Bull. Environ. Contam. Toxicol. (1998)60:659-666

Bulletin of Environmental Contamination and Toxicology

Exposure of Juvenile Rainbow Trout *(Oncorhynchus mykiss)* to Methoxychlor Results in a Dose-Dependent Decrease in Growth and Survival But Does Not Alter Male Sexual Differentiation

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Received: 7 January 1998/Accepted: 17 February 1998

The proestrogenic pesticide methoxychlor (MXC) [1,1,1-trichloro-2,2-bis (*p* - methoxyphenyl) ethane], a substitute for DDT, has been used as a larvicide to control various insect pests. Although MXC is relatively non-toxic to mammals, it has been shown to bioaccumulate and its primary metabolite exhibits estrogenic activity (Duax and Weeks 1980). Since MXC is an environmentally persistent compound with reported estrogenic properties it may have numerous adverse effects on non-target aquatic organisms such as fish.

Several fish appear to be sensitive to MXC as the 96-hr LC_{50} for various teleosts ranges from 10 to 260 µg/L (Heming et al. 1989). Although reported levels of MXC in river systems are not lethal to native fishes (Lockhart et al. 1977), laboratory studies suggest that MXC may have various sublethal effects. Investigators report decreased growth and reduced fecundity in various teleosts (Holdway and Dixon 1985; 1986; Holdway et al. 1987; Kumar and Pant 1988). Additionally, compared to adults, juveniles may be much more sensitive to environmental contaminants since this is a period of rapid growth, development, and differentiation (Lee et al. 1975; Laale 1981; Holdway and Dixon 1986).

Salmonids may be especially sensitive to the estrogenicity of MXC since gonadal steroids appear to play a critical role in sexual differentiation in these fish. It has been shown that the expected sexual phenotype can be reversed by addition of exogenous androgens or estrogens at the time of gonadal differentiation (Johnstone et al. 1978; Goetz et al. 1979; Donaldson and Hunter 1982). Because little attention has been given to the effects of organochlorlne pesticides on salmonids, the objective of this study was to determine if chronic MXC exposure effects male juvenile rainbow trout (*Oncorhynchus mykiss*) growth, survival or sexual differentiation.

MATERIALS AND METHODS

Kainbow trout eggs were obtained from Mt. Lassen Trout Farms (Red Bluff, CA); all Y-bearing sperm was obtained from Dr. Gary Thorgaard (Washington State University, Pullman, WA) to generate populations of all genetically male trout. Eggs were fertilized by methods previously described (Nilsson and Cloud 1992) and embryos were incubated in plastic boxes equipped with mesh bottoms in a Heath incubator (Heath Techna, Kent, WA) at 11-12° C.

Beginning 24 days post-fertilization (1 week prior to 100% hatching) eyed embryos (the stage of retinal pigmentation) were partitioned into 7 treatment groups; each group had 2 replicates and each replicate had 80 embryos. Fish were immersed in 1L aerated aged tap water (untreated), water containing the highest concentration (0.5 mL/L) of DMSO (dimethyl sulfoxide) used as a vehicle (untreated and DMSO-treated fish are referred to collectively as "control" fish), or 2.5, 5, or 10 mg/L 95% pure MXC (Sigma Chemical Co., St. Louis, MO) for 2 hr periods every other day for a

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total of 14 exposures. Since technical grade MXC has several contaminants, purified MXC was used in this study to examine the effects of its active compound. Following immersion, fish were thoroughly rinsed, returned to the incubator and checked daily for mortality. Final mortality rates were calculated following the dipping regimen.

At the time of swim-up (approximately 60 days post-fertilization), surviving fry from each treatment group were transferred to individual 5 gallon aquaria at 11-12° C. Aquaria were equipped with Penguin BIO-wheel power filters with flow rates of 110 gal/hr and water changes were made every 5 days. Fish were fed Rangen trout starter pellets (Buhl, ID) to satiation and excess food was removed dally. Trout were fed the following diets 2 to 3 times per day. Untreated control groups were fed a diet without any chemical additives and the DMSO control groups were fed a diet containing 8 mL/kg DMSO. The 2.5 mg/L MXC groups were given either 200 (2.5/200) or 400 mg/kg MXC (2.5/400) in the diet, the 5 mg/L MXC groups received 400 mg/kg MXC (5/400), and the 10 mg/L MXC groups were fed either 400 (10/400) mg/kg or 800 mg/kg MXC (10/800). Since gonadal differentiation in rainbow trout occurs between hatching and the time of first feeding, the exposure regimen to MXC encompassed this critical period. Food was prepared by dissolving MXC and DMSO in 70% ethanol. The solution was sprayed onto the food, and the food was dried overnight (allowing for evaporation of the ethanol) and stored in darkness at -20° C. Fish densities and feeding rates for all groups were kept constant throughout the study.

To keep stocking densities equal between treatment groups, a subsample of fish from those groups with more than 18 fish was sacrificed on days 75, 100, and 114 post-fertilization. The experiment was terminated 140 days post-fertilization. Trout were sacrificed by an overdose of MS-222 (tricaine methanesulfanate) and fork length, wet weight, liver weight, and gonad weight recorded. Additionally, hepatosomatic indices [(liver weight/total body weight) x 100] and gonadosomatic indices [(gonad weight /total body weight) x 100] were calculated to normalize liver and gonad size relative to body weight. For histological observation, gonad samples were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin. Samples were sectioned to a thickness of 6 µm, stained with hematoxylin and eosin and examined via light microscopy to determine sexual morphology of the gonads.

Data have been summarized and are expressed as the mean ± SD. Wet weights, fork lengths, percent mortalities, hepatosomatic indices, and gonadosomatic indices were analyzed by a one-way ANOVA ($p<0.05$) followed by Duncan's new multiple range test. Analysis of variance showed no significant differences between replicates of the same treatment group; therefore, replicate measurements for individual treatment groups were pooled and total means were utilized to analyze the effect of MXC exposure. Percent mortality dam were transformed for statistical analysis; data were transformed back to percent mortality for graphical illustration.

RESULTS AND DISCUSSION

Chronic exposure of juvenile rainbow trout to MXC significantly decreased overall growth. At 140 days post.-fertilization, untreated and DMSO-treated fish were longer and weighed more than all MXC-treated groups (Fig. 1). This response appeared dose-dependent because both weights ($r= -0.90$) and lengths ($r = -0.92$) were highly correlated with MXC concentration. On days 75, 100, and 114, a subsample of fish from untreated, DMSO, 2.5/200 and 2.5/400 MXC groups were collected and weighed (Fig. 2). In general, a trend similar to that observed at 140 days post-fertilization was noted. The mean growth rates (mg/d) from 45 through 140 days post-fertilization of the treatment groups shown in Fig. 2 are shown in Table I. As shown in Fig. 2 and Table I, at all sample periods, except for 114 days post-fertilization, MXC-treated fish were smaller and had a slower rate of growth compared to control groups; the small sample size at 114 days post-fertilization may account for this apparent deviation in absolute weight and growth rate.

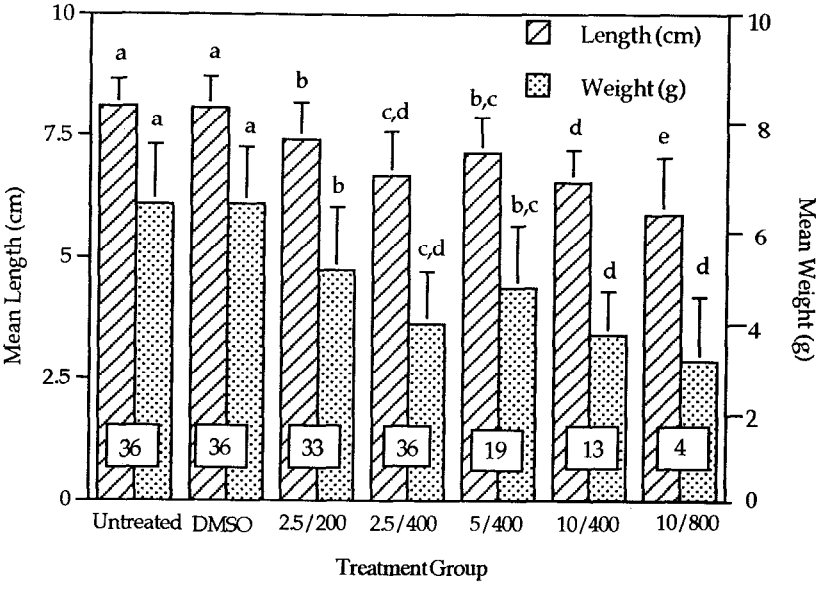

Figure 1. Mean fork lengths and wet weights of rainbow trout sacrificed 140 days post-fertilization reared at 11-12 °C. Each value represents the mean ± SD. Numbers within each bar represent the sample size for that group. Different letters indicate that means are significantly different at $p<0.05$. Treatment groups include: untreated, (DMSO) 0.5 mL/L dip + 8 mL/kg DMSO diet, (2.5/200) 2.5 mg/L dip + 200 mg/kg MXC diet, (2.51400) 2.5 mg/L dip + 400 mg/kg MXC diet, (5/400) 5 mg/L dip + 400 mg/kg MXC diet, (10/400) 10 mg/L dip + 400 mg/kg MXC diet, and (10/800) 10 mg/L dip + 800 mg/kg diet.

The reduction in weight of MXC-treated rainbow trout was observed as early as 45 days post-fertilization, prior to initiation of feeding. Following 11 exposures to MXC, excluding the 2.5 mg/L MXC group ($p = 0.056$), MXC-treated fish weighed significantly less than control fish. Additionally, MXC concentration and weight were highly correlated even at this early stage of development ($r = -0.96$). Methoxychlor exposure delayed initiation of exogenous feeding. Control groups began eating around 59 days post-fertilization, while MXC-treated groups did not begin to feed until 70 days post-fertilization. Moreover, groups treated with MXC fed less aggressively compared to control fish; control fish actively swam to the surface to obtain food, whereas MXC-treated fish passively waited on the bottom for food.

The decreased growth observed in rainbow trout is consistent with previous findings in which it was reported that MXC decreased growth in larval white sucker and immature flagfish (Holdway and Dixon 1986; Holdway et al. 1987); however, unlike previous studies, in this investigation MXC concentration and growth were highly correlated suggesting that weight gain may be used as an indicator of MXC toxicity on juvenile rainbow trout. The weight differences between control and MXC-treated fish may reflect a difference in feeding rates. In accordance with studies completed on larval white sucker (Holdway et al. 1987), in this investigation, visual observations indicated that control fish began feeding nearly 2 weeks earlier than MXC-treated fish and were more aggressive eaters compared to MXC-treated fish. The delay in first feeding, however, should not have resulted in such a drastic effect on final weights and lengths since the experiment continued for more than two months once all groups were eating. Further, the difference in size between control and MXC-treated fish was noted as early as 45 days post-fertilization. At this time, fry had not yet initiated feeding thus all fish were equal relative to nutrient availability.

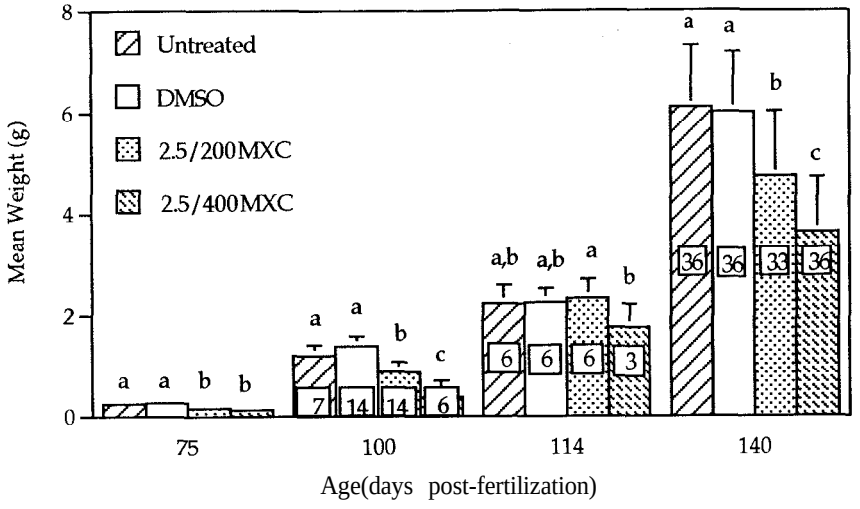

Figure 2. Mean wet weights of rainbow trout sacrificed 75, 100, 114, and 140 days post-fertilization. Data represent means ± SD. Sample sizes are indicated within the bars for each group. For 75 days post-fertilization sample sizes were 15 for untreated, 12 for DMSO, 19 for 2.5/200, and 15 for 2.5/400. Different letters indicate that means are significantly different at $p<0.05$.

Table I. Mean growth rates (mg/day) of juvenile rainbow trout from 45 days post-fertilization through 140 days post-fertilization exposed to varying amounts of MXC.

Group	45->75 days	75->100 days	100->114 days	114->140 days	Total Rate (45->140)
Untreated	5.0	38	73	149	63
DMSO	5.5	45	61	144	62
2.5/200 MXC	2.3	29	102	92	49
2.5/400 MXC	1.3	18	84	75	38

Heming et al. (1988) suggested that exposure of alevin rainbow trout to 30 µg/L technical grade MXC did not significantly effect the rate of yolk absorption, the yolk-to-tissue conversion efficiency, or the rate of tissue growth. However, data from this study suggests that MXC-treated fish may be less efficient at metabolizing nutrients compared to control fish. The wet weight data at 45 days post-fertilization indicate that MXC may affect the efficiency by which alevins are able to convert yolk nutrients into growth. These fish had no exogenous food source, yet control fish were significantly larger than MXC-treated fish. Additionally, at nearly all sample periods MXC-treated fish had a slower growth rate (mg/d) compared to control fish indicating that MXC exposure may adversely effect the rate of tissue growth in trout.

Exposure of juvenile rainbow trout to DMSO (the carrier-control group) appeared to have a slightly positive effect on fish growth since at nearly all sample periods DMSO-treated fish were larger than untreated fish. The effects of DMSO exposure on various organisms remains controversial; some studies indicate that DMSO is beneficial while others claim it is harmful. For example, high doses of DMSO appear to produce various toxic effects including liver degeneration, edema, hemolysis, and death (Rubin 1983). On the other hand, DMSO is often used as a cryoprotectant; it acts as an antioxidant specifically protecting against hydroxyl radical-mediated tissue

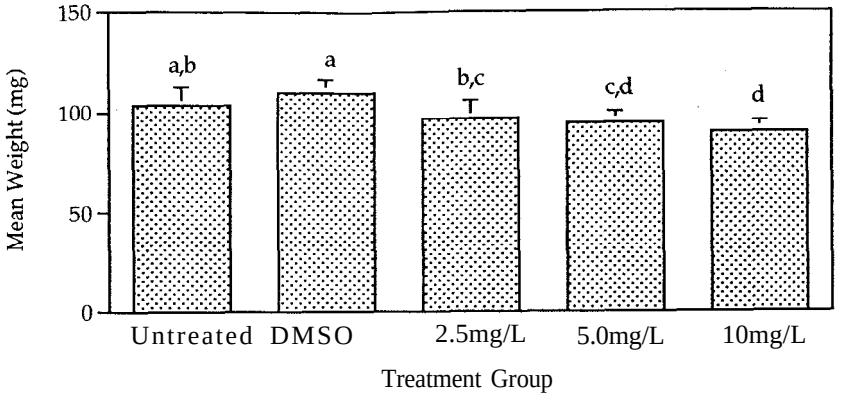

Figure 3. Mean wet weights of rainbow trout sacrificed 4.5 days post-fertilization. Data represent means ± SD. Sample sizes for each group were 10 and different letters indicate means are significantly different at $p<0.05$.

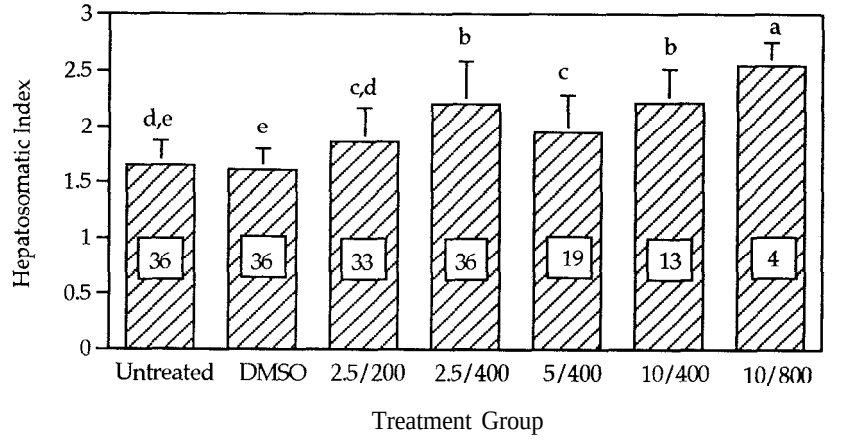

Figure 4. Mean hepatosomatic indices of rainbow trout at 140 days post-fertilization. Data represent means ± SD. Sample sizes are indicated within the bars for each group. Different letters indicate means are significantly different at $p<0.05$. The same groups were analyzed as in Fig. 1.

injury, and it is anti-inflammatory, protects against ischemia and has various other reported therapeutic effects (Shlafer 1983). Since MXC is highly insoluble in water, DMSO was the solvent of choice for this investigation. Because DMSO has been shown to have several augmentary effects, and since DMSO- treated fish in this experiment were larger than untreated fish, suspension of MXC in DMSO may have masked potential deleterious effects of MXC on fish growth.

In contrast to the effect of MXC on overall growth, MXC-treated rainbow trout had increased hepatosomatic indices when compared to control fish (Fig. 4). An exception to this trend was the lack of significant difference between the untreated group and the 2.5/200 MXC group ($p = 0.052$). The increase in hepatosomatic indices may be an indication that MXC-treated fish were using much of their energy for detoxifying the pesticide. Hansell and Ecobichon (1974), have reported that liver weights in rats were significantly increased following exposure to several pesticides

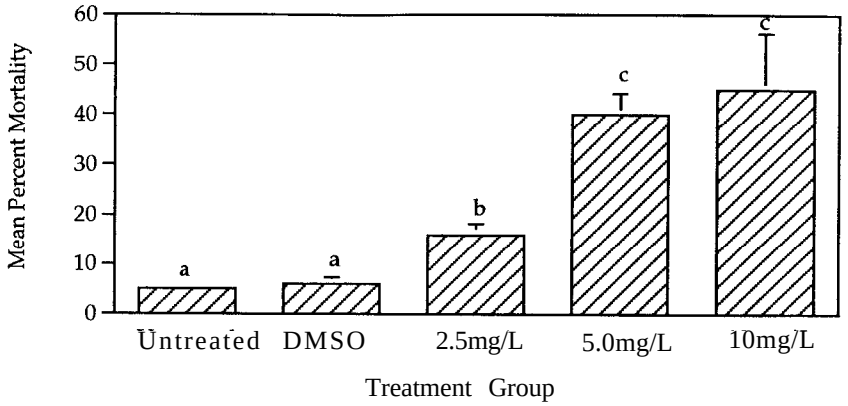

Figure 5. Mean percent mortalities for rainbow trout 56 days post-fertilization. Untreated, DMSO, and 5 mg/L MXC groups had 2 replicates of 80 fish, while the 2.5 and 10 mg/L MXC groups had 4 replicates of 80 fish. Data represent means ± SD. Different letters indicate that means are significantly different at $p<4.05$.

including o,p'-DDT. Since o,p' -DDT is structurally very similar to MXC, and since the liver is the primary detoxifying organ, the greater hepatosomatic indices found in MXC-treated fish is consistent with this conclusion. The increased energy required for detoxification may be another reason for the reported growth reduction in MXC-treated rainbow trout.

The mortalities for each group caused by dipping alone, as fry had not begun feeding by this time, were calculated at 56 days post-fertilization (following 14 exposures to MXC) (Fig. 5). Mortality and MXC concentration were highly correlated ($r = 0.92$) suggesting that embryonic and juvenile mortality may be a sensitive indicator of MXC toxicity. Mortality data for the entire duration of the experiment were not calculated because several of the groups showed an increased die-off (up to 80% in certain groups) upon transferring to individual 5 gallon aquaria. Since this fatality appeared random relative to treatment group, it was concluded that factors other than MXC exposure were responsible for this observation. These results contradict a previous study in which it was suggested that MXC had no effect on the survival of rainbow trout alevins (Heming et al. 1988). The doses and regimen of MXC exposures in this investigation, however, were chronic and higher which may account for the discrepancy in the reported mortalities.

In contrast to the adverse effects caused by MXC relative to growth and survival, the gonads of MXC-treated and untreated fish were histologically similar (Fig. 6). Only the gonad of a single MXC-treated fish (5/400) is shown because the gonads of all MXC-treated fish were comparable. This figure clearly shows that gonads of genotypic male fish (XY) exposed to MXC during the period of sexual differentiation are testes. The only difference observed between testes of MXC-treated and untreated fish is that there appears to be slightly more connective tissue in the MXC-treated testes. Gonads from all fish, regardless of treatment, were testes with clearly defined germ cells. In addition, there was not a significant difference in gonadosomatic indices between any of the treatment groups.

Methoxychlor exposure physically appears to parallel the effects of exogenous estradiol-17 β (E_2 administration in rainbow trout. Orally administered E_2 has been shown to decrease growth (Johnstone et al. 1978), increase mortality, and cause liver hypertrophy in juvenile trout (Herman and Kincaid 1988). However, the effects of MXC on male sexual differentiation and subsequent gonadal development differ from the effects of E_2. It is well documented that exposure of genotypic male

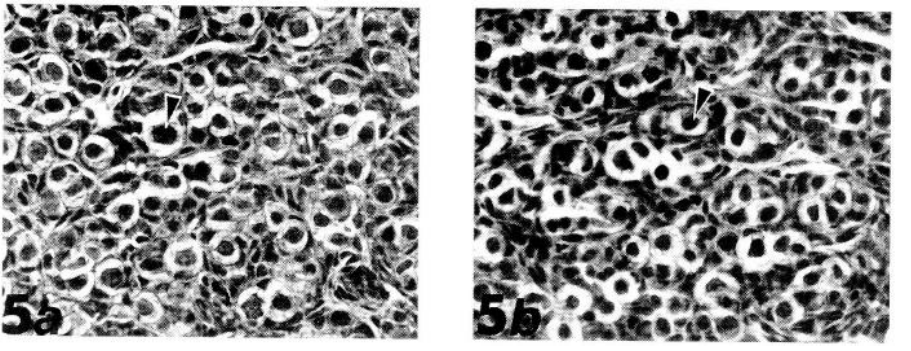

Figure 6. Longitudinal sections of genotypic male (XY) rainbow trout gonads at 140 days post-fertilization viewed at a magnification of 520x. Control (a) and 5/400 MXC-treated (b) gonads are both testes with visible germ cells (arrows) and are virtually indistinguishable.

rainbow trout to exogenous E_2 during the critical period of gonadal differentiation will sex reverse these fish into phenotypic females. In this study, genotypic male fish were exposed to 95% pure MXC during this critical period yet these fish had gonads (testes) similar to untreated fish. It should be noted, however, that at this stage of development it is not possible to determine whether the testicular function of MXC-treated fish would be altered at sexual maturity.

Although it is not possible at this time to resolve the apparent insensitivity of the male reproductive system to MXC, we offer a few speculative interpretations. First, MXC itself does not elicit potent estrogenic activity, rather the metabolite HPTE [2,2-bis (*p* -hydroxyphenyl)-1, 1, 1 -trichloroethane] does (Duax and Weeks 1980). During the period of sexual differentiation rainbow trout fry may lack the hepatic enzymes required to metabolize MXC to HPTE and are therefore unresponsive to the estrogenic effects of MXC. Commercially available MXC, on the other hand, contains several contaminants with estrogenic activity and it may have a completely different effect on testicular development compared to pure MXC. Second, investigators using rodents as a model organism commonly use 100 times more MXC than E_2 to generate estrogenic responses in the reproductive system. In this study the highest MXC treatment was only 40 times greater than the amount of E_2 required to sex reverse male rainbow trout. Since the highest treatment group in this study (10/800) only had 4 surviving fish at 140 days post-fertilization, using a concentration of MXC 100 fold greater would have most likely resulted in 100% mortality. Third, MXC may have estrogenic properties and it may bind to the estrogen receptor in rainbow trout, yet it may not elicit the exact responses as the natural ligand E_2 nor lead to complete phenotypic sex reversal of genetically male trout. Finally, in contrast to several other species studied, 95% MXC may not elicit estrogenic responses in rainbow trout. Although the rainbow trout exposed to MXC in this study showed a response similar to rainbow trout treated with exogenous E_2 relative to growth, survival, and hepatosomatic indices, the lack of a response relative to male gonadal differentiation following MXC treatment may suggest that the physical similarities resulting from MXC and E_2 exposure may be due to the toxicity of both of these chemicals rather than the proposed estrogenicity of MXC.

In summary, chronic exposure of juvenile rainbow trout to MXC decreased growth and increased mortality in a dose-dependent manner. The reduced growth rate was evident prior to initiation of feeding (45 days post-fertilization) and continued throughout the duration of the experiment. In contrast, MXC did not disrupt male sexual differentiation or early testicular development. Thus, though MXC-treated fish physically resemble E_2-treated fish, it is not clear whether MXC has estrogenic effects in juvenile rainbow trout.

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Bulletin of Environmental Contamination and Toxicology

Erratum

"Toxicity of Cadmium and Copper in *Chlamydomonas reinhardtii* Wild-Type (WT 2137) and Cell Wall Deficient Mutant Strain (CW 15)" by M. N. V. Prasad, K. Drej, A. Skawińska, K. Strzalka

[Bull. Environ. Contam. Toxicol. 60:306-311 (1998)]

K. Strzalka's name was misspelled in this article. The publisher regrets the error.