Fundamental Toxicological Sciences (Fundam. Toxicol. Sci.)
Vol.9, No.6, 173-178, 2022

The Japanese Society of Toxicology

Fundamental Toxicological Sciences
URL : http://www.fundtoxicolsci.org/index_e.html

Letter

Assessment of genotoxicity of 2-[2-(acetylamino)-4-[bis(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2H-benzotriazole (PBTA-6) in zebrafish (*Danio rerio*) using the comet assay and micronucleus test

Yuya Deguchi[1], Tomoyasu Toyoizumi[2], Hiroaki Nagaoka[1] and Shuichi Masuda[3]

[1]Faculty of Pharmaceutical Science, Nagasaki International University, 2825-7 Huis Ten Bosch-cho, Sasebo, Nagasaki 859-3298, Japan
[2]Shizuoka Prefectural Research Institute of Agriculture and Forestry, 678-1 Tomigaoka, Iwata, Nagasaki 438-0803, Japan
[3]Graduate School of Integrated Pharmaceutical and Nutritional Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan

(Received September 27, 2022; Accepted October 7, 2022)

ABSTRACT — This study aimed to evaluate the genotoxicity of 2-[2-(acetylamino)-4-[bis(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2H-benzotriazole (PBTA-6) in fish. Adult zebrafish (*Danio rerio*) were exposed to PBTA-6 solution of 0.3 to 10 mg/L for 96 hr. Genotoxicity was evaluated using alkaline single-cell gel electrophoresis (comet assay) and micronucleus (MN) tests on blood and gills. Although PBTA-6 showed no positive response to MN induction, it caused a concentration-dependent increase in DNA damage. Fish exposed to PBTA-6 (10 mg/L) for 96 hr followed by a 96 hr recovery period in tap water were also examined; DNA damage decreased to the level before treatment within 96 hr. To compare the cytochrome P450 (CYP) activity in different tissues, CYP1A (ethoxyresorufin-*O*-deethylase; EROD and methoxyresorufin-*O*-demethylase; MROD) and CYP2B (penthoxyresorufin-*O*-dependentylase; PROD and benzyloxyresorufin-*O*-debenzylase; BROD) activities were analyzed in liver and gill microsomes. The results showed that PBTA-6 markedly induced PROD activity in gills and caused DNA damage, but the results show that this damage could be reverted if the exposed fish are returned to tap water.

Key words: Zebrafish, PBTA-6, Genotoxicity

INTRODUCTION

2-Phenylbenzotriazole (PBTA) compounds are generated during the dyeing process in the textile industry and can enter rivers systems, as is the case in Japan (Morisawa *et al*., 2003; Ohe *et al*., 2006). Eight PBTA compounds (PBTA-1, PBTA-2, PBTA-3, PBTA-4, PBTA-5, PBTA-6, PBTA-7, and PBTA-8) showed strong genotoxicity toward *Salmonella typhimurium* TA98 and YG1024 with metabolic activation (Ohe *et al*., 1999; Shiozawa *et al*., 2000; Watanabe *et al*., 2001; Oda *et al*., 2008). Recently, PBTA-9 was found in textile effluent in Brazil (Vendemiatti *et al*., 2021) and showed genotoxicity in mice (Rodrigues *et al*., 2021). As PBTA compounds are detected in river water it is important to evaluate its genotoxicity in fish. Among the techniques used to detect genotoxicity, the

Correspondence: Yuya Deguchi (E-mail: deguchi@niu.ac.jp)

micronucleus (MN) test has been used to study genotoxicity studies in fish (Al-Sabti and Metcalfe, 1995; Hayashi *et al*., 1998). It detects MN resulting from chromosomal breakage during cell division or chromosome loss events during anaphase damage (Kirsch-Volders *et al*., 2003). The MN test is often performed using alkaline single-cell gel electrophoresis (comet assay). The comet assay has shown to be a rapid, simple, and sensitive technique for measuring and analyzing DNA damage in individual cells caused by genotoxic agents (Fairbairn *et al*., 1995), and it has been widely used in studies on the genotoxicity of environmental chemicals in fish (Toyoizumi *et al*., 2008; Cavas, 2011). Although the MN test and comet assay can show genotoxicity of 2-[2-(acetylamino)-4-[bis(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-6), a PBTA compound, in goldfish (*Carassius auratus*) using intraperitoneal injection (Masuda *et al*., 2004), it is also useful for evaluating the genotoxicity of the exposure model. Therefore, this study examined the genotoxicity in zebrafish (*Danio rerio*), which are well characterized genetically and are a valuable vertebrate model for studying carcinogenesis (Amatruda *et al*., 2002). The objective of this study was to evaluate the genotoxicity of zebrafish exposed to a PBTA-6 solution using the comet assay and MN test. The enzyme activity of cytochrome P450 (CYP) was also measured. CYP-based activation is generally regarded as an indispensable prerequisite for genotoxicity by aromatic amines, and the CYP activity in fish clouds can be used as a biomarker to investigate the presence of dioxin-like substances (Kais *et al*., 2017). In this study, CYP1A and CYP2B activities were determined using ethoxyresorufin-*O*-deethylase (EROD) and methoxy resorufin-*O*-demethylase (MROD), penthoxy resorufin-*O*-depenthylase (PROD), and bezyloxy resorufin-*O*-debenzylase (BROD) activities.

MATERIALS AND METHODS

Materials

PBTA-6 (CAS Registry No. 392274-07-6) was synthesized as previously described (Watanabe *et al*., 2001). Fetal bovine serum (FBS) and ethidium bromide were purchased from Gibco BRL (Grand Island, NY, USA) and Merck (Darmstadt, Germany), respectively. Low melting point (LMP) agarose, normal melting point (NMP) agarose, 7-ethoxyresorufin, 7-methoxyresorufin, 7-penthoxyresorufin, 7-benzyloxyresorufin, and resorufin were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Fish

Five-month-old sexually mature zebrafish of the AB strain with an average weight of 300 mg were maintained at the Research Center for Environmental Risk, National Institute for Environmental Studies in Japan. Prior to the experiment, male fish were acclimatized for two weeks in a well-aerated aquarium that was maintained at 24−28°C with a 14 hr light/10 hr dark cycle.

Treatment of fish

Experiment 1: Fish were exposed to 1 L solutions containing 0.3, 1, 3, and 10 mg/L of PBTA-6 in glass beakers under static conditions. Benzo[a]pyrene (BaP) was used as a positive control. BaP was dissolved in dimethyl sulfoxide (DMSO) and exposed to a 1 mg/L solution. The negative control group was only exposed to the solvent carrier (DMSO 100 μL/L). Five fish were used in each group, and the test solutions were exchanged every two days. The fish were sampled after 96 hr; blood and gills were collected, and DNA damage and MN induction were examined.

Experiment 2: Fish were exposed to 1 L solutions containing 3 and 10 mg/L of PBTA-6 in glass beakers under static conditions. The negative control group was only exposed to the solvent carrier (DMSO 100 μL/L). Five fish were used in each group, and the test solutions were exchanged every two days. Fish were sampled directly after 96 hr or 96 hr followed by a 48 and 96 hr recovery period in tap water (i.e., 96 hr + 48 hr and 96 hr + 96 hr). The exposure concentrations were selected based on the results from the previous study in Experiment 1. Blood and gills were collected, and DNA damage and MN induction were examined. CYP activity was also analyzed in liver and gill microsomes after 96 hr of exposure in a 10 mg/L solution.

Comet assay

The comet assay was performed using the method described by Deguchi *et al*. (2008). Blood was collected by cutting off the tail and dropping it onto a clean glass slide. The gills were transferred to 100 μL of 30 mM EDTA-2Na containing 0.9% KCl and broken with forceps. The tissue clumps and gill arches were removed and discarded. Comet images were analyzed using a fluorescence microscope (magnification 200 ×) equipped with a CCD camera. Two slides were prepared for each fish, and 50 cells per slide were examined. The tail moment of the DNA was measured using a Comet analyzer (Youworks, Tokyo, Japan).

MN test

The MN test was performed using the method described

by Deguchi *et al*. (2008). Two slides were prepared for each fish. At least 1,000 erythrocytes per slide were observed under a fluorescence microscope (magnification 400 ×), and the number of micronucleated cells was recorded. The MN frequency was calculated as the number of micronucleated cells per 1,000 cells.

CYP activity assay

The EROD, MROD, PROD, and BROD activities of the liver or gill microsomes were measured using the method described by Deguchi *et al*. (2008). Liver and gills were collected from five fish, and each tissue was pooled. Microsomes and 25 μL of NADPH generation system (100 mM $MgCl_2$, 10 mM NADP, 100 mM G-6-P, and 1000 U/mL G-6-P DH) were pre-incubated for 5 min at 30°C (total volume: 200 μL), and 50 μL of each substrate (25 μM of 7-ethoxyresorufin, 25 μM of 7-methoxyresorufin, 25 μM of 7-pethoxyresorufin, or 25 μM of 7-benzyloxyresorufin) was added to start the reaction. After 5 min, 250 μL of ice-cold ethanol was added to stop the reaction. The mixture was then centrifuged at 15,000 rpm for 3 min at 4°C. The fluorescence of the supernatant was measured using a fluorescence microplate reader (Thermo Fisher Scientific, MA, USA) at an excitation wavelength of 544 nm and emission wavelength of 590 nm. Enzyme activity was expressed as the rate of resorufin formation and calculated based on the fluorescence of a standard curve of resorufin standards.

Statistical analyses

Dunnett's test after one-way ANOVA was used to evaluate the significance of the differences in the tail moment in the comet assay and frequency of micronucleated cells in the MN test between the control group and the group exposed to the PBTA-6 solution. Unpaired Student's t-test was used to evaluate the significance of the differences in the CYP activity assay between the control group and the group exposed to the PBTA-6 solution. Statistical significance was set to indicate statistical significance.

RESULTS AND DISCUSSION

As shown in Fig. 1, PBTA-6 was determined to cause a concentration-dependent increase in DNA damage, which showed a significant increase after 96 hr of exposure to the 10 mg/L solution. MN did not increase in either tissue type (data not shown). In our previous study, a significant increase in DNA damage in the blood and MN in the gills was observed in goldfish following intraperitoneal injection (Masuda *et al*., 2004). In this study, different species were used, and the route of exposure was different. In addition, MN was detected as a result of either chromosomal breakage during cell division or chromosome loss events in anaphase damage (Kirsch-Volders *et al*., 2003); therefore, more time might be required to detect MN. To examine this hypothesis, genotoxicity tests were performed by allowing some recovery periods (48 and 96 hr). Fish were exposed to PBTA solutions (3 and 10 mg/L), which were chosen based on the results shown in Fig. 1, and sampled after 96 hr, or after 96 hr followed by 48 and 96 hr of recovery in tap water (i.e., 96 hr + 48 hr and 96 hr + 96 hr). As shown in Fig. 2(a), DNA damage in the blood and gills decreased after exposure to tap water. The comet assay can also be used to evaluate DNA repair in damaged cells. This study confirmed that DNA damage in the blood and gills decreased to the level before treatment within 96 hr. As shown in Fig. 2(b), the frequency of MN in the blood increased slightly at 96 hr + 48 hr and 96 hr + 96 hr, and the frequency of MN in the gills increased slightly at 96 hr + 48 hr; however, the differences were not statistically significant. PBTA-6 did not significantly increase the *rpsL* mutant frequency in the zebrafish gill or hepatopancreas (Amanuma *et al*., 2008). Since the results show that DNA damage decreased after exposure to tap water, the genotoxicity of PBTA-6 in fish may be weak and easy to recover under these conditions.

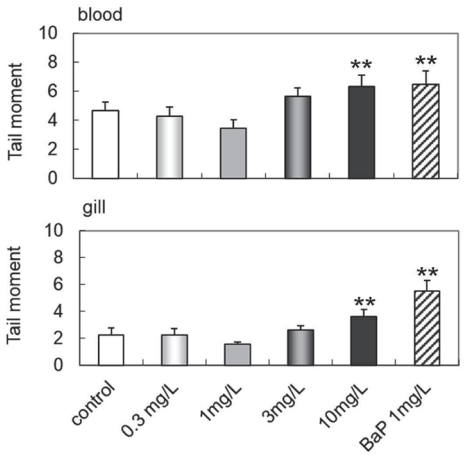

Fig. 1. Cellular DNA damage in the blood (upper graph) and gills (lower graph) of the zebrafish exposed to the PBTA-6 solution. Fifty cells were counted per fish. The mean values were obtained from 250 cells. The bars represent the SEM values. Tail moment = Tail distance x Ratio (using the Comet analyzer). Significant difference is denoted by '**'; $p < 0.01$ vs control (by Dunnett's test after one-way ANOVA).

It has been reported that PBTA-6 shows strong genotoxicity in *Salmonella typhimurium* TA98 and YG1024

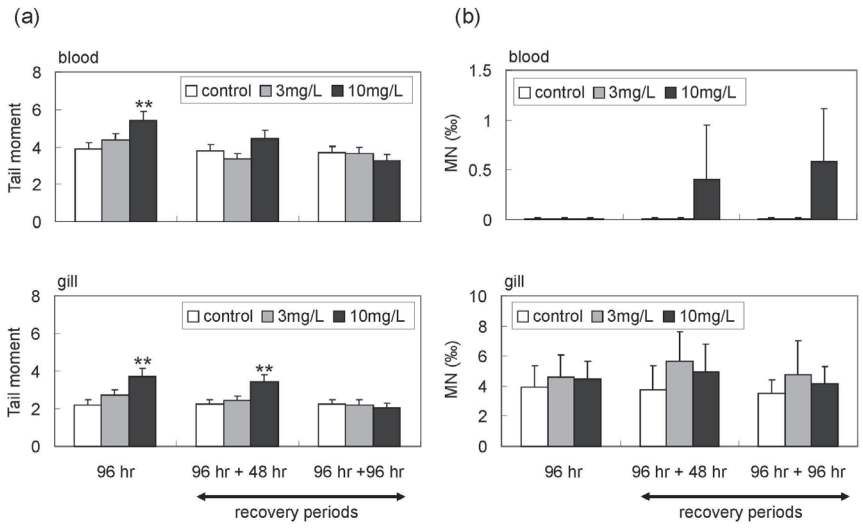

Fig. 2. Fish were sampled after 96 hr or 96 hr followed by a 48 and 96 hr recovery period in tap water (i.e., 96 hr + 48 hr and 96 hr + 96 hr). (a) Cellular DNA damage in the blood (upper graph) and gills (lower graph) of the zebrafish exposed to the PBTA-6 solution. Fifty cells were counted per fish. The mean values were obtained from 250 cells. The bars represented the SEM values. Significant difference is denoted by '**'; $p < 0.01$ vs control (by Dunnett's test after one-way ANOVA). (b) The frequency of MN in the blood (upper graph) and gills (lower graph) of the zebrafish exposed to the PBTA-6 solution. The mean values were obtained from five fish. The bars represent the SD values.

with metabolic activation (Watanabe *et al*., 2001). 2-Phenyl-benxotriazole compounds can be activated by CYP1A1 using AutoDock Vina software (Mena-Ulecia and MacLeod-Carey, 2018); therefore, this study analyzed CYP activity in the liver and gill microsomes after 96 hr of exposure to the 10 mg/L solution. Fig. 3 shows the EROD, MROD, PROD, and BROD activities in both tissues. PBTA-6 markedly induced PROD activity in the gills but showed no or only weak induction in the liver. Generally, EROD activity is analyzed in liver microsomes, but gills are found to be more sensitive than the liver in exposure experiments (Jonsson *et al*., 2006, 2009). Although PROD seems to be predominantly catalyzed by CYP2B in mammals (Burke *et al*., 1994) and phenobarbital (PB) is known to be a CYP2B inducer in rats, PROD activity was not increased by PB, but EROD activity was increased in primary cultures of rainbow trout hepatocytes (Sadar *et al*., 1996). In contrast, high correlations were observed between EROD and PROD activities in cod (*Gadus morhua*) and bullrout (*Myoxocephalus Scorpius*; Ruus *et al*., 2002). It is generally accepted that PBTA-6 is catalyzed by CYP1A1 (Mena-Ulecia and MacLeod-Carey, 2018); therefore, PROD might be catalyzed by CYP1A in zebrafish. PROD activity in fish is unclear, and further investigation is required to elucidate this activity.

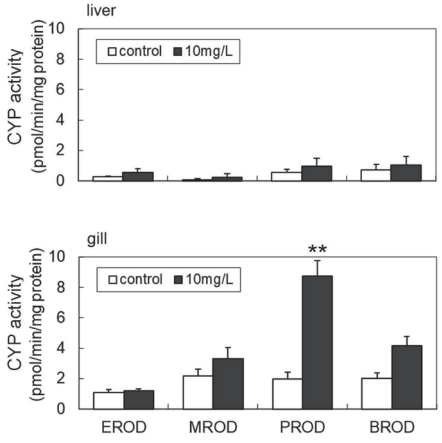

Fig. 3. Activity of four enzymes (EROD, MROD, PROD, and BROD) in the liver (upper graph) and gills (lower graph) of the zebrafish exposed to the PBTA-6 solution. Tissue samples were very small, therefore, pooled liver and gills were used, and the activity was measured as the total activity of five fishes. The mean values were obtained from triplicate measurements. The bars represent the SD values. Significant difference is denoted by '**'; $p < 0.01$ vs control (by unpaired Student's t-test).

In conclusion, this study shows that PBTA-6 exposure markedly induced PROD activity in gills and caused an increase in DNA damage in the blood and gills of zebrafish. Significant DNA damage was observed with PBTA-6, but this could be reverted if exposed fish are returned to tap water.

ACKNOWLEGEMENTS

We would like to thank Dr. Keiji Wakabayashi of the University of Shizuoka for providing PBTA-6, Dr. Yasunobu Aoki of the National Institute for Environmental Studies for providing zebrafish, and Dr. Naohide Kinae for helpful discussions.

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Al-Sabti, K. and Metcalfe, C.D. (1995): Fish micronuclei for accessing genotoxicity in water. Mutat. Res., **343**, 121-135.

Amanuma, K., Tone, S., Nagaya, M., Matsumoto, M., Watanabe, T., Totsuka, Y., Wakabayashi, K. and Aoki, Y. (2008): Mutagenicity of 2-[2-(acetylamino)-4-[bis(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2H-benzotriazole (PBTA-6) and benzo[a]pyrene (BaP) in the gill and hepatopancreas of rpsL transgenic zebrafish. Mutat. Res., **656**, 36-43.

Amatruda, J.F., Shepard, J.L., Stern, H.M. and Zon, L.I. (2002): Zebrafish as a cancer model system. Cancer Cell, **1**, 229-231.

Burke, M.D., Thompson, S., Weaver, R.J., Wolf, C.R. and Mayer, R.T. (1994): Cytochrome P450 specificities of alkoxyresorufin O-dealkylation in human and rat liver. Biochem. Pharmacol., **48**, 923-936.

Cavas, T. (2011): In vivo genotoxicity evaluation of atrazine and atrazine-based herbicide on fish Carassius auratus using the micronucleus test and the comet assay. Food Chem. Toxicol., **49**, 1431-1435.

Deguchi, Y., Wu, N.X., Toyoizumi, T., Masuda, S., Nagaoka, H., Watanabe, T., Totsuka, Y., Wakabayashi, K. and Kinae, N. (2008): Application of a new bioassay technique using goldfish for assessment of water toxicity. Environ. Toxicol., **23**, 720-727.

Fairbairn, D.W., Olive, P.L. and O'Neill, K.L. (1995): The comet assay: a comprehensive review. Mutat. Res., **339**, 37-59.

Hayashi, M., Ueda, T., Uyeno, K., Wada, K., Kinae, N., Saotome, K., Tanaka, N., Takai, A., Sasaki, Y.F., Asano, N., Sofuni, T. and Ojima, Y. (1998): Development of genotoxicity assay systems that use aquatic organisms. Mutat. Res., **399**, 125-133.

Kais, B., Schiwy, S., Hollert, H., Keiter, S.H. and Braunbeck, T. (2017): In vivo EROD assays with the zebrafish (Danio rerio) as rapid screening tools for the detection of dioxin-like activity. Sci. Total Environ., **590-591**, 269-280.

Kirsch-Volders, M., Sofuni, T., Aardema, M., Albertini, S., Eastmond, D., Fenech, M., Ishidate, M. Jr., Kirchner, S., Lorge, E., Morita, T., Norppa, H., Surralles, J., Vanhauwaert, A. and Wakata, A. (2003): Report from the *in vitro* micronucleus assay working group. Mutat. Res., **540**, 153-163.

Jonsson, E.M., Abrahamson, A., Brunstrom, B. and Brandt, I. (2006): Cytochrome P4501A induction in rainbow trout gills and liver following exposure to waterborne indigo, benzo[a]pyrene and 3,3′,4,4′,5-pentachlorobiphenyl. Aquat. Toxicol., **79**, 226-232.

Jonsson, M.E., Brunstrom, B. and Brandt, I. (2009): The zebrafish gill model: induction of CYP1A, EROD and PAH adduct formation. Aquat. Toxicol., **91**, 62-70.

Masuda, S., Deguchi, Y., Masuda, Y., Watanabe, T., Nukaya, H., Terao, Y., Takamura, T., Wakabayashi, K. and Kinae, N. (2004): Genotoxicity of 2-[2-(acetylamino)-4-[bis(2-hydroxyethyl) amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2H-benzotriazole (PBTA-6) and 4-amino-3, 3′-dichloro-5,4′-dinitrobiphenyl (ADDB) in goldfish (Carassius auratus) using the micronucleus test and the comet assay. Mutat. Res., **560**, 33-40.

Mena-Ulecia, K. and MacLeod-Carey, D. (2018): Interactions of 2-phenyl-benzotriazole xenobiotic compounds with human Cytochrome P450-CYP1A1 by means of docking, molecular dynamics simulations and MM-GBSA calculations. Comput. Biol. Chem., **74**, 253-262.

Morisawa, T., Mizuno, T., Ohe, T., Watanabe, T., Hirayama, T., Nukaya, H., Shiozawa, T., Terao, Y., Sawanishi, H. and Wakabayashi, K. (2003): Levels and behavior of 2-phenylbenzotoriazole-type mutagens in the effluent of a sewage treatment plant. Mutat. Res., **534**, 123-132.

Oda, Y., Watanabe, T., Terao, Y., Nukaya, H. and Wakabayashi, K. (2008): Genotoxic activation of 2-phenylbenzotriazole-type compounds by human cytochrome P4501A1 and N-acetyltransferase expressed in Salmonella typhimurium umu strains. Mutat. Res., **654**, 52-57.

Ohe, T., Takeuchi, N., Watanabe, T., Tada, A., Nukaya, H., Terao, Y., Sawanishi, H., Hirayama, T., Sugimura, T. and Wakabayashi, K. (1999): Mutation spectra in Salmonella TA98, TA100, and TA104 of two phenylbenzotriazole mutagens (PBTA-1 and PBTA-2) detected in the Nishitakase River in Kyoto, Japan. Mutat. Res., **429**, 189-198.

Ohe, T., Mizuno, T., Morisawa, T., Kiritani, S., Suzuki, S., Takehana, H., Kasetani, S., Watanabe, T., Nukaya, H., Shiozawa, T., Terao, Y. and Wakabayashi, K. (2006): Mutagenicity and levels of 2-Phenylbenzotriazole (PBTA)-type mutagens in sewage effluent, river water, sediment and drinking water collected from the Yodo river system, Japan. Genes Environ., **28**, 108-119.

Rodrigues, T.A., Fernandes, F.H., de Souza, V.J., Prediger, P., Camparotto, N.G., Sousa, R.N., Aragao, U.G. and Favero, S.D. (2021): Mutagenicity of a novel 2-phenylbenzotriazole (nonchlorinated 2-phenylbenzotriazole-9) in mice. Environ. Mol. Mutagen., **62**, 471-477.

Ruus, A., Sandvik, M., Ugland, K.I. and Skaare, J.U. (2002): Factors influencing activities of biotransformation enzymes, concentrations and compositional patterns of organochlorine contaminants in members of a marine food web. Aquat. Toxicol., **61**, 73-87.

Sadar, M.D., Ash, R., Sundqvist, J., Olsson, P.E. and Andersson, T.B. (1996): Phenobarbital induction of CYP1A1 gene expression in a primary culture of rainbow trout hepatocytes. J. Biol. Chem., **271**, 17635-17643.

Shiozawa, T., Tada, A., Nukaya, H., Watanabe, T., Takahashi, Y., Asanoma, M., Ohe, T., Sawanishi, H., Katsuhara, T., Sugimura, T., Wakabayashi, K. and 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.

Toyoizumi, T., Deguchi, Y., Masuda, S. and Kinae, N. (2008): Genotoxicity and estrogenic activity of 3,3′-dinitrobisphenol A in goldfish. Biosci. Biotechnol. Biochem., **72**, 2118-2123.

Vendemiatti, J.A., Camparotto, N.G., Vidal, C., Cristale, J., Agapito, E.V., Oliveira, A.C., Rodrigues, E.A., Montagner, C.C., Umbuzeiro, G.A. and Prediger, P. (2021): New benzotriazoles generated during textile dyeing process: Synthesis, hazard, water occurrence and aquatic risk assessment. J. Hazard. Mater., **403**, 123732.

Watanabe, T., Nukaya, H., Terao, Y., Takahashi, Y., Tada, A., Takamura, T., Sawanishi, H., Ohe, T., Hirayama, T., Sugimura, T. and Wakabayashi, K. (2001): Synthesis of 2-phenyl benzotriazole-type mutagens, PBTA-5 and PBTA-6, and their detection in river water from Japan. Mutat. Res., **498**, 107-115.