (E_{ArNH+} - E_{ArNH2}) - (E_{PhNH+} - E_{PhNH2})$$

Similarly the relative $ArNH^-$ formation energy relative to $PhNH_2$ is defined by

$$\Delta\Delta E_{ArNH-} = (E_{ArNH-} - E_{ArNH2}) - (E_{PhNH-} - E_{PhNH2})$$

3.4. Prioritization

Cleavage products were allocated to one of the following priorities: priority 1 (P1) are potential mutagens to test in highest priority, priority 2 (P2) are other potential mutagens to test, priority 3 (P3) are substances for which Ames test results could be found in a database, or substances for which the prediction was borderline, priority 4 (P4) are predicted to be non-mutagens, and priority P5 are substances for which quantum mechanics calculation failed. Cleavage products were clustered according to the substituents found on the aryl ring in order to select substances representative of the structural diversity.

Substances in priority group 1 (P1) were selected from structures for which $\Delta\Delta E_{ArNH+} < -15$ kcal/mol, which do not contain sulfonic acid, sulfonamide, sulfonic ester, or 2-aminophenols. Whenever possible, we chose substances with $\Delta\Delta E_{ArNH-} < 0$ kcal/mol. Additionally, at most two representatives of each structural cluster were selected. Substances with $\Delta\Delta E_{ArNH-} > 0$ or in clusters with more than two substances, and the 2-aminophenols were assigned to P2. Substances with $\Delta\Delta E_{ArNH+} > +15$ kcal/mol and which did not contain any other obvious toxicophores (e.g. nitro) were assigned to P4. Substances for which the quantum mechanics calculation took too long or failed were classified as P5. All the other substances (substances with Ames test results available, $\Delta\Delta E_{ArNH+}$ between −15 and + 15 kcal/mol, or containing sulfonic acids, sulfonamide or sulfonic ester) were assigned to P3.

4. Results

4.1. Prediction of mutagenicity potential by bentzien

4.1.1. Validation dataset

Energies of the substances in the Bentzien dataset were calculated as described above. The truth table and statistics are reported in Tables 1 and 2. The sensitivity (93%) is identical to the one reported by Bentzien. The specificity calculated in our own validation test is slightly higher than the one reported by Bentzien (72% vs 62%). Overall, the accuracy of our implementation is comparable to that reported by Bentzien.

4.1.2. Validation set with 15 kcal/mol gray zone

Bentzien introduced a gray zone by excluding substances for which $\Delta\Delta E_{ArNH+}$ is in the range ± 15 kcal/mol. The purpose of this gray zone is to avoid predicting borderline substances for which $\Delta\Delta E_{ArNH+}$ is close to 0 and for which energy calculation errors might overweight the energy of stabilization of the ArNH + cation.

The accuracy with the gray zone is slightly higher than that of predictions without a gray zone (92% vs 87%). However, with this gray zone, half of the substances are excluded from the prediction; they are not classified as mutagenic or non-mutagenic.

4.1.3. Cleavage products

The energies of cleavage products were calculated as described above. 397 cleavage products were processed, and energies could be calculated for 366 substances (Supplementary Table 1). The 31 substances for which energy was not calculated were either large substances (MW > 500 Da) for which the calculation took too long or metal complexes. Among the 366 predicted substances, 179 substances (48.9%) had $\Delta\Delta E_{ArNH+}$ smaller than −15 kcal/mol, 68 substances (18.6%) had $\Delta\Delta E_{ArNH+}$ between −15 kcal/mol and 0 kcal/mol, 57 substances (15.6%) had $\Delta\Delta E_{ArNH+}$ between 0 and + 15 kcal/mol and 62 substances (16.9%) had $\Delta\Delta E_{ArNH+}$ greater than +15 kcal/mol. We used the prediction method with the ± 15 kcal/mol grey zone because it was the most accurate on the validation dataset.

Table 1
Truth Tables for Ames Predictions using different grey zones.

	No grey zone All compounds predicted		Grey zone ±15.0 kcal/mol Compounds within ±15.0 kcal/mol are excluded from prediction	
	Predicted Ames positive	Predicted Ames negative	Predicted Ames positive	Predicted Ames negative
Ames positive experimental	165	13	105	4
Ames negative experimental	21	53	6	11

Table 2
Summary statistics for ames prediction.

	No grey zone	Grey zone ±15.0 kcal/mol
Sensitivity	93%	96%
Specificity	72%	65%
Accuracy	87%	92%
Compounds in grey zone	0%	50%

Aiming at the prioritization of the substances for subsequent testing it was more important to increase accuracy than to assign a prediction to the 125 (34.1%) substances falling in the grey zone.

4.2. Prioritization and selection of test substances

Thirty cleavage products were assigned to P1, 91 to P2, 216 to P3, 53 substances among those having known Ames results. Twenty-nine cleavage products were assigned to P4 and 31 cleavage products to P5.

There were several challenges in the selection process of the test substances for the Ames test: i) principal availability of the substances by suppliers, ii) sufficiently high level of purity (≥95%) of the substances, and iii) an affordable price. We came up with the selection of 5 P1 substances, 13 P2 substances and 5 P4 substances, in total 23 substances.

4.3. Ames test

Four substances out of the total 23 substances gave positive results in the Ames screening test: 4,6-Diaminoresorcinol dihydrochloride (CASRN 15791-87-4) was positive in the plate incorporation test in TA98 (+S9) and in TA100 (±S9). 1,1-Bis(4-aminophenyl)cyclohexane (3282-99-3) was positive in experiment I in TA100 (+S9). 1,2,4-Triaminobenzene dihydrochloride (615-47-4) was positive in TA98 (-S9) and slightly positive in TA98 (+S9), while the outcome in experiment II was clearly positive in TA98 (±S9). 2,2'-dimethylbenzidine (84-67-3) was positive in TA100 (+S9). The individual results of the Ames positive AAs are given in the Supplement.

Due to borderline results in the first experiment, a pre-incubation assay was performed for four substances. While the positive finding for 1,2,4-triaminobenzene dihydrochloride (615-47-4) could be confirmed in experiment II (see above), the other substances (4-amino-2-methylphenyl)dimethylamine (2776-118), N4-ethyl-N4-(2-hydroxyethyl)-2-methyl-1,4-phenylenediamine sulfate (25646-77-9), and 4-amino-3-hydroxy-N-(2-methoxyphenyl)-2-naphthamide (23342-49-6) were found to be negative in experiment II. 4-Amino-3-hydroxy-N-(2-methoxyphenyl)-2-naphthamide revealed a more than twofold induction of revertants in TA100 without metabolic activation only at the highest tested dose in experiment II (Supplement). Consequently, it did not fulfill the criteria for a positive result (see section 2.3).

1,2,4,5-Benzentetraamine tetrahydrochloride (4506-66-5) was found to be negative. Cytotoxic effects could be observed at doses ≥1000 μg/plate (Supplement). The high cytotoxicity could impair the detection of potential mutagenic effects of this substance in the Ames test.

The five selected substances from P4 (2,5-dichlorosulfanilic acid (88-50-6), aniline-2,5-disulfonic acid (98-44-2), 4-amino-1,3-benzenedisulfonic acid (137-51-9), 3-amino-4-chloroben zenesulfonic acid (98-36-2), 2-amino-1,5-naphthalenedisulfonic acid (117-62-4) were confirmed to be negative in the experiment. They all contain one or two sulfonate groups.

4.4. Compilation of mutagenic AAs

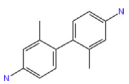
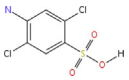
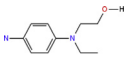
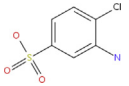
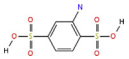
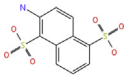
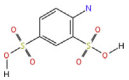
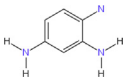
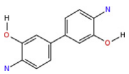
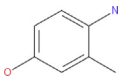
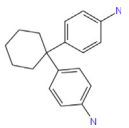
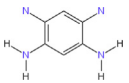
A complemental query for indications of mutagenicity in our database resulted in 33 additional AAs showing positive Ames test results next to the four AAs for which we obtained positive Ames test results in this study. These 33 additional AAs are part of the Kazius Mutagenicity Dataset (Kazius et al., 2005) basically referring to the CCRIS database (Table 4). Supplementary Table 2 depicts positive test results with information of the strains, test conditions and references. Additionally, three further substances with point of concern were found in our previous publication (Brüschweiler et al., 2014). Two of these three substances are also listed as *Salmonella* assay positive in the CPDB (Fig. 1). Overall, we identified a total of 40 different potential cleavage products of azo dyes being mutagenic substances, as indicated in different sources (Table 4). Seventeen substances, i.e. 43% of all positive AAs, were found to contain at least one nitro group. Four out of these 17 substances have two nitro groups. Six out of 40 positive substances have alkylgroups on the aromatic rings. Eleven out of 40 positive substances have diamino groups, one has a triamino group. Six out of 40 positive substances have a halogen (Br or Cl) on the aromatic ring. Only one positive substance has a sulfonate group (o-aminobenzenesulfonic acid).

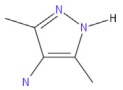
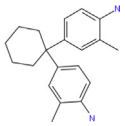
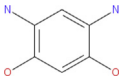
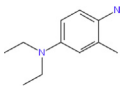
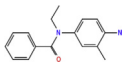
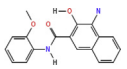
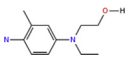
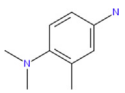
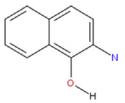
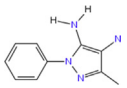
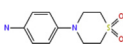
The identified 40 non-regulated mutagenic AAs are potential cleavage products of approximately 180 parent azo dyes (Supplementary Table 3). They represent 38% of all azo dyes in our database. This illustrates that that mutagenicity of AAs being potential cleavage products of azo dyes is of much higher concern than originally expected. The cleavage products with the highest number of parent compounds (n > 10) are: p-nitroaniline (CASRN 100-01-6; 30 parent compounds), aniline (62-53-3, 142-04-1; 23), 2-chloro-4-nitroaniline (121-87-9; 15), p-phenylenediamine (dihydrochloride) (624-50-3, 106-50-3; 13), and 1,4-naphthalenediamine (2243-61-0; 13).

5. Discussion and conclusions

Various computational models have been developed to predict the mutagenic potency of AAs. Computational models have focused on the stability of the nitrenium ion (Ford and Griffin, 1992; Bentzien et al., 2010; McCarren et al., 2011), anion formation energy (Shamovsky et al., 2012), hydrophobicity (Benigni et al., 2000, 2007), and expert rule-based models (e.g. Gadaleta et al., 2016). *In silico* predictivity of AA genotoxicity and carcinogenicity has proven

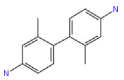
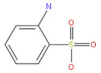
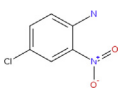
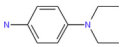
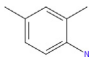
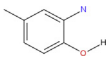
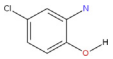
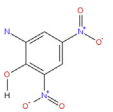
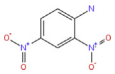
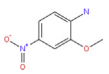
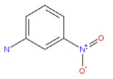
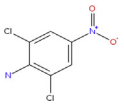
Table 3
Ames Screening test results.

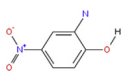
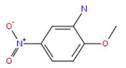
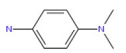
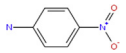
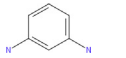
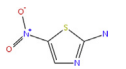
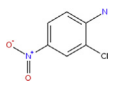
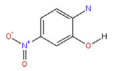
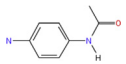
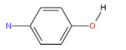
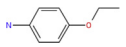
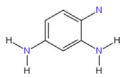
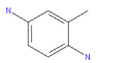
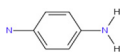
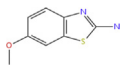
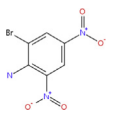
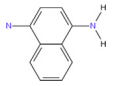
CASRN tested	Chemical name	Chemical structure *	Concentration range tested	Plate incorporation test (Experiment I)					Pre-incubation test (Experiment II)					Overall test results
				TA98 − S9	TA98 + S9	TA100 − S9	TA100 + S9	Remarks	TA98 − S9	TA98 + S9	TA100 − S9	TA100 + S9	Remarks	
84-67-3	4-amino-2,2-dimethyl(1,1'-biphenyl)-4-ylamine		3-5000 μg/plate	neg	neg	neg	pos							**pos**
88-50-6	2,5-dichlorosulfanilic acid		3-5000 μg/plate	neg	neg	neg	neg							neg
92-65-9	2-(4-amino(ethyl)anilino) ethanol		3-5000 μg/plate	neg	neg	neg	neg							neg
98-36-2	3-amino-4-chlorobenzenesulfonic acid		3-5000 μg/plate	neg	neg	neg	neg							neg
98-44-2	aniline-2,5-disulfonic acid		3-5000 μg/plate	neg	neg	neg	neg							neg
117-62-4	2-amino-1,5-naphthalenedisulfonic acid		3-5000 μg/plate	neg	neg	neg	neg							neg
137-51-9	4-amino-1,3-benzenedisulfonic acid		3-5000 μg/plate	neg	neg	neg	neg							neg
615-47-4	1,2,4-triaminobenzene dihydrochloride		3-5000 μg/plate exp. I, 1−1000 μg/plate exp. II without activation, 0.3−1000 μg/plate exp. II TA98 with activation, 3−2500 μg/plate exp. II TA100 with activation	pos	slightly pos	neg	neg		pos	pos	neg	neg		**pos**
2373-98-0	3,3'-dihydroxybenzidine		3-5000 μg/plate	neg	neg	neg	neg							neg
2835-99-6	4-amino-3-methylphenol		3-5000 μg/plate	neg	neg	neg	neg							neg
3282-99-3	1,1-bis(4-aminophenyl) cyclohexane		3-5000 μg/plate	neg	neg	neg	pos							**pos**
4506-66-5	1,2,4,5-benzenetetraamine tetrahydrochloride		3-5000 μg/plate	neg	neg	neg	neg	very cytotoxic at ≥ 1000 μg/plate						neg

5272-86-6	3,5-dimethyl-1H-pyrazol-4-amine		3-5000 μg/plate	neg	neg	neg	neg						neg
6442-08-6	4-(1-(4-amino-3-methylphenyl)cyclohexyl)-2-methylphenylamine		3-5000 μg/plate	neg	neg	neg	neg						neg
15791-87-4	4,6-diaminoresorcinol dihydrochloride		3-5000 μg/plate	neg	pos	pos	pos						**pos**
2051-79-8	N4,N4-diethyl-2-methyl-1,4-benzendiamine hydrochloride		3-5000 μg/plate	neg	neg	neg	neg						neg
5856-00-8	N-(4-amino-3-methylphenyl)-N-ethylbenzamide		3-5000 μg/plate	neg	neg	neg	neg						neg
23342-49-6	4-amino-3-hydroxy-N-(2-methoxyphenyl)-2-naphthamide		3-5000 μg/plate exp. I, 0.3–1000 μg/plate exp. II without metabolic activation, 3–5000 μg/plate exp. II with metabolic activation	neg	neg	neg	neg	neg	neg	pos (only at one dose)	neg		neg
25646-77-9	N4-ethyl-N4-(2-hydroxyethyl)-2-methyl-1,4-phenylenediamine sulfate		3-5000 μg/plate exp. I, 10–5000 μg/plate exp. II	neg	neg	neg	neg	neg	neg	neg	neg		neg
27746-11-8	(4-amino-2-methylphenyl) dimethylamine		3-5000 μg/plate exp. I, 10–5000 μg/plate exp. II	neg	slightly pos	neg	neg	neg	neg	neg	neg		neg
41772-23-0	2-amino-1-naphthol hydrochloride		3-5000 μg/plate	neg	neg	neg	neg						neg
52943-88-1	3-methyl-1-phenyl-1H-pyrazole-4,5-diamine		3-5000 μg/plate	neg	neg	neg	neg						neg
105297-10-7	4-(4-aminophenyl) thiomorpholine 1,1-dioxide		3-5000 μg/plate	neg	neg	neg	neg						neg

*: hydrogen atoms attached to nitrogen and oxygen are mostly not drawn in the chemical structure.
neg: negative.
pos: positive.

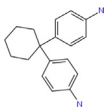
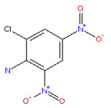
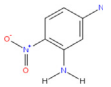
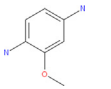
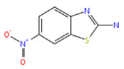
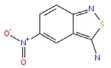
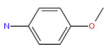
Table 4
Compilation of the identified mutagenic AAs.

CASRN	Chemical name	Chemical Structure [a]	Structural characteristics	Kazius/ CCRIS	CPDB	Brüschweiler et al. (2014) [*]	Ames screening (this study)	Ames test (Jung et al., 1992)	Salmonella/ microsome (Jung et al., 1992)	Germ cell mutagenicity (CLP regulation)	Germ cell mutagenicity (C&L inventory)	Carcinogenicity (CLP regulation)	Carcinogenicity (C&L inventory)
62-53-3; 142-04-1	aniline			active [f]				inactive	inactive	Muta. 2 (H341)	Muta. 2 (H341)	Carc. 2 (H351)	Carc. 2 (H351)
84-67-3; 198487-76-2	2,2'-dimethylbenzidine		benzidine, alkyl				active						
87-62-7	2,6-xylidine; 2,6-dimethylaniline		alkyl	inactive	active	point of concern [e]						Carc. 2 (H351)	Carc. 2 (H351)
88-21-1; 13846-13-4;	o-aminobenzenesulfonic acid		sulfate	active				inactive/ weak					
89-63-4	2-nitro-4-chloroaniline		nitro, halogen	active									
93-05-0; 2198-58-5;	N,N-diethyl-p-phenylenediamine		diamino	active									
95-68-1	2,4-dimethylaniline		alkyl	active		point of concern		active	active		Muta. 2 (H341)		
95-84-1	2-amino-4-methylphenol		alkyl, phenol	active									
95-85-2	2-amino-4-chlorophenol		phenol, halogen	active				weak	weak		Muta. 2 (H341)		Carc. 2 (H351)
96-91-3	4,6-dinitro-2-aminophenol		dinitro, phenol	active									
97-02-9	2,4-dinitroaniline		dinitro	active									
97-52-9	2-amino-5-nitroanisole		nitro, methoxy	active		point of concern					Muta. 2 (H341)		Carc. 2 (H351)
99-09-2	m-nitroaniline		nitro	active							Muta. 2 (H341)		
99-30-9	2,6-dichloro-4-nitroaniline		nitro, halogen	active	active								

99-57-0	2-amino-4-nitrophenol		nitro, phenol	active	active	point of concern			Muta. 2 (H341)	Carc. 2 (H351)
99-59-2	m-nitro-o-anisidine		nitro, methoxy		active	point of concern [c]				
99-98-9	N,N-dimethyl-p-phenylenediamine		diamino	active						
100-01-6	p-nitroaniline		nitro	active	active	point of concern				
108-45-2	m-phenylenediamine		diamino	active	active			Muta. 2 (H341)	Muta. 2 (H341)	
121-66-4	2-amino-5-nitrothiazole		nitro, thiazole	active	active	point of concern				Carc. 1B (H350); Carc. 2 (H351)
121-87-9	2-chloro-4-nitroaniline		nitro, halogen	active						
121-88-0	2-amino-5-nitrophenol		nitro, phenol	active	active	point of concern				Carc. 2 (H351)
122-80-5	p-aminoacetanilide		diamino, acetanilide	active						
123-30-8	p-aminophenol		phenol	inactive		point of concern [b]			Muta. 2 (H341)	Muta. 2 (H341)	
156-43-4	p-phenetidine		ethoxy	active					Muta. 2 (H341)	Muta. 2 (H341)	Carc. 1B (H350)
615-47-4; 615-71-4	1,2,4-triaminobenzene (dihydrochloride)		triamino				active			
615-50-9; 95-70-5; 25376-45-8	2-methyl-p-phenylenediamine; 2,5-diaminotoluene		diamino, alkyl	active		point of concern			Muta. 2 (H341)	Carc. 1B (H350); Carc. 2 (H351)
624-18-0; 106-50-3	p-phenylenediamine (dihydrochloride)		diamino	active	active					
1747-60-0	6-methoxy-2-benzothiazolamine		benzothiazole, methoxy	active		point of concern				
1817-73-8	2-bromo-4,6-dinitroaniline		dinitro, halogen	active						
2243-61-0	1,4-naphthalenediamine		diamino, naphthalene	active						

(*continued on next page*)

Table 4 (*continued*)

CASRN	Chemical name	Chemical Structure [a]	Structural characteristics	Kazius/ CCRIS	CPDB	Brüschweiler et al. (2014) *	Ames screening (this study)	Ames test (Jung et al., 1992)	Salmonella/ microsome (Jung et al., 1992)	Germ cell mutagenicity (CLP regulation)	Germ cell mutagenicity (C&L inventory)	Carcinogenicity (CLP regulation)	Carcinogenicity (C&L inventory)
3282-99-3	1,1-bis(4-aminophenyl) cyclohexane		bis(aminophenyl)				active						
3531-19-9	6-chloro-2,4-dinitroaniline		dinitro, halogen	active							Muta. 2 (H341)		
5131-58-8	4-nitro-m-phenylenediamine		diamino, nitro	active		point of concern							
5307-02-8	2,5-diaminoanisole		diamino, methxy	active									
6285-57-0	2-amino-6-nitrobenzothiazole		nitro, benzothiazole	active		point of concern							
6393-01-7	4-amino-2,5-dimethylaniline		diamino, alkyl	active									
14346-19-1	3-amino-5-nitro-2,1-benzisothiazole		nitro, benzisothiazole	active		point of concern							
16523-31-2; 15791-87-4	4,6-diaminoresorcinol (dihydrochloride)		diamino, phenol				active				Muta. 2 (H341)		
20265-97-8; 104-94-9	p-anisidine (hydrochloride)		methoxy	active	active [d]						Muta. 2 (H341)		Carc. 1B (H350)

*: 3,4-Dichloroaniline (CASRN 95-76-1) was not considered from the Brüschweiler et al. (2014) publication. *In vitro* genotoxicity tests were negative for gene and chromosome mutations. However, there is limited evidence for a mutagenic potential mainly due to a weakly positive SCE test *in vitro* and a positive test for induction of spindle damage *in vitro*. The clearly negative *in vivo* micronucleus tests indicate that this potential is unlikely to be expressed *in vivo* (ECB, 2006).

[a] Hydrogen atoms attached to nitrogen and oxygen are mostly not drawn in the chemical structure.

[b] Active in *E. coli* wp2uvra and *mouse lymphoma* L5178Y.

[c] Carcinogenic in rat and mouse [CCRIS].

[d] IARC: no data are available in humans. Inadequate evidence of carcinogenicity in animals. Overall evaluation: Group 3: The agent is not classifiable as to its carcinogenicity to humans/data lacking.

[e] Active in in vitro chromosomal aberration test in CHO cells.

[f] Also in *mouse lymphoma* assay with L5178Y, CHO cells.

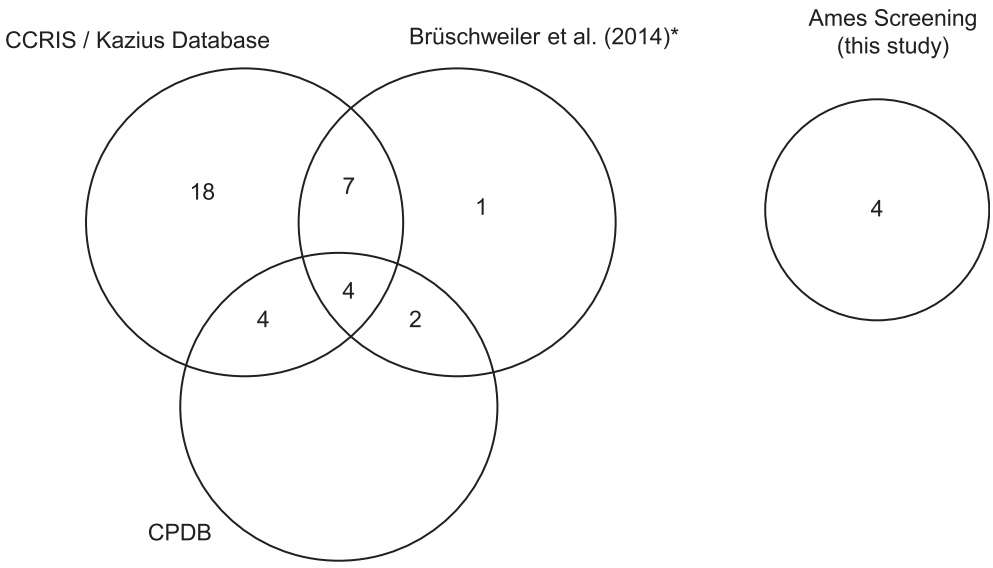


Fig. 1. Sources for the identification of mutagenic AAs as azo dye cleavage products used in clothing textiles *: 3,4-Dichloroaniline (CASRN 95-76-1) was not considered from the Brüschweiler et al. (2014) publication. In vitro genotoxicity tests were negative for gene and chromosome mutations. However, there is limited evidence for a mutagenic potential mainly due to a weakly positive SCE test *in vitro* and a positive test for induction of spindle damage *in vitro*. The clearly negative *in vivo* micronucleus tests indicate that this potential is unlikely to be expressed *in vivo* (ECB, 2006).

to be still a major challenge (Williams et al., 2015) what could be confirmed in this study by comparing the predictions with the experimental results. From the 18 substances assigned to priority group P1 or P2, only four substances (22%) were found to be mutagenic in the Ames screening test (Table 3). As a conclusion, our prioritization system based on the Bentzien method can be regarded as a very conservative method for a preliminary screening to select substance candidates for testing. It can only be validated empirically whether a substance is positive in the Ames test or not.

Since AAs are an important class of intermediates in drug synthesis and are metabolites of various drugs, there are great ongoing efforts by pharmaceutical companies to enlarge the AA mutagenicity database, share the data and develop more predictive models (Ahlberg et al., 2016; Elder et al., 2015). Pharmaceutical companies set up a consortium dedicated to the investigation of genotoxicity of aromatic amines that performs Ames tests with drug relevant AAs. However, coverage of AAs as cleavage products from azo dyes in textile industry was found to be very small in the CIGAA database with only 11 matches (A.-L. Werner, Lhasa Limited, Leeds UK, personal communication).

Ahlberg et al. (2016) have compiled a set of activating and deactivating functional groups. A well known deactivating group is sulfonate independent on its position to the amino-group. Sulfonated AAs, in contrast with some of their unsulfonated analogues, have generally no or a very low genotoxic potential (Jung et al., 1992). This was confirmed in this study: five sulfonated AAs for which no experimental mutagenicity test data could be found, were shown to be negative in the Ames screening test.

Nitro group substituted AAs are the pre-dominant class of mutagenic AAs in this evaluation, comprising 17 out of the 40 identified substances, i.e. 43% of all positive AAs. Bronaugh and Maibach (1985) studied the percutaneous absorption of nitroaromatic compounds *in vivo* and *in vitro* in human and monkey. They could observe rapid absorption of all substances tested and assumed that dermal absorption of nitro anilines might be as considerable as for other AAs.

From the identified 40 mutagenic AAs in this study, only 4 (10%) are classified as mutagens according to the CLP regulation (EC, 2008, 2015, 2016a, 2016b) and 13 substances (32.5%) corresponding to the assignment by notifiers (ECHA, 2016). Two substances (5%) have a classification as a carcinogen under the CLP regulation and 10 substances (25%) according to the classification by notifiers. Prospectively, clarification regarding different carcinogenic classifications by the notifiers and consequently harmonization of classification may follow. Until today, 22 known carcinogenic AAs and their corresponding parent azo dyes are prohibited in the EU. Recently, the European Commission performed a fast-track consultation on a possible restriction of more hazardous substances (CMR 1A and 1B) in textile articles and clothing for consumer use (EC, 2016c). Beside the regulated AAs, the list contains 6 arylamines, 2 azo dyes, and 6 dyes/colorants with carcinogenic potential, which may cleave to carcinogenic aromatic amines or are carcinogenic, mutagenic or toxic to reproduction. None of the AAs proposed for restriction was found in our database.

AAs which are prohibited in clothing textiles in Annex XVII of REACH must be classified as carcinogen category 1A or 1B and/or as mutagen category 1A or 1B. The mutagens and carcinogens classified as category 2 identified within the present study do not have to be restricted based on the existing REACH regulation. We notice that substances in clothing textiles needs more comprehensive data requirements regarding the mutagenicity for their restriction due to their carcinogenic and/or genotoxic effects. Substances for other consumer products such as food contact materials (FCMs) for instance, cannot be authorized, when they show positive Ames test results unless there is an overruling negative *in vivo* genotoxicity test result (EFSA, 2008, 2011, 2016). For most of the AAs representing cleavage products of azo dyes in textiles, no such comprehensive genotoxicity dataset is available. Data requirements depend on the production amounts being mostly low in Europe and therefore, the data on mutagenicity is not available or insufficient. Dyes for textiles on the European market are mostly produced in Asia (e.g. China, India, Bangladesh, Indonesia). Clothing textiles on the European market were mostly dyed in those Asian countries, and are then imported to Europe. As potential cleavage products of azo dyes in textile articles, AAs principally do not fall under the REACH criteria for the requirement for genotoxicity testing. If

genotoxicity testing is performed for azo dyes, the OECD testing guideline 471 mentions that using a reductive metabolic activation may be more appropriate. The standard method was established by Prival et al. (1984) using uninduced hamster liver S9 that is able to reduce the azo bond. However, it still has to be demonstrated whether the uninduced hamster liver S9 is able to bioactivate AAs to the same extent as the Aroclor1254-or phenobarbital/β-naphthoflavone-induced rat liver S9.

Environment Canada & Health Canada (2016) tried to quantify the health risks of consumers wearing textiles for several carcinogenic AAs (e.g. 2-napthylamine, o-toluidine, 2,4-diaminotoluene) based on a model developed by Zeilmaker et al. (1999). For the exposure estimate, they used the measured content of AAs after azo dye reduction per kg textile and calculated a leaching rate over 20 wears. They made several conservative assumptions such as 100% dermal absorption and 10% probability that a given AA is present in textile materials and is released. They compared the estimated exposure with a toxicological reference value (BMDL10 or NOAEL) and calculated a margin of exposure which was large enough in all cases. For that reason the health risk was considered low. However, the content of other mutagenic AAs might be much higher than in the assessment by Health Canada. In our previous paper, we could measure contents of up to 134 mg/kg for mutagenic AAs (4-ethoxyaniline) (Brüschweiler et al., 2014). It should be taken into account that the additional exposure to AAs might take place by skin contact via hair dyes, rubber products, azo colorants in various consumer products like textile and leather clothings, from toys as well as from tattoos. Another important source of consumer exposure to AAs is tobacco smoke (Platzek, 2010; Sabbioni and Hauri, 2016).

AAs are commonly used as intermediates in the synthesis of azo dyes (Freeman, 2013). For that reason, considerable occupational exposure has to be expected. Workers can also be exposed during the dying process of textiles in water baths, particularly in low-wage countries with suboptimal occupational safety standards. Beside consumer protection, these considerations are also important in environmental protection. Up to 50% of annual dye production reaches the environment either directly as effluent or due to loss occurring during dying process or the washing of textiles. Formation of AAs during environmental degradation of azo dyes has been shown to cause environmental pollution (Rawat et al., 2016).

As stated by IARC (2010), "product safety should be an essential consideration in molecular design of dyes for consumer products such as clothing textiles. In this regard, the raw materials employed in the manufacture of synthetic dyes should not involve compounds known to pose health risks. This would include a large group of AAs that are either cancer-suspect agents or established mutagens in the standard *Salmonella* mutagenicity assay. It is clear therefore, that dye design must take into consideration the likely genotoxicity of the potential metabolites generated in mammalian systems. In case of azo dyes, the enzyme-mediated formation of genotoxic AAs as metabolites must be considered, since it is possible that the intact dye is safe but not all of its metabolites."

The focus of this study was on the results of the Ames assay. It was out of scope to evaluate the overall genotoxicity status of the listed mutagenic AAs. This would have required more detailed literature search and a weight-of-evidence approach. This might be a next step in the evaluation of this whole issue.

6. Conclusions

In this study, we identified 40 different AAs which are found to be mutagenic, primarily in the Ames test, and are potentially released as cleavage products from approximately 180 parent azo dyes used in clothing textiles. For that reason, not only exposure to single AAs but also combined exposure to different mutagenic AAs in textiles should be taken into account in the overall exposure and risk assessment in the future, as mutagenic properties of AAs depict a much higher concern than previously expected. It might be of concern not only for consumers wearing these coloured clothing textiles, but to greater extent also for workers in chemicals industry during dye production and workers in textile dying in countries with low occupational safety standards. As long as improvement of *in silico* tools predicting their mutagenicity is still ongoing, empirical validation regarding the mutagenicity of AAs is recommended at this stage.

Declaration of interest

Nothing to declare. This study was sponsored by the Federal Food Safety and Veterinary Office (FSVO). Cédric Merlot is founder and CEO of the consulting company LeadOp Computing Sarl and was contractor. He performed the *in silico* predictions in this study.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.yrtph.2017.06.012.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.yrtph.2017.06.012.

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