Bull. Environ. Contam. Toxicol. (2002) 68:630–636

DOI: 10.1007/s00128-001-0301-x

Bulletin of Environmental Contamination and Toxicology

Toxicity of Chloroanilines and Effects on Superoxide Dismutase Activities in Serum of Crucian Carp (*Carassius auratus*)

W. Li, D. Yin, A. Zhang, L. Wang

State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing 210093, People's Republic of China

Received: 3 July 2001/August: 21 December 2001

Chloroanilines (CA) are widely used as chemical intermediates in the synthesis of herbicides, azo dyes and pharmaceuticals. They are also degradation products of some herbicides and pesticides. CA may be found in the water environment as contaminant, which bring danger to growth, development and propagation of aquatic organisms, furthermore, potential harm to human being (Lucia et al. 1998; Bozena et al. 1998). Due to their toxicity both to invertebrates and vertebrates and its high production rate, CA are included in the European Union priority list 1 of chemicals. Therefore, there is great significance to evaluate the potential toxicity of these compounds to organisms especial aquatic animals. Quantitative structure-activity relationship (QSAR) is an important tool to obtain the quantitative correlation of molecular structure with biological activity and to predict the biological activities for novel compounds, which is crucial for ecological risk assess of compounds (Kiyoshi et al. 1999; Yoshiaki et al. 2000).

Free radical formation has been implicated as a contributing factor in the deleterious effects of many environmental pollutants (Pedrajas et al. 1995). The toxic effects of organic compounds are clearly mediated by reactive oxygen species (ROS) as shown for lipid peroxidation, DNA damage in domesticated mouse hepatocytes (Klaunig et al. 1995), lipid peroxidation and peroxisomal changes in fish (pedrajas et al. 1998). Superoxide dismutases (SOD) play a key role in the defense against the toxic effects of ROS, which can clean up $O_2^{\cdot-}$ to protect cell from lesions and keep the balance between oxide and anti-oxide. The activities of antioxidant enzymes in organisms may alter if they are exposed to contamination. Therefore, the changes of SOD activities may indirectly reflect the presence of toxic contaminants. SOD, as a biomarker for oxidative stress, was used to predict the harm of organic contaminants to aquatic organisms (Pedrajas et al. 1998).

Correspondence to: L. Wang

In this paper, the acute toxicity tests of CA to crucian carp were conducted. These chemicals were 2-chloro-4-nitroaniline, 4-chloro-3-nitroaniline, 2-chloro-5-nitroaniline, 2,4-dichloroaniline, 3,4-dichloroaniline and 2,5-dichloroaniline. The toxicity of CA was predicted by using 3-D QSAR analysis. Furthermore, crucian carp were exposed to CA for 48 hr, evaluating the effects of CA on SOD activities in serum and elucidating the eco-toxicity of CA to antioxidant defenses of aquatic organisms. The relationship between toxic action model and chemical structure was discussed. This paper may give some consults for ecological risk assesses of CA and their analogues.

MATERIALS AND METHODS

2-chloro-4-nitroaniline (A.R.), 4-chloro-3-nitroaniline (A.R.), 2-chloro-5-nitroaniline (A.R.), 2,4-dichloroaniline (A.R.), 3,4-dichloroaniline (A.R.), 2,5-dichloroaniline (A.R.) were purchased from Acros (USA). All test chemicals had a purity of 98% or greater. SOD Diagnostic Kit was purchased from Nanjing Jiancheng Bioengineering institute (China).

Crucian carp (*Carassius auratus* 32.5±9.1 g, 10.2±1.0 cm) were purchased from Fisheries Research Institute of Jiangsu Province and maintained in de-chloric water for 7 d before tests.

In acute toxicity tests, the pretest was conducted with water and acetone controls before five test concentrations were established for each test chemical. For each treatment and control, ten crucian carp were placed in a 50 L glass tank with 40 L test solution in two replicates. Half of test solution was changed daily, and the crucian carp were not fed during the tests. All experiments were conducted at 15 ±1 ℃(water temperature), pH 7.4±0.3 and 12 /12 hr light/dark. The crucian carp were observed at exposure times of 24, 48, 72 and 96 hr, and the dead crucian carp were recorded and removed. 96-hr LC_{50} value was calculated using Trimmed Spearman-Karber Method (version 1.5, from U.S. EPA). 3-D QSAR (CoMFA) was conducted using SYBYL (version 6.22, from Tripos of U.S.).

SOD activity in serum of crucian carp was determined using the SOD Diagnostic Kit. Utilizing an identical design and conditions to that of the acute toxicity test described earlier, 20 crucian carp were exposed to each chemical test concentration. For each test chemical, the nominal concentrations were treated as the following: 2-chloro-4-nitroaniline (3.3, 6.7, 10.0 mg/L), 4-chloro-3-nitroaniline (0.5, 1.0, 1.5 mg/L); 2-chloro-5-nitroaniline (1.0, 5.0, 10.0 mg/L), 2,4-dichloroaniline (0.5, 2.5, 4.5 mg/L), 3,4-dichloroaniline (1.5, 2.5, 3.5 mg/L), 2,5-dichloroaniline (0.5, 1.3, 2.0 mg/L). After 48 hr exposure, the blood of crucian

carp was collected into Eppendorf cuvette using the Watson Method (Watson et al. 1989), and the serum was separated from blood by 10000 rpm for 7 min at 4℃.

SOD activity unit is defined as: the SOD quantum in 1 mL solution, which includes serum sample, response solution and indicator, is looked as 1 nitrite unit (NU) when the inhibiting ratio of SOD is to 50% (Ji et al. 1991). SOD activity was calculated using the following formula:
SOD activity (NU/mL)= (control OD_{550}— test OD_{550})÷ (control OD_{550}) ÷50% ×diluting multiple

All data were checked using statistical analysis. The differences between control group and treated groups were determined (t-test) using STATGRAPHIC with 95% confidence limits. Data represented as mean±SD.

RESULTS AND DISCUSSION

96-hr LC_{50} values of six CA to crucian carp were shown in Table 1. Based on 96-hr LC_{50} values, the rank order of chemicals from most toxic to least toxic was: 4-chloro-3-nitroaniline > 2,5-dichloroaniline > 3,4-dichloroaniline > 2-chloro-4-nitroaniline > 2,4-dichloroaniline > 2-chloro-5-nitroaniline. Compared to water control, acetone had no significant effect of toxicity on CA. 3-D QSAR analysis of these chemicals was conducted to study the relationships between molecular structure and biological activity (toxicity). For 3-D QSAR analysis, toxicity was transformed to negative logarithmic form in m*M*/L for each chemical. Table 2 was obtained from the CoMFA analysis of 6 compounds, in which the actual values were obtained by using Trimmed Spearman-Karber Method mentioned above, and the calculated values were obtained from the CoMFA analysis. The relationship between actual 96-hr LC_{50} values and the main component index was shown in Figure 1 ($y = 0.287 x + 1.447$, $R^2 = 0.919$), and the actual 96-hr LC_{50} values were close to the calculated LC_{50} values ($R^2 = 0.907$), as shown in Figure 2. The result showed that it was feasible to predict the toxicity (LC_{50} value) of chloroanilines by using 3-D QSAR analysis.

Table 1. 96-hr LC_{50} of compounds to crucian carp

	LC_{50}(mg/L)	-lgLC_{50}(m*M*/L)
2-chloro-4-nitroaniline	6.99 (6.21～7.86)	1.39(1.34～1.44)
4-chloro-3-nitroaniline	2.58(2.20～3.03)	1.82(1.76～1.89)
2-chloro-5-nitroaniline	8.63 (7.68～9.71)	1.30(1.25～1.35)
2,4-dichloroaniline	7.79 (6.60～9.19)	1.32(1.25～1.39)
3,4-dichloroaniline	6.08 (4.71～ 7.84)	1.43(1.32～1.54)
2,5-dichloroaniline	5.23 (4.44～6.15)	1.49(1.42～1.56)

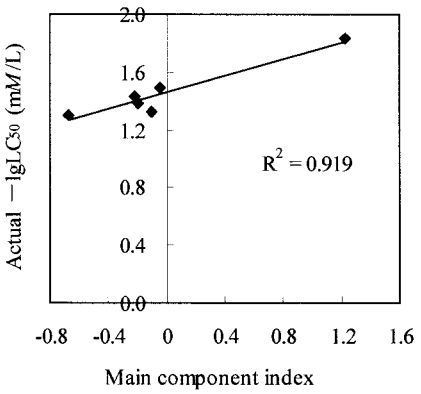

Figure 1. The relationship between $-\lg LC_{50}$ and the main component index.

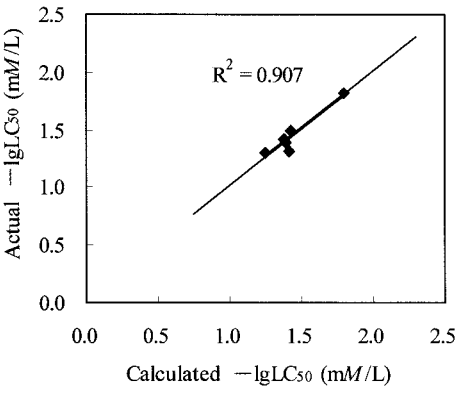

Figure 2. The relationship between actual $-\lg LC_{50}$ and calculated $-\lg LC_{50}$

Table 2. CoMFA correlation statistics for 6 compounds

	Component	Actual	Calculated	Residual
2-chloro-4-nitroaniline	-0.191	1.39	1.39	-0.0024
4-chloro-3-nitroaniline	1.230	1.82	1.80	0.03
2-chloro-5-nitroaniline	-0.675	1.30	1.25	0.05
2,4-dichloroaniline	-0.099	1.32	1.42	-0.10
3,4-dichloroaniline	-0.219	1.43	1.38	0.05
2,5-dichloroaniline	-0.045	1.49	1.43	0.06

Standard Error of Estimate 0.060; % Variance 0.919; R squared 0.919;
F values (n1=1, n2=4) 45.551; Prob.of R2=0 (n1=1, n2=4) 0.003

SOD activities in serum were measured after crucian carp were exposed to six CA for 48 hr, as shown in Table 3. There were some changes for SOD activities of treated groups compared to the control group. 3,4-dichloroaniline and 2,5-dichloroaniline had more significant influence on SOD activities than the other four CA. Free radicals play an important role in toxicity of environmental chemicals. Some pesticide chemicals may induce oxidative stress leading to generation of free radicals and alteration in antioxidants or oxygen free radical (OFR) scavenging enzyme system. Lipid peroxidation had been suggested as one of the molecular mechanisms involved in pesticide-induced toxicity (Banerjee et al. 1999). Oxygen free radical enzymatic scavengers like superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) etc., may protect the system from adverse effect of OFR_S (Khrer 1993). This research result showed that six CA could affect SOD activities in serum of crucian carp, which indicated

that CA could participate in redoxcycling in organisms.

Table 3. Effects of CA on SOD activities in crucian carp serum

	Concentrations (mg/L)	SOD activities (NU/mL)
Water control		98.7±8.2
Acetone control		98.6±11.7
2-chloro-4-nitroaniline	3.3	101.0±9.5 *
	6.7	94.3±4.0 *
	10.0	88.4±6.5 **
4-chloro-3-nitroaniline	0.5	100.6±2.9 *
	1.0	96.2±8.7 *
	1.5	89.4±5.5 **
2-chloro-5-nitroaniline	1.0	101.6±9.9 *
	5.0	96.4±11.7 *
	10.0	85.8±12.6 **
2,4-dichloroaniline	0.5	106.0±13.3 *
	2.5	95.9±4.8 *
	4.5	93.8±8.4 *
3,4-dichloroaniline	1.5	80.8±17.5 **
	2.5	64.0±10.3 **
	3.5	88.1±9.8 **
2,5-dichloroaniline	0.5	69.8±12.6 **
	1.3	65.1±13.7 **
	2.0	64.1±11.9 **

Crucian carp did not die in the course of tests, n =20;
There is no statistical difference between water control and acetone control;
* No-statistical difference ($P>0.05$) from control group;
** Statistical difference ($P<0.05$) from control group.

This research showed that the effects of 2-chloro-4-nitroaniline, 4-chloro-3-nitroaniline and 2-chloro-5-nitroaniline on SOD activities in crucian carp serum were similar, as shown in Table 3. Namely, under the low concentrations (3.3, 0.5, 1.0 mg/L), SOD activities were slightly induced, and with the concentration increasing, activities decreased step by step, and at the high concentrations (10.0, 1.5 10.0 mg/L) SOD activities were inhibited most. 2,4-dichloroaniline had similar effects on SOD activities compared to the three chloro-nitroanilines mentioned above.

The effects of 2,5- dichloroaniline and 3,4-dichloroaniline on SOD activities were

similar, and SOD activities were significantly ($P<0.05$) inhibited at different exposing concentrations, as shown in Table 3. 2,4-dichloroaniline had different effects on SOD activities compared to 3,4-dichloroaniline and 2,5-dichloroaniline, which was related to the acute toxicity of these three dichloroanilines. In this research, the acute toxicity test showed that the toxicity order of three dichloroanilines was: 2,5-dichloroaniline > 3,4-dichloroaniline > 2,4-dichloroaniline. Among these three dichloroanilines, 2,5-dichloroaniline was the most toxic in acute toxicity assay, and its inhibition on SOD activities was also the most significant ($P<0.05$).

Crucian carp were exposed to chlorobenzene, 1,3-dichlorobenzene, 1,4-dichlorobenzene, p-chlorotoluene for 96 hr and 168 hr, and SOD activities in liver did not show a distinct change compared to the control group (Yin et al. 2000). In another research, the SOD activities in the liver of *Boleophthalmus pectinirostris* were significantly induced under the exposure of 30 μg/L benzo(a) pyrene(BaP) for 7 d (Feng et al. 2000).

These research results showed that SOD, as a sensitive indicator, could be used to indicate different bio-response on organisms of chemicals.

In this research, SOD activities in crucian carp serum were affected significantly after being exposed to five CA for 48 hr, and the bio-response was quick and sensitive. 2,4-dichloroaniline was an exception, and no significant inhibition was observed at all concentrations in this research. Instead of liver, kidney,spleen, serum was used as the research tissue, because it presented advantages such as easiness and quickness of collection, no need to mill and freeze. Therefore, it is effective for SOD, as a sensitive biomarker, to indicate contaminants' oxidative stress on aquatic organisms in water environment.

Acknowledgements. This work was financially supported by the Natural Science Fund of China.

REFERENCES

Bozena S-S, Danuta T (1998) Microalgal ecotoxicity test with 3,4-dichloroaniline. Chemosphere 37:2975-2982

Banerjee BD, Seth V, Bhattacharya A (1999) Biochemical effects of some pesticides on lipid peroxidation and free-radical scavengers. Toxicol Lett 107:33-47

Yin DQ, Sun JF, Li WM (2000) Effects of four chlorinated chemicals on liver superoxide dismute (SOD) activities Carp (*Carassius auratus*). Hohai Univ

(Natural Sciences) 28 (Suppl.):107-110 (in Chinese)
Ji JP, Wu ZB, Liu QS (1991) An ultramicroanalytic and rapid method for determination of superoxide dismutase activity. Nanjing Railway Med Coll 10 :27-30 (in Chinese)
Khrer JP (1993) Free radical as mediator of tissue injury and disease. Crit Rev Toxicol 23:21-48
Kiyoshi H, Masamoto A, Kimito F (1999) 3-D QSAR study of insecticidal neonicotinoid compounds based on 3-way partial least squares model. Chemom Intellig Lab Sys 47:33-40
Klaunig JE, Xu Y, Bachowski S (1995) Oxidative stress in nongenotoxic carcinogenesis. Toxicol Lett 82-83:683-691
Lucia G, Amadeu S (1998) Acute effects of 3,4-dichloroaniline on blood of male wistar rats. Chemosphere 37:619-632
Pedrajas JR, Peinado J, Lopez-Barea J (1995) Oxidative stress in fish exposed to model xenobiotics.Oxidatively modified forms of Cu,Zn-superoxide dismutase as potential biomarkers. Chemico-Biological Interactions 98:267-282
Pedrajas JR, Gavilanes F, Lopez-Barea J, Jose Peinado (1998) Incubation of superoxide dismutase with malondialdehyde and 4-hydroxy-2-nonenal forms new active isoforms and adducts. An evaluation of xenobiotics in fish. Chemico-Biological Interactions 116:1-17
Feng T, Zheng WY, Hong WS (2000) The effects of Benzo (a) pyrene on antioxidant defenses in the liver of *Boleophthalmus pectinirostris*. Marine Sciences 24: 27-30(in Chinese)
Watson C F, Baer K N, Benson W H (1989) Dorsal gill incision: a simple method for obtaining blood samples in small fish. Environmental Toxicology and Chemistry 8:457-461
Yoshiaki N, Craig W, Christophe M (2000) 3-D QSAR analysis of inhibition of murine soluble epoxide hydrolase (MsEH) by benzoylureas, arylureas, and their analogues. Bioorg Med Chem 8:2663-2673