Environmental Contamination and Toxicology

Identification of a Water Pollutant, 2-Amino-6,7-dichlorobenzothiazole, at a River near a Textile Industrial Complex in Korea

J.-H. Kwon,[1] J.-W. Kwon,[2] K. Kim,[1] Y.-H. Kim[1]

[1] Environmental Chemistry Laboratory, Korea Research Institute of Chemical Technology, Post Office Box 107, Yusong, Taejon, 305-600, Korea
[2] National Veterinary Research and Quarantine Service Pusan Regional Office 620-2, Amnam-dong, Seo-gu, Pusan, 602-030, Korea

Detection of unregulated chemicals in the public waterways is a prime concern of environmental scientists and government authorities charged with preventing unexpected impact on human health and ecosystem by emerging chemical contaminants in the environment. This aspect is specifically important in developing countries where the number of chemicals under regulation is very limited and the government is under consistent pressure by the public to increase the numbers to the level of developed countries. In developing countries, protection of aquatic life is rarely considered as a component in the decision-making process on which chemicals to regulate, while industrialized countries incorporate this aspect into the effluent water quality standards (US EPA, 2001a).

One of the easiest ways of incorporating a chemical onto the existing list of water quality standards is concern about the occurrence of accidents of emergency character caused by a specific chemical. To cope with emergency situation in the public waterways, biological warning systems using fish, daphnia, and algae are established and under operation in industrialized countries and some developing countries like Korea. However, a chemical causing chronic effect on aquatic organisms at low concentrations and further indirectly affecting human health by bioaccumulation and bioconcentration in the ecosystem can not be easily characterized and regulated. Therefore, limited number of scientists have been involved in detecting the chemicals causing chronic and persistent impact on human health and the aquatic ecosystem by integrating the bioassays and chemical analysis at specific sites of environmental concern.

Mutagenic activity, shown by *in vitro* and *in vivo* methods, is an easy marker for detection and identification of carcinogens in the environment. Several of these type of chemicals have been identified in the public water samples and raised concern of the public and academia in the world. For example, MX [3-chloro-4-(chloromethyl)-5-hydroxy-2(5H)-furanone] was detected after chlorination of drinking water (Hemming et al. 1986; Kronberg et al. 1988). Known mutagens such as PAHs (Sayato et al. 1993) and heterocyclic amines (Ohe. 1997; Ono et al. 2000) were identified in river waters. Recently, a group of Japanese scientists found novel PBTA-type mutagens in river water which accounted for a significant proportion of the mutagenic activity of the river water (Nukaya et al.

Correspondence to: Y.-H. Kim

1997; Oguri et al. 1998; Shiozawa et al. 2000). Mutagenicity in river water and industrial effluent has also been reported in Korea (Lee et al., 1991; Otsu et al. 1998; Park et al. 1997). However, tracking down the chemicals in the river waters which have this property has not been attempted.

Recent field surveys conducted in Kumho River, a tributary river of Nakdong River, by the laboratory of the authors showed consistent and apparently positive signals in the Ames mutagenicity test (*Kwon et al. 2002*). Therefore, chemical structural identification was attempted to characterize the significance of the chemical with regard to the impact on aquatic life and on human health. In the process, we incidentally identified and report herewith the chemical structure of an aquatic contaminant which has not been reported in the literature as a chemical of environmental concern. Even if it did not show mutagenic activity with the Ames mutagenicity test, the environmental significance of the presence of this chemical in public waterways might need further scientific attention for possible risk to humans and the ecosystem.

MATERIALS AND METHODS

Water was sampled using XAD-2 resin (Serva Feinbiochemica GmbH & Co, Heidelberg, Germany) adsorption method (LeBel et al. 1987) for the isolation of a mutagen originated from the wastewater treatment plant. XAD-2 resin was used after washing with n-hexane, acetone, ethyl acetate, and methanol for 24 hrs in a soxhlet extractor, sequentially. Then, 60 g of XAD-2 resin was packed into a column, 2.3 cm diameter, with glass wool filter in both ends.

Methanol eluates of the sample were applied to HP1100 liquid chromatography on a C18 column (5 μm particle size, 3.9 mm × 300 mm; Waters Corporations, Milford, MA) and eluted with the following gradient system of methanol: 0-60 min, linear gradient of 0-100%; 60-80 min, 100%; 80-100 min, linear gradient of 100-0%, at a flow rate of 1 ml/min. Mutagenicity of fractions of 0-30 min, 5 min interval from 30 min to 60 min, and 60-100 min was tested using *Salmonella typhimurium* TA98 with S9 mixture because our preliminary study revealed that the 30-60 min fraction showed mutagenicity. All fractions were dissolved in 100 μL of dimethylsulfoxide (Aldrich Chem. Co. Inc., WI, USA) and treated with direct plate-incorporation method, according to Maron and Ames (1983) using *Salmonella typhimurium* TA98, obtained from Molecular Toxicology Inc. (MD, USA) in the presence of S9 mixture. S9 mix was prepared using Aroclor 1254-induced rat liver homogenate (S9). The S9 mixture contained 25 μL of S9 in a total volume of 0.5 mL. 2-Aminoanthracene (WAKO Pure Chem. Ind., Ltd., Osaka, Japan) was used as the positive control.

Isolation of a mutagen, whose retention time was 43 min, was accomplished as follows. The evaporated residues were dissolved in 50 μL of methanol and injected into the C18 column with a slight gradient of methanol from 40% to 45% in 10 mM phosphate buffer at a flow rate of 1 ml/min. A fraction of 52-60

min was then purified in 55% methanol mobile phase at a flow rate of 1 ml/min. Purity of the isolated chemical was confirmed on the C18 column with the same mobile phase at a flow of 1 ml/min. All HPLC procedures were carried out at ambient temperature, and the UV absorbance of the eluate was monitored at 260 nm.

The minimum amount of the chemical needed for structural determination using proton-NMR was obtained from the river water sample by concentrating with XAD-2 resin trap apparatus. 300 g of XAD-2 resin, pretreated following the procedures mentioned earlier, was packed in 10 columns (60 cm long, 5.3 cm inner diameter). 10 columns were then installed at the site-4 and the water suctioned at an average flow rate of 500 ml/min for 10 hours. 300 L of water was passed through each column. Each XAD-2 column was eluted with 3-bed volume (1.5 L) of methanol. The extracts were then combined and evaporated to dryness, and the residue (2.8 g) was dissolved in 30ml of methanol. Each 2ml of dissolved residue was applied to a CAPCELL PAK C18 column (5 μm particle size, 10 × 250 mm; Shiseido Co. Ltd., Tokyo) with TSP P4000 liquid chromatography with the same gradient system used in the first isolation procedure. The flow rate was adjusted to 5 ml/min. Small amount of the isolated chemical was used as a standard marker.

The fraction at the retention time of 44-49 min, containing the chemical, was collected and evaporated. The dried residue (76 mg) was dissolved in 15 ml of methanol and applied again to the CAPCELL PAK C18 column with a slight gradient of methanol from 40% to 45% in 10mM phosphate buffer at a flow rate of 5 ml/min, and the 45-55 min fraction was collected and evaporated. The residue dissolved in 1 ml of methanol was finally purified by HPLC with methanol mobile phase. The mobile phase of 75% methanol was pumped in isocratically at a flow rate of 5 ml/min, and the chemical was found at the retention time of 14 min. The above process was repeated twice to give 1.3 mg of the chemical with more than 99 % purity, calculated by the peak areas in UV absorbance at 260 nm.

UV absorption specra were measured with a HP1100 series diode array detector. ^{1}H-NMR spectra were taken as solution in chloroform-d with a Bruker AMX500 (500 MHz) Fourier transform spectrophotometer. Chemical shifts are shown in ppm using tetramethylsilane as an internal standard. High resolution mass spectra were measured using Autospec mass spectrometer (Micromass, Britain) equipped with a direct inlet system. Electron impact ionization was used in 200°C with the ionization energy of 70 eV.

A mixture of 2-amino-5,6-dichlorobenzothiazole and 2-amino-6,7-dichlorobenzothiazole was kindly provided by the Nippon Kayaku company (Tokyo, Japan). The gradient systems used for the isolation procedures were applied to a CAPCELL PAK C18 column for the analysis of the mixture. The reference chemical mixture was also analyzed by GC-MS using a HP5971A Mass selective detector run in electron impact mode at 70 eV with a HP5890

series II gas chromatography. ^{1}H-NMR spectra of the mixture were taken with a solution in chloroform-*d* with a Bruker AMX500 (500 MHz) Fourier transform spectrophotometer.

RESULTS AND DISCUSSION

Figure 1 shows the HPLC profile of UV absorbance at 260 nm and mutagenicity. Two major mutagenic fractions with retention times of 30-35 min and 40-45 min were observed. 40-45 min fraction was selected as the priority concern because of the highest activity and the lowest water solubility. By further separation of the 40-45 min fraction using phosphate buffer, major mutagenicity appeared in the fraction at the retention time of 52-60 min, and this fraction was further purified on the same column with 55% methanol mobile phase to the point that no noticeable peak of impurities was seen in the HPLC condition. The UV spectrum of the isolated mutagenic chemical is shown in Figure 2.

At the completion of semipreparative procedure using 3000 L river water, 1.3 mg of the chemical was obtained. The UV spectrum was identical to that of the isolated chemical from the initial adsorbate to XAD-2 resin (Figure 2). The mass spectrum of the chemical exhibited a molecular ion peak at m/z 218 and two isotopic peaks at m/z 220 and 222. The ratio of the molecular ion peak and two isotopic peaks indicates the existence of two chlorine atoms. Subsequent high-resolution mass spectrometry indicated the molecular formular to be $C_7H_4Cl_2N_2S$ (measured molecular weight: 217.9467, calculated molecular weight: 217.9472) (Figure 3). The existence of nitrogen and sulfur atom in the molecule was checked using gas chromatography-thermionic specific detector and gas chromatography-flame photometric detector in sulfur mode, respectively Figure 4 shows the ^{1}H-NMR spectrum of the chemical in chloroform-*d*, indicating the presence of four protons in the molecule, which is in agreement with the high-resolution mass spectrometry data. The broad signal at 5.30 (2H) ppm suggests the presence of amine (NH$_2$) group. Two doublets at 7.33 (1H, J=8.5 Hz) and 7.36 (1H, J=8.7 Hz) ppm suggest the presence of two aromatic protons.

It was speculated that the isolated chemical is a breakdown compound from industrial dye or an intermediate used in the synthesis of dyes. It was almost concluded that the isolated chemical would not be a commercial dye itself, because it has relatively low molecular weight and it does not have significant absorbance in the visible range.

Most sulfur containing dyes also have oxygens in the molecule as a SO_3 form. This category could be excluded from the candidate structure due to the absence of oxygen in the formula. Among the widely used sulfur containing dyes, benzothiazole-based azo dye was thought as a possible skeleton without an oxygen. The presence of a benzothiazole derivative in the environmental sample could have been originated from the reduction of azo dyes in the anaerobic environment (Nam and Renganathan 2000; Weber and Wolfe 1987; Weber and
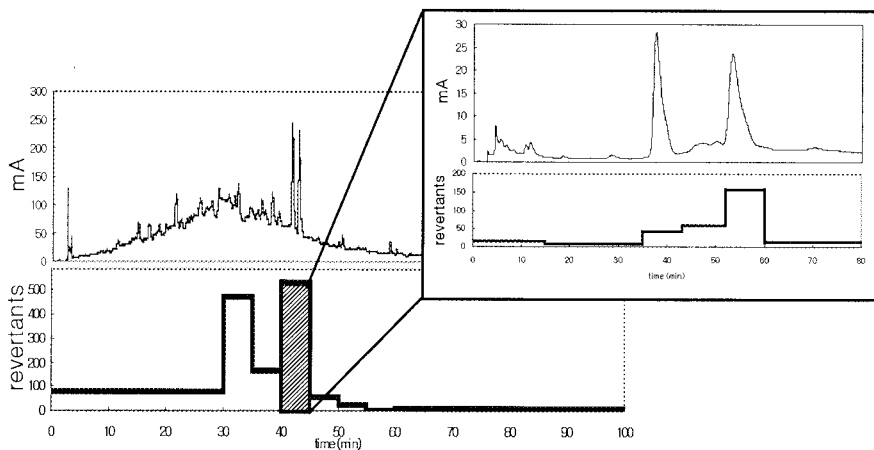
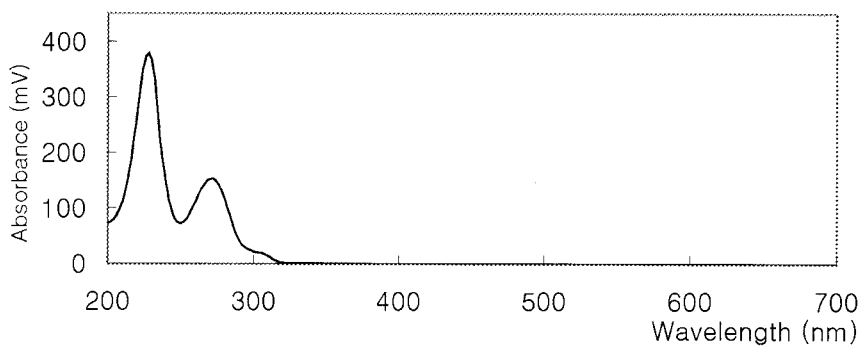**Figure 1**. HPLC profile of mutagens in river water and Ames test results

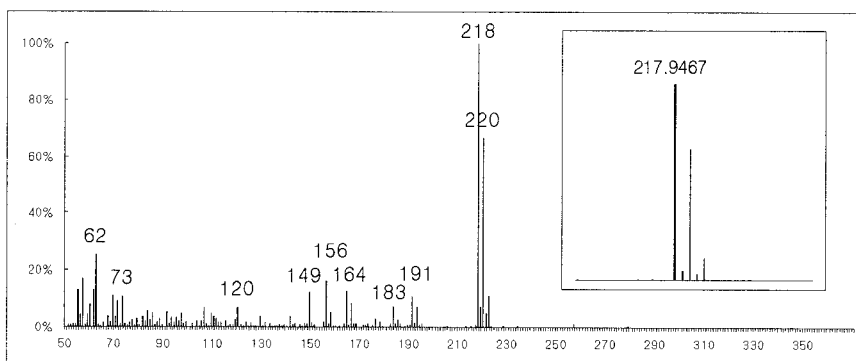
Figure 2. UV spectrum of the isolated chemical

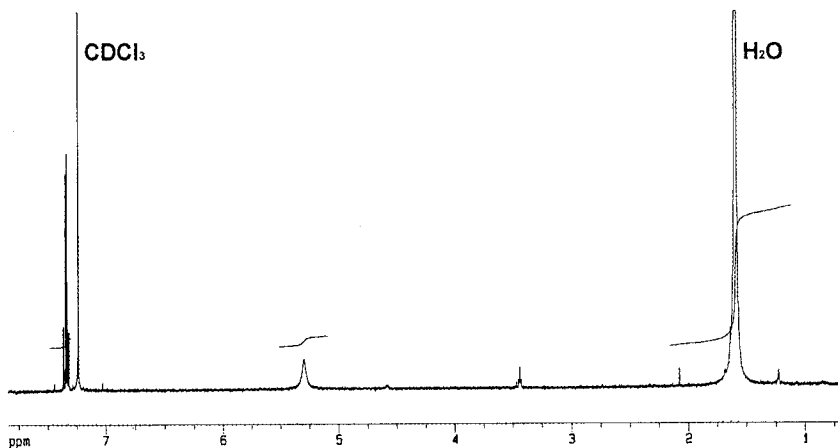**Figure 3.** Mass spectrum and the high-resolution mass spectrum of the isolated chemical

Figure 4. ^{1}H-NMR spectrum of the isolated compound in chloroform-*d*
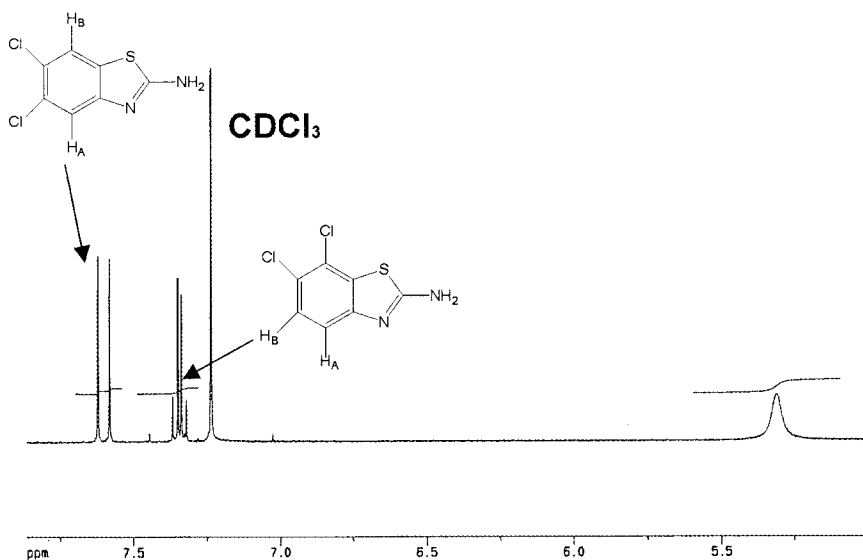

Figure 5. The ^{1}H-NMR spectrum of the reference chemical mixture in chloroform-*d*

Adams 1995; Yen et al. 1991) or plausible intermediate used for the synthesis of a dye and escaped from the wastewater treatment process (Alaimo 1971; Peters et al. 1992; Peters and Chisowa 1996). Dichlorinated 2-aminobenzothiazole, containing 2 prontons in amino group and two aromatic proton, was in good agreement with the NMR data. Among the six possible isomers, 4,5-, 4,7-, and 6,7-substituted isomers were selected as final candidates, because two aromatic

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protons should be located as neighbors judging from its doublet spectra.

The reference chemical mixture of 5,6- and 6,7-iso The mixture of 2-amino-5,6-dichlorobenzothiazole and 2-amino-6,7-dichlorobenzothiazole is known to be in commercial use as an intermediate for the synthesis of benzothiazole-based azo dyes such as disperse red 153. The reference chemical mixture, kindly provided by the Nippon Kayaku company, was easily separated into two peaks, having almost identical intensity in the chromatographic conditions of HPLC and GC. The retention times of the chemical mixture were similar to those of the isolated chemical and the other noticeable peak, neighboring peak of the isolated chemical in the first separation in various HPLC conditions. Figure 5 shows the ^{1}H-NMR spectrum of the reference chemical mixture in chloroform-d. Two singlets at 7.62 (1H) and 7.58 (1H) ppm represents two aromatic proton from 2-amino-5,6-dichlorobenzothiazole and two doublets at 7.33 (1H, J=8.6Hz) and 7.36 (1H, J=8.6Hz) ppm, the same chemical shifts from the isolated chemical, represents another two aromatic protons from 2-amino-6,7-dichlorobenzothiazole. Broad signal at 5.30 (4H) ppm is also in agreement with the signal of the isolated chemical.

The reference chemical mixture of 5,6- and 6,7-isomer was separated successfully with the semi-preparatory HPLC. The isolated 6,7-isomer was tested for mutagenicity by the Ames method using TA98 strain with metabolic activation. However, the pure chemical did not show mutagenicity (Table 1). It was concluded that the mutagenicity of the fractionated sample was due to a trace amount of unidentified mutagen in the fraction, not the significant peak identified in this study. It was confirmed using HPLC that the known PBTA-type of mutagens did not match with the retention time of the fraction. Further study is under way for identification of the mutagenic substance(s).

Table 1. Mutagenic activity of 2-amino-6,7-dichlorobenzothiazole by Ames test with S9 mix

Dose (μg/plate)	Revertants
0	37 ± 3
8	34 ± 4
40	35 ± 1
200	25 ± 2
1000	23 ± 1
2000	21 ± 3
5000	7 ± 3
0.4 μg 2-AA	434

At present, physico-chemical and toxicological information of 2-amino-6,7-dichlorobenzothiazole is not available in the literature. Although 2-amino-6,7-dichlorobenzothiazole is shown to be non-mutagenic by itself, possible neurotoxicity was reported (US EPA, 2001b). This aspect should further be investigated whether the amount estimated in this study could be significant

source of harm to human and the ecosystem. Furthermore, as the source of the contaminant is not known, search for the origin of the chemical would be necessary in the near future.

REFERENCES

Alaimo RJ (1971) The preparation and characterization of 2-amino-5,6-dichloro and 2-amino-6,7-dichlorobenzothiazole. J Heterocycl Chem 8:309-310

Hemming J, Holmbom B, Reunanen M, Kronberg L (1986) Determination of the strong mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone in chlorinated drinking and humic waters. Chemosphere 15:549-556

Kronberg L, Holmbom B, Reunanen M, Tikkanen L (1988) Identification and quantification of the Ames mutagenic compound 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone and its geometric isomer (E)-2-chloro-3-(dichloro methyl)-4-oxobutenoic acid in chlorine-treated humic water and drinking water extracs. Environ Sci Technol 22:1097-1103

LeBel GL, Williams DT, Benoit FM (1987) Use of large-volume resin catridges for the determination of organic contaminants in drinking water derived from the great lakes. In: Malaiyandi M, Suffet IH (ed) Organic Pollutants in Water – Sampling, Analysis, and Toxicity Testing. American Chemical Society, Washington, DC. p 309-325.

Lee S-K, Shim J-S, Kim Y-H, Roh J-K (1991) Ecological and mutagenicity evaluation of industrial effluents with aquatic organisms (*Oryzias laptise, Daphnia magna, Selenastrum capricornutum*) and Ames test with *Salmonella*. J Korean Soc Water Qual 7:100-109 (In Korean with English abstract)

Maron DM, Ames BN (1983) Revised methods for Salmonella mutagenicity test. Mut Res 113:173-215

Nam S, Renganathan V (2000) Non-enzymatic reduction of azo dyes by NADH. Chemosphere 40:351-357

Nukaya H, Yamashita J, Tsuji K, Terao Y, Ohe T, Sawanishi H, Katsuhara T, Kiyokawa K, Tezuka M, Oguri A, Sugimura T, Wakabayashi K (1997) Isolation and chemical-structure determination of novel aromatic mutagen in water from the Nishitakase river in Kyoto. Chem Res Toxicol 10: 1061-1066

Oguri A, Shiozawa T, Terao Y, Nukaya H, Yamashita J, Ohe T, Sawanishi H, Katsuhara T, Sugimura T, Wakabayashi K (1998) Identification of a 2-phenylbenzotriazole (PBTA) -type mutagen, PBTA-2, in water from the Nishitakase river in Kyoto. Chem Res Toxicol. 11: 1195-1200

Ohe T (1997) Quantification of mutagenic/carcinogenic heterocyclic amines, MeIQx, Trp-P-1, Trp-P-2 and PhIP, contrivuting highly to genotoxicity of river water. Mut Res 393:73-79

Ono Y, Somiya I, Oda Y (2000) Identification of a carcinogenic heterocyclic amine in river water. Wat Res 34:890-894

Otsu R, Horikawa K, Min BY (1998) Mutagenicity of river water in Korea. Bull Environ Contam Toxicol 60:615-619

Park Y-K, Lee C-H, Lee S-H, Ha K-S, Kim S-H, Chung Y-S, Han S-K, Utsumi H (1997) Application of Ames Salmonella test to Nakdong river in Korea. Mizu Kankyo Gakkaishi 20:763-767 (In Japanese)

Peters AT, Tsatsaroni ET, Xisai M (1992) Hetarylazo disperse dyes derived from 5,6-dichloro- and 6,7-dichloro-2 aminobenzothiazoles. Dyes Pigm 20:41-51

Peters AT, Chisowa E (1996) Disazo disperse dyes derived from 5,6(6,7)-dichloro-2-aminobenzothiazole. Dyes Pigm 31:131-139

Sayato Y, Nakamuro K, Ueno H, Goto R (1993) Identification of polycyclic aromatic hydrocarbons in mutagenic adsorbates to a copper-phthalocyanine derivative recovered from municipal river water. Mut Res 300:207-213

Shiozawa T, Tada A, Nukaya H, Watanabe T, Takahashi Y, Asanoma M, Ohe T, Sawanishi H, Katsuhara T, Sugimura T, Wakabayashi K, Terao Y (2000) Isolation and identification of a new 2-phenylbenzotriazole-type mutagen (PBTA-3) in the Nikko river in Aichi, Japan. Chem Res Toxicol 13:535-540

US EPA (2001a) Application requirements for new and existing POTWs, 40 CFR 122.21 (j) (5), Washington, D. C.

US EPA (2001b) TSCA New Chemicals Program Chemical Categories, p 23, Washington, D. C.

Weber EJ, Adams RL (1995) Chemical- and sediment-mediated reduction of the azo dye disperse blue 79. Environ Sci Technol 29:1163-1170

Weber EJ, Wolfe NL (1987) Kinetic studies of the reduction of aromatic azo compounds in anaerobic sediment/water systems. Environ Toxicol Chem 6:911-919

Yen CPC, Perenich TA, Baughman GL (1991) Fate of commercial disperse dyes in sediments. Environ Toxicol Chem 10:1009-1017