

Subacute Toxicity of Wood Preservatives, DDAC and BAAC, in Several Aquatic Organisms

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We investigated the subacute toxicity of 2 wood preservatives, DDAC (principal component: 37.5% didecyldimethyl ammonium chloride) and BAAC (principal components: 40% DDAC, 13.4% boric acid), in a number of aquatic invertebrates: a green alga (*Selenastrum capricornutum*), 2 cladocerans (*Ceriodaphnia dubia* and *Daphnia magna*), 2 fishes (*Danio rerio* and *Oryzias latipes*), and photobacteria (*Microtox*[®]). DDAC and BAAC inhibited biological functions in all of the organisms tested; the order of sensitivity was green alga > cladocerans > fish. Growth of *S. capricornutum* was inhibited by both preservatives at very similar levels of exposure. DDAC inhibited reproduction in *C. dubia* at lower levels of exposure than for *D. magna*, and the reverse was true for BAAC. Both DDAC and BAAC reduced the survival rate of *D. rerio* at lower exposure levels than for *O. latipes*. However, phylogenetic class differences in sensitivity to the preservatives were much greater than species differences.

Key words — biological test, wood preservative, chronic toxicity, daphnia, green algae, fish

INTRODUCTION

Wood remains a staple construction material despite the development of many sophisticated alternatives. According to the latest investigation, the outdoor use of wood products has increased in Ja-

pan because these products harmonize well with the environment.¹⁾ Wood is considered to be an environmentally friendly material because it is a renewable natural resource. However, its organic nature means that it is often easily decomposed by microorganisms^{2,3)} and insects.^{4,5)} To avoid or delay this damage, wood products are sometimes treated with preservatives.^{6,7)} Although these preservative treatments are beneficial in wood used for outdoor facilities, such as decks, fence posts, boardwalks, bridges, check-dams, and slope protection work, users of preserved wood products have raised environmental concerns over their toxicity, especially to aquatic organisms.⁸⁾ There is a possibility that the use of preservatives in park furniture and wooden houses could affect human health.

Ecotoxicological risk assessment is an essential step in the development of environmentally benign wood preservatives. In the European Biocidal Products Directive, a detailed field method has been prepared for the ecotoxicity assessment of rainwater wash-off from treated timber.⁹⁾ Run-off water was ecotoxicologically treated with organisms from 3 trophic levels (*Vibrio fischeri*, *Kirchneriella subcapitata*, and *Daphnia magna*).¹⁰⁾

We would like to see more ecotoxicological data gathered on the active ingredients in wood preservatives before environmental emissions from preservative-treated wood are analyzed in the field.¹¹⁾

DDAC and BAAC are 2 of the wood preservatives approved by the Japan Housing and Wood Technology Center. They are widely used for impregnation of wood used outdoors, including wooden building materials. Wood preserved with DDAC or BAAC can be used in the outer shells of houses and can therefore be exposed to wind and rain. Influence of these pharmaceuticals, however, leached from those lumbers to environment has not been examined. The purpose of this study was to determine the acute and subacute toxicity of DDAC and BAAC to *Microtox*[®] bacteria (*V. fischeri*), a green alga (*Selenastrum capricornutum*), cladocerans (*Ceriodaphnia dubia* and *D. magna*), and fish (Japanese Medaka fish, *Oryzias latipes*; and Zebrafish, *Danio rerio*). These test organisms have been utilized extensively for the evaluation of specific compounds and effluents, and in establishing water quality criteria. We calculated the IC₂₅ (the concentration at which a reduction of 25% in survival or reproduction is observed), the no-observed-effect concentration (NOEC), and the lowest-observed-effect concentration (LOEC). The predicted no-effect-concentration (PNEC) could not

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be calculated because the concentrations of these chemicals in nature have not been investigated. The biological tests performed are described briefly below.

Algal Growth Inhibition Testing: The unicellular green alga *S. capricornutum* (ATCC 22662) is often used as a test organism.^{12,13} Algal growth inhibition testing is performed to investigate the effects of chemicals on the growth of algae, which are primary producers in the aquatic ecosystem.

Water Flea Reproduction Testing: Water fleas have short life cycles and high sensitivity to environmental contaminants. The results of reproduction testing on these species are highly reproducible. Therefore, water flea reproduction tests are suitable for assessing the long-term effects of ambient water containing particular chemicals. The Organization for Economic Cooperation and Development (OECD) and Japan have adopted *D. magna* as a test organism, and Environment Canada and the United States Environmental Protection Agency (USEPA) uses *C. dubia*. We conducted our experiments in accordance with the methods for testing both fleas so that we could compare the results.

Toxicity Testing on Early Life Stages of Fish: This test is performed to investigate the effects (hatch/growth/survival) of test substances on fish continuously exposed from hatching to the larval or fry stages, which are thought to be the most sensitive to toxicity.¹⁴ It is known that if NOECs are obtained from testing at these stages, then similar results will be obtained for the whole life cycle of the test fish. Medaka are often used in Japan, and Zebrafish are used in Europe. We compared the toxicity of DDAC and BAAC in accordance with the test methods for both Medaka and Zebrafish.

Bacterial Bioluminescence Inhibition Testing (Microtox® Test): This acute test, which was developed by the Microbics company (now Azur Environmental, Carlsbad, CA, U.S.A.), takes advantage of the phenomenon that toxins reduce the luminescence of luminescent marine bacteria. With this test it is easy to obtain consistent data. Although this test is not used by the OECD, it was recommended in an USEPA-American Society for Testing and Materials (ASTM) document, and is used worldwide.¹⁵⁻²⁰

In summary, we conducted an algal growth in-

hibition test, 2 types of water flea reproduction tests, 2 types of fish early-life-stage toxicity test, and a Microtox test. From the results of our tests we were able to clarify the toxicity of DDAC and BAAC in the aquatic organisms tested.

MATERIALS AND METHODS

Chemicals — DDAC was obtained from Xyence Corporation, Tokyo. This formulation contained 37.5% DDAC as the active ingredient. The remaining 62.5% consisted of alcohol, glycol, defoamer, and water as inert ingredients. BAAC was obtained from Koshii Preserving Co. Ltd., Osaka, Japan. This formulation contained 40.0% DDAC and 13.6% boric acid as active ingredients. The remaining 46.4% of the formulation consisted of alcohol, glycol, defoamer, and water as inert ingredients. All other chemicals used came from Wako Chemical Co., Tokyo, Japan. DDAC and BAAC were dissolved in ultra-purified methanol.

Biological Tests —

Green Algal Growth Inhibition Test: *Selenastrum capricornutum* Printz (NIES-35 strain) was obtained from axenic unialgal cultures at the National Institute for Environmental Studies (NIES), Tsukuba, Japan. Conical flasks (50 ml) covered with silicon caps (Shin-etsu Chemical Co. Ltd., Tokyo, Japan) were used for the culture and assay. All tests were conducted under sterile conditions with algae cultured in sterile media. The flasks were shaken automatically at 100 rpm at $24 \pm 1^\circ\text{C}$ under illumination at 4000 ($\pm 10\%$) lux. To obtain the preculture, a few drops (0.1 ml) of stock culture were inoculated into a 50-ml flask containing 20 ml of growth medium C.²¹ These flasks were incubated for 3 days. Equal volumes (0.1 ml) of precultured algae were added to 20 ml of medium C freshly prepared to set up stable conditions for algal growth. Forty-eight hours after the beginning of incubation, 1 to 5 ml of cultured algae was added to medium C. The final volume was adjusted to 20 ml, containing 4×10^4 cells/ml. Flasks containing 20 ml of the test solution with various doses of each chemical were prepared for assay. The effects of each preservative were measured by exposing the algae in their exponential growth phase to various concentrations of each preservative under test conditions, and then measuring their growth rates every 24 hr for 72 hr from the start of testing. Comparisons were made with the results obtained from control (no toxicant) groups, and the

effects were indicated by 25% effective concentration (EC₂₀) and NOEC. Six concentrations of DDAC or BAAC solution (100, 50, 25, 12.5, 6.25, and 0 mg/l) were prepared in triplicate. At the start of the assay, 1 ml of precultured algae was inoculated into each flask. After 72 hr, the cell density was measured with a Coulter Counter ZM (Coulter Electronics Ltd. Fullerton, CA, U.S.A.). The rate of growth inhibition was calculated by dividing the numbers of cells in the cultures containing the various concentrations of preservative with that of the untreated control culture. Regression lines were compared statistically according to the method of Sokal and Rohlf.²²⁾

Water Flea Reproduction Testing —

C. dubia Reproduction Test: *C. dubia* were cultured and kept at 25 ± 1°C under a 16-hr light/8-hr dark photoperiod. The water used for culture of *C. dubia* was a mixture of 33% Evian water (Calpis Co. Ltd., Tokyo, Japan) and 67% Volvic water (Mitsubishi Co., Tokyo, Japan) (v/v). Both waters are commonly marketed as mineral waters in Japan. 0.3 ml of *S. capricornutum* (4 × 10⁴ cells/ml) and 0.3 ml of YCT (a mixture of yeast, Cerophyll, and trout chow used as food for *C. dubia*)^{23,24)} were added to each 400-ml culture beaker every day.

Survival and reproduction tests on *C. dubia* were continued for 7 days in accordance with the methods proposed by the USEPA.²⁴⁾ The percentage of living adults and the mean number of young produced by a female were calculated. Six concentrations of each preservative were prepared, as were the diluents and control vehicle. Dilutions were made with fresh culture water. Ten replicate glass chambers (50 ml), each containing 1 *C. dubia* in 40 ml water, were used for each concentration. These chambers were tightly closed with Teflon caps to prevent volatilization of the test chemicals, and the water quality was measured every day. Solutions were renewed daily. Water hardness, pH, and dissolved oxygen concentration were 110 mg/l, 7.0 to 7.5, and 80% to 99%, respectively. Temperature was maintained at 24 ± 1°C. Testing was continued until 60% of the control animals had completed 3 broods (usually 6 to 7 days). For a test to be valid, we required 80% survival of controls and ≥ 15 young per female over the 7-day test period.

D. magna Reproduction Test: *D. magna* was obtained from NIES. Survival and reproduction tests on *D. magna* were continued for 21 days in accordance with the methods proposed by the OECD.²⁵⁾ The percentages of living adults were calculated, and

the mean numbers of neonatal fleas were counted. *D. magna* less than 24 hr old were used at the start of the test. They were exposed to various concentrations of the test substance according to the OECD test conditions, then observed and fed daily during 21 days. Water, light, temperature, feeding, and other test conditions were the same as those used in the *C. dubia* testing. The medium was changed every 1 or 2 days, and neonatal fleas were counted every day. The total number of fleas born over the 21 days in the test fluids of each concentration and the total number born in the control group were statistically processed to determine the IC₂₅, the LOEC, and the NOEC.

Early-life-Stage Testing in Medaka and Zebrafish

— Medaka fish were obtained from NIES. Zebrafish were taken from a line of commercial fish that had been kept for several years in our laboratory. Breeding conditions and keeping procedures were carried out in accordance with those suggested by Piron²⁶⁾ and Neilson.²⁷⁾ The bloodstock fish were fed twice with Tetramin (Herrenteich, Germany) and once with live *Artemia* each day. After being kept under the same of the test condition (water quality, temperature, feeding, light cycle *etc.*) for over 2 weeks, the fish were moved to the stock aquaria and separated by sex. Each mating pair was placed together until the day after the female had laid her eggs. Eggs at the blastula stage were collected approximately 4 hr after fertilization. They were rinsed with clean water to remove feces and then transferred to 50-ml glass flasks containing 40 ml of DDAC or BAAC solution. Six concentrations of DDAC (2.5, 1.25, 0.625, 0.313, 0.156, and 0 mg/l) and BAAC (1.0, 0.5, 0.25, 0.125, 0.0625, and 0 mg/l) were prepared in triplicate. Control flasks filled with tapwater that had been passed through a charcoal filter were also used in each test. The test solution was provided with sufficient oxygen at pH 7.0 ± 0.5, and NaOH or HCl was added to keep the hardness at 200 mg/l as CaCO₃. The 20 eggs was added to each flask. During the test period, 80% of the water containing chemical in each flask was changed every day. Live eggs were counted daily.

Testing was performed in a climate chamber at 25 ± 1°C and with a 16-hr light/8-hr dark photoperiod. The fry were not fed during the test period, which was terminated after more than 9 days (Zebrafish) and 14 days (Medaka) from hatching, when all of the fry had died.²⁸⁾

Microtox Test — Reagents, the freeze-dried bioluminescent bacterium *V. fischeri*, and other required

Table 1. Biological Influences of DDAC and BAACUnits: $\mu\text{g/l}$.

Test	Duration	DDAC			BAAC		
		IC ₂₅	NOEC	LOEC	IC ₂₅	NOEC	LOEC
Green algal growth inhibition test	72 hr	10.0 ± 24.4	25	50	24.5 ± 7.4	25	50
	96 hr	46.7 ± 21.0	50	100	54.0 ± 7.0	50	100
Water flea reproduction test (<i>C.dubia</i>)	7 days	73.7 ± 29.6	83	167	208.0 ± 27.0	137	274
Water flea reproduction test (<i>D.magna</i>)	21 days	211.2 ± 6.8	125	250	139.4 ± 11.3	68.5	137
Fish early life stage test (Zebra fish)	9 days	439.7 ± 9.9	312.5	625	304.9 ± 6.7	250	500
Fish early life stage test (Medaka fish)	14 days	1563.0 ± 14.5	1250	2500	2360.0 ± 120.0	2000	4000
Microtox	15 min	930.8 ± 305.3	366	731	1352.4 ± 465.2	414	827

Results of Microtox test are shown with IC₂₀ ± S.D. and others are shown with IC₂₅ ± S.D. S.D.: Standard deviation.

test solutions (including dilution water and reconstitution water) were purchased from Azur Environmental. The experiment was carried out in accordance with the test conditions and operating protocol of the Microtox acute toxicity test.²⁹⁾ Luminescence was measured with a Microtox Model 500 Photometer (Azur Environmental) in acute mode. Various concentrations of DDAC solution (2.925, 1.463, 0.731, 0.366, and 0 mg/l) or BAAC solution (6.62, 3.31, 1.65, 0.827, 0.414 and 0 mg/l) were prepared as test solutions in duplicate.

Statistical Analyses — Test results were analyzed statistically by hypothesis testing. The data were tested for normality and homogeneity of variance.

Dennett's method^{24,30)} was used to compare the treatment mean with the control mean to determine the IC₂₅ (the inhibitory concentration at which a reduction of 25% in survival or reproduction was observed), which is commonly used as the endpoint.²⁴⁾ The NOEC and LOEC for reproduction and growth were determined by one-way ANOVA and Dunnett's test.³¹⁾

RESULTS

Table 1 presents the biological effects of each lumber preservative in terms of IC₂₅, NOEC, and LOEC. The sensitivity of the aquatic organism to DDAC and BAAC tested was in the order of green alga > water flea > fish \approx bacteria (acute). The preservative concentrations at which biological effects were recorded in fish were 10 or more times those for the alga. The growth inhibitory effects of DDAC and BAAC on the alga are depicted in Fig. 1. Both chemicals at very similar levels of exposure inhibited algal growth. The effects of each preservative on reproduction in the 2 water fleas are shown in Figs. 2-A and 2-B. *C. dubia* was more sensitive than

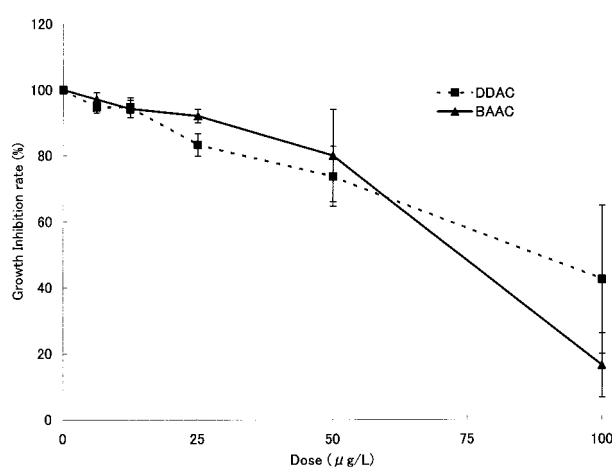


Fig. 1. Effects of DDAC and BAAC on Growth of a Green Alga (*S. capricornutum*)

D. magna to DDAC, but *D. magna* was more sensitive than *C. dubia* to BAAC. The sensitivity of Zebrafish to both chemicals was higher than that of Medaka (Figs. 3-A, 3-B) in terms of survival rate. However, differences in sensitivity to both chemicals between classes were much greater than differences between species.

DISCUSSION

There have been few evaluations of the ability of wood preservatives to leach into the environment and adversely affect animal species.³²⁾ Lee and Son 33 have reported the ecotoxicological effects of wood treated with chromated copper arsenate (CCA) preservative.³³⁾ However, these authors performed only an acute toxicity test and obtained results only for high concentrations. Our examinations and evaluations used subacute toxicity tests, which are considered more sensitive than acute toxicity tests. The biological effects of an emulsified tar oil fraction

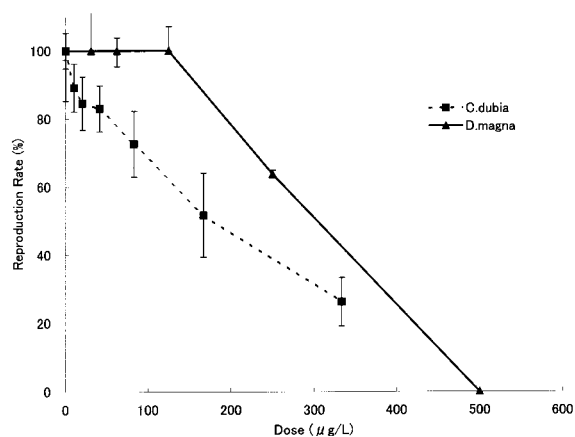


Fig. 2-A. Effects of DDAC on Reproduction of *C. dubia* and *D. magna*

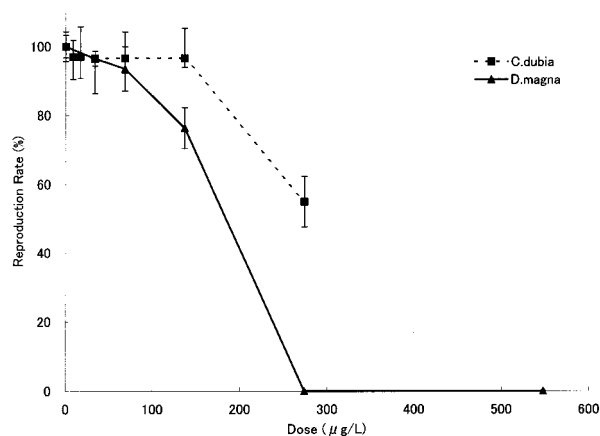


Fig. 2-B. Effects of BAAC on Reproduction of *C. dubia* and *D. magna*

used for wood preservation have also been reported.³⁴⁾ These researchers compared the effects on the tar oil fraction on the growth of an alga, on acute lethality in rotifer (*Brachionus* sp.) and crustaceans (*Streptocephalus* sp.), and in the Microtox test. Their aquatic toxicity tests indicated high sensitivity in the Microtox test and low sensitivity of rotifers, crustaceans and, to a lesser extent, the alga, to exposure to tar oil leachates. The differences between these and our results could be explained by the differences in the preservatives tested. As we had attempted to simulate the leaching of our 2 compounds into the environment, we expected that they would affect the green alga first.

When we evaluate the influence of a wood preservative on the environment we need to check the biological influence of the leachate containing that preservative. The effects of the complex mixture of active ingredients and wood components need to be

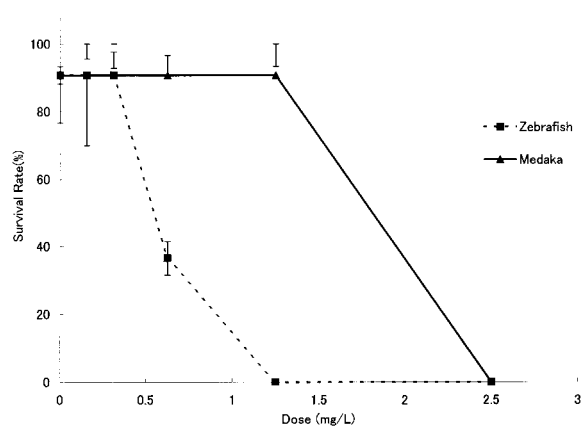


Fig. 3-A. Effects of DDAC on Survival of Medaka and Zebrafish

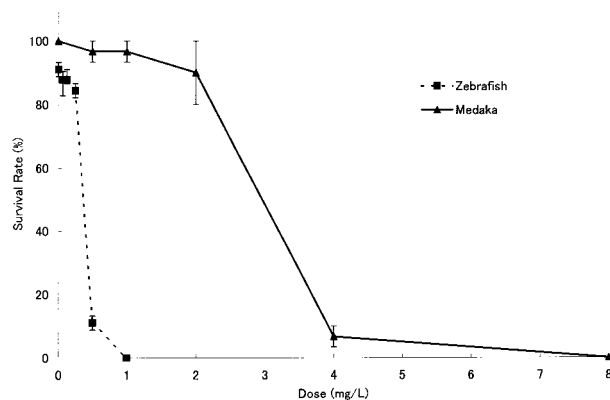


Fig. 3-B. Effects of BAAC on Survival of Medaka and Zebrafish

investigated. However, in the very few reports that have been published on the basic aquatic ecotoxicology of wood preservatives used in Japan, only the aquatic ecotoxicity of the wood preservative itself has been measured Melcher.¹¹⁾

Our results indicate that the green alga *S. capricornutum* is a suitable species for use in investigations of the effects of wood preservatives on aquatic organisms. Having considered the discussions of the Wood Preservative Working Group of the European Biocidal Products Directive,⁹⁾ we are therefore conducting environmental risk assessment of treated wood by examining the effects of leachate in water and rainwater.

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