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Toxicity of Sodium Molybdate and Sodium Dichromate to *Daphnia magna* Straus Evaluated in Acute, Chronic, and Acetylcholinesterase Inhibition Tests

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As a result of a widespread application in numerous industrial processes, chromium is a contaminant of many environmental systems. Chromium and their compounds are toxic to both invertebrates and vertebrates and, for this reason, there has been a search for suitable and less toxic alternatives. Molybdenum compounds have been studied as alternative to chromium compounds for some industrial applications. The toxicity of chromium is well known but the effects of molybdenum and molybdenum mining on natural populations and communities of freshwater invertebrates have not often been studied. However, chromium, and molybdenum (and their compounds) are included in the same list (List II) of European Union dangerous substances. In this study, the acute and chronic effects of sodium molybdate and sodium dichromate to *Daphnia magna* Straus were evaluated. Furthermore, *in vitro* and *in vivo* effects of these two metals on acetylcholinesterase (AChE) activity of *D. magna* Straus were investigated. LC_{50} values determined at 48 h were 0.29 and 2847.5 mg L^{-1} for chromium (as sodium dichromate) and molybdenum (as sodium molybdate), respectively. No significant *in vitro* effects of both metals on AChE were found. However, both toxicants inhibited AChE *in vivo* at concentrations under the respective 48-h LC_{50} values. Both sodium dichromate and sodium molybdate inhibited the reproduction and growth of *D. magna*, but the concentrations inducing significant effects were different for the two chemicals. Sodium molybdate had significant lower toxicity to *D. magna* Straus than sodium dichromate. © 2000 Academic Press

Key Words: **sodium molybdate; sodium dichromate; acute and chronic effects; AChE, *Daphnia magna*.**

1. INTRODUCTION

Heavy metals are widely recognized as highly toxic and dangerous to organisms (Mance, 1987). As a result of widespread application in numerous industrial processes, chromium is a contaminant of many environmental systems (Cohen *et al*., 1993). Industrial uses of chromium include metal finishing industry, production of paints and pigments, tanning, wood preservation, chromium chemicals production, pulp and paper production, and metal smelting (Pawlisz *et al*., 1997). One of the major uses of environmentally hazardous chemicals is in the metal finishing industry. Sodium dichromate is the most important of the industrial chromium chemicals; it is used as the starting compound for almost all chromium compounds (Anger *et al*., 1986) and is firmly established in the metal finishing field (Biestek and Weber, 1976). One of the environmental problems of these industries is the elimination of hexavalent chromium compounds, which are used extensively for the passivation of zinc, and for zinc-based coatings and surfaces. The elimination of these toxic chemicals from metal processing, particularly in the automobile industry where chromates are widely used for the protection of zinc-plated parts, is considered a priority within the European Union (SI, 1997).

Molybdenum compounds are used in catalysis, luberfization, flame retardancy and smoke suppression, pigments, agriculture, and corrosion inhibition (Sebenik *et al*., 1990). At high concentrations, molybdenum is toxic to many organisms, including mammals and freshwater invertebrates (Khangarot, 1991). The effects of molybdenum or molybdenum mining on natural populations or communities of freshwater invertebrates have not often been studied (Whiting *et al*., 1994). Based on toxicological studies from the literature, some authors have been studying alternatives to chromium hexavalent compounds for passivation of zinc coatings based on molybdenum compounds (Tang *et al*, 1994; Wharton *et al*., 1996; Almeida *et al*., 1998). Despite the differences of toxicity between chromium and molybdenum reported in the literature, both metals (and their compounds) are included in the List II of European dangerous substances directive (CEC, 1976).

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Daphnia magna is widely used as a test organism in aquatic toxicology (Adema, 1978; Meer *et al*., 1988; Soares *et al*., 1992). Bioassays involving this species are required for the assessment of the potential impact of new chemicals on the aquatic environment (Soares *et al*., 1991). Acute and chronic bioassays with this species are the most widely used test methods for the toxicity assessment of chemical compounds and effluents. In acute tests the measured parameter is death, while in chronic tests the inhibition of normal reproduction and growth are the most used endpoints for toxicity evaluation. However, chronic tests are particularly time-consuming and expensive. For this reason, in recent decades there have been efforts to develop and validate alternative methods to conventional toxicity tests (Blaise *et al*., 1997; Tylor *et al*., 1998).

Enzymes are known to play a crucial role in vital functions of all organisms. The inhibition of cholinesterases has been widely used as a specific biomarker for organophosphate and carbamate pesticides, being considered a "gold standard biomarker" (Peakall, 1994). However, studies published in recent years indicate that some metals (including chromium) might also inhibit these enzymes, under both *in vivo* and/or *in vitro* conditions (Labrot *et al*., 1996; Payne *et al*., 1996; Guilhermino *et al*., 1998).

In this study, the toxicity of sodium dichromate and sodium molybdate to *D. magna* was evaluated using conventional 48-h acute tests, *in vivo* and *in vitro* acetylcholinesterase (AChE) inhibition assays, and 21-day chronic tests. Furthermore, the effect of the color of sodium dichromate in the results of an *in vitro* AChE inhibition test was also investigated.

2. MATERIALS AND METHODS

In the following sections total chromium and molybdenum concentrations refer to the concentrations of the metallic element in the medium.

Parent animals were cultured in ASTM hard water (ASTM, 1980) with an organic additive (Baird *et al*., 1989), in groups of 10 animals per 1000 ml of medium, and fed with the algae *Chlorella vulgaris* (0.322 mg carbon/daphnia/day). The photoperiod was 16 h light : 8 h dark and the temperature was $20 \pm 1^{\circ}C$.

2.1. Acute and Chronic Tests

Tests were carried out in ASTM hard water (ASTM, 1980), with animals from a single clone (clone A, *sensu*; Baird *et al*., 1989) and initiated with third- to fifth-brood neonates (<24 h). In acute tests, organisms were not fed during the experiments and no organic additive was used. For conventional acute tests, 20 animals were used per treatment, in groups of 5 per 100 ml of test solution. Each test was carried out for 48 h. The criterion of toxic effect was death, recognized by immobilization after stimulation by a bright light. In chronic tests, the organisms were cultured in ASTM medium with an organic additive (Baird *et al*., 1989), one animal per 1000 ml of medium, and fed with the algae *C. vulgaris* (0.322 mg carbon/daphnia/day). Each test was carried out for 21 days. Animals were transferred to newly prepared test solutions three times a week (i.e., every other day). One control and six toxicant concentrations with 10 replicates for each treatment were used. Endpoints were reproduction, growth, and mortality. In both acute and chronic tests, temperature and photoperiod were as described above.

2.2. In Vitro AChE Inhibition Test

Tests were performed as described in Guilhermino *et al.* (1996) with the following modifications: animals were cultured for 48 h in the presence of food; homogenates were stored at $-20^{\circ}C$ for a maximum of 1 week. The incubation procedure was performed according to Herbert *et al.* (1995/1996). In each experiment, homogenate samples (495 µl) were incubated at 20°C with 5 µl of each test solution, for 30 min. For each compound, a water control and five concentrations of the metallic element were used (12.5, 25, 50, 75, and 100 mg L^{-1}). Three replicates per treatment were used. The activity of AChE was determined, in triplicate, by the Ellman method (Ellman *et al*., 1961) adapted to microplate (Herbert *et al*., 1995/1996). The protein content of the samples was determined according to the Bradford technique (Bradford, 1976) with the modifications described by Herbert *et al.* (1995/1996). The activity of AChE in each sample was presented as the mean of the three determinations performed and expressed in units (U) per milligram of protein (1 U = 1 nmol of substrate hydrolyzed per minute). A Labsystems Multiskan MS microplate reader was used.

Sodium dichromate solutions presented an orange color interfering with both AChE and protein determinations. For this reason, a correction test for color interference as described above but without *Daphnia* homogenate was performed in parallel. The objective of this procedure is to account for eventual effects due to the color of the chromium solutions.

2.3. In Vivo AChE Inhibition Tests

Tests were carried out according to Guilhermino *et al.* (1996) with the following modifications: 60 animals were used per treatment, in groups of 20 per 1000 ml of test solution. Neonates were cultured for 48 h. Preparation of homogenates and the determinations of AChE activity and protein content were performed as described above for the *in vitro* AChE inhibition tests.

TABLE 1
Range of Concentrations of Molybdenum and Chromium Tested in Conventional Acute, Chronic, *in Vivo*, and *in Vitro* AChE Inhibition Tests

	Chromium (mg L^{-1})	Molybdenum (mg L^{-1})
Conventional acute tests	0.075–0.6	1500–48,000
In vivo AChE Inhibition tests	0.025–0.3	250–3000
In vitro AChE Inhibition tests	12.5–100	12.5–100
21-day chronic tests	0.0125–0.125	50–250

Note. Values are total concentrations of each metallic element.

2.4. Chemicals and Test Solutions

Acetylthiocholine, acid dithiobisnitrobenzoate, and γ-bovine globulins were purchased from Sigma. Bradford reagent was from Bio-Rad, and all the other chemicals were from Merck. For all the tests, stock solutions were prepared in nanopure water (conductivity <5 μS/cm). The range of concentrations tested for each chemical is summarized in Table 1.

2.5. Data Analysis

LC_{50} values for conventional acute tests and EC_{50} values for *in vivo* AChE inhibition assays were determined by probit analysis (Finney, 1971). Data from chronic tests (growth and reproduction) were analyzed with one-way ANOVA. NOEC and LOEC were determined by Dunnett's test (Zar, 1996). Only the organisms for which death occurred after delivery of the first brood and before the end of the tests were considered for statistical analysis. EC_{50} values for reproduction and growth were also calculated using probit analysis (Finney, 1971). NOEC and LOEC values for mortality were determined using Fisher's Exact test (EPA, 1989) and 21-day LC_{50} values were calculated by probit analysis (Finney, 1971). Data from AChE inhibition tests were analyzed using a hierarchical (nested) analysis of variance. NOEC and LOEC were determined by Dunnett's test (Zar, 1996). The significance level was 0.05.

TABLE 2
48-h LC_{50} Values of Metallic Elements (Chromium and Molybdenum) for *Daphnia magna* and Their Corresponding 95% Confidence Limits

Chemical (mg L^{-1})	48-h LC_{50}	95% CL
Chromium	0.29	0.269–0.315
Molybdenum	2847.5	2838.7–2857.0

3. RESULTS

In both acute and chronic tests, oxygen levels were always above 7.0 mg L^{-1} and pH variation was always lower than 1 U.

3.1. Acute Tests

The 48-h LC_{50} values determined for each metal element with the correspondent 95% confidence limits are presented in Table 2.

3.2. Chronic Tests

Each test organism was checked for body length at birth and at the end of the test, as well as for the total number of offspring (total reproduction). Significant effects in total growth and reproduction were found between the different chromium concentrations and the control (total growth: $F = 74.48$; $df = 6,63$; $P < 0.05$) (reproduction: $F = 174.68$; $df = 6,63$; $P < 0.05$). For both reproduction and growth, LOEC was 0.0125 mg L^{-1} and NOEC was 0.025 mg L^{-1} (Table 3). For mortality, LOEC was 0.01 mg L^{-1} and NOEC 0.075 mg L^{-1} (Fisher Exact test: $A = 10$; $B = 10$; $a = 10$; $b = 6$; $P = 0.043$).

Relative to total growth, significant effects were also observed between all the molybdenum concentrations and the control (total growth: $F = 35.52$; $df = 5,52$; $P < 0.05$) (reproduction: $F = 26.6$; $df = 5,54$; $P < 0.05$). For reproduction and growth, the LOEC was 50 mg L^{-1} and NOEC was

TABLE 3
Sublethal (Total Growth and Reproduction) and Lethal Toxicity Endpoints Determined in Chronic Tests for Chromium and Molybdenum (Total Concentrations of Metallic Elements)

Chemical (mg L^{-1})	Total growth			Reproduction			Mortality		
	NOEC	LOEC	EC_{50}	NOEC	LOEC	EC_{50}	NOEC	LOEC	EC_{50}
Chromium	0.0125	0.025	0.233 (0.232–0.234)	0.0125	0.025	0.047 (0.042–0.053)	0.075	0.01	0.524 (0.405–0.677)
Molybdenum	50	75	204.1 (203.7–204.2)	50	75	102.1 (97.1–107.3)	75	100	255.1 (255.0–256.2)

Note. 95% confidence limits are in parentheses.

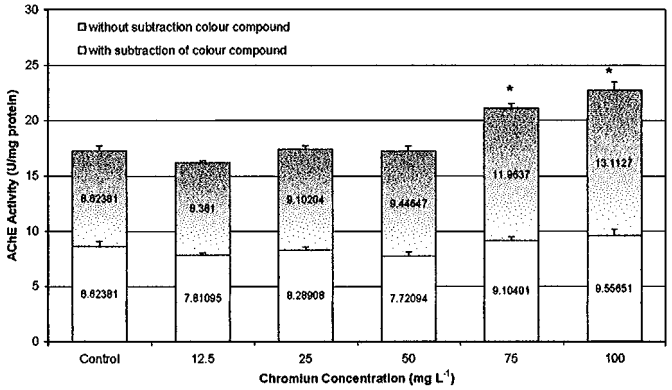

FIG. 1. Columns with points indicate the results of AChE activity without subtraction of the effect due to the color of chromium solution. White columns presents the *in vitro* effect of chromium on AChE activity with the subtraction of the effect of the compound color. Data are expressed as the mean of three samples (three measurements per sample) ± SEM. (* significantly different at $P \leq 0.05$).

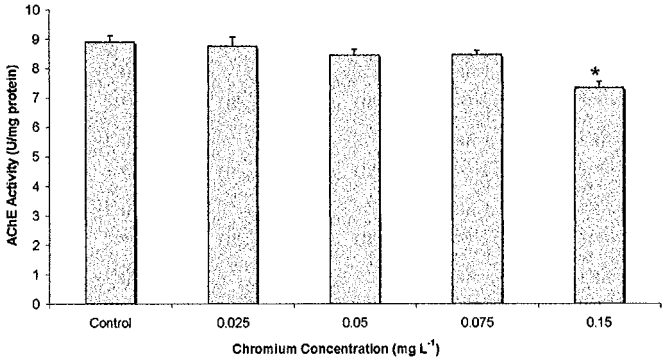

FIG. 3. *In vivo* effects of chromium on AChE activity. Data are expressed as the mean of three samples (three measurements per sample) ± SEM. (* significantly different at $P \leq 0.05$).

75 mg L^{-1} (Table 3). Significant differences were also found in mortality (Fisher Exact test: $A = 10$; $B = 10$; $a = 10$; $b = 6$; $P = 0.043$; LOEC = 100 mg L^{-1}, NOEC = 75 mg L^{-1}.

3.3. *In Vitro AChE Inhibition Tests*

The results obtained in the *in vitro* AChE tests suggest that chromium significantly increases the activity of the enzyme ($F = 16.15$; $df = 5, 12$; $P < 0.05$). However, the color of chromium solutions indicated interferences with the determination of both AChE activity and protein content (Fig. 1). After removal of the color effect, results indicate that chromium did not induce significant alterations in AChE activity. No significant effects of molybdenum on AChE were found ($F = 3.89$; $df = 5,12$; $P > 0.05$) (Fig. 2).

3.4. *In Vivo AChE Inhibition Tests*

Chromium significantly inhibited AChE activity *in vivo* (Fig. 3) ($F = 10.97$; $df = 4, 10$; $P < 0.05$) with a LOEC value of 0.15 mg L^{-1} and a NOEC value of 0.075 mg L^{-1}. Molybdenum, in *in vivo* conditions, significantly inhibited AChE activity (Fig. 4) ($F = 12.76$; $df = 4, 10$; $P < 0.05$). The NOEC was 750 mg L^{-1} and the LOEC, 1500 mg L^{-1}.

EC_{50} values for metallic elements determined by the *in vivo* AChE inhibition tests are provided in Table 4.

4. DISCUSSION

Considerable differences were found between the toxicities of chromium and molybdenum compounds to *D. magna*. Sodium molybdate revealed a lower toxicity for *D. magna* in all tests performed. The results of acute tests for chromium element obtained in this work are similar to corresponding 48-h LC_{50} reported in the literature by other

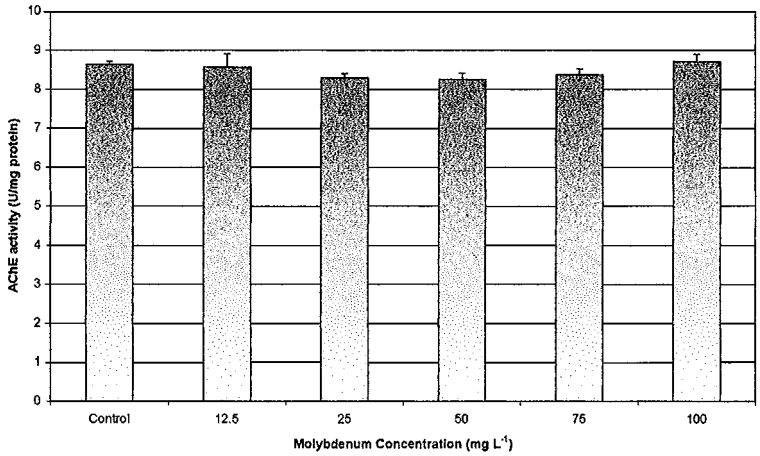

FIG. 2. *In vitro* effects of molybdenum on AChE activity. Data are expressed as the mean of three samples (three measurements per sample) ± SEM.

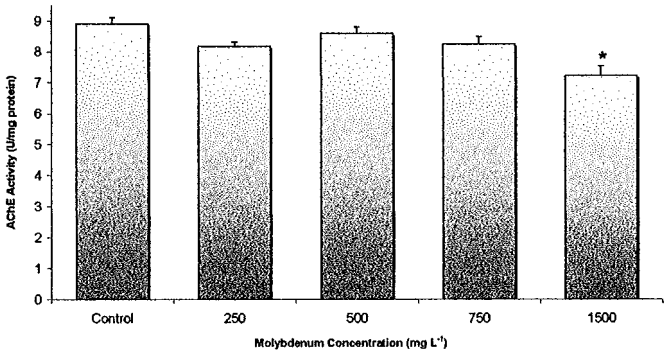

FIG. 4. *In vivo* effects of molybdenum on *in vivo* AChE activity. Data are expressed as the mean of three samples (three measurements per sample) ± SEM. (* significantly different at $P \leq 0.05$).

TABLE 4
NOEC, LOEC, and EC_{50} Values (mg L^{-1}) (Concentrations of Metallic Elements) Determined from *in Vivo* AChE Inhibition Tests

Chemical	NOEC	LOEC	EC_{50}
Chromium	0.075	0.15	0.632 (0.617–0.647)
Molybdenum	750	1500	26,492 (22,258–31,531)

Note. 95% confidence limits are in parentheses.

authors (0.42 mg L^{-1}) (0.229 mg L^{-1}) (Baird *et al.*, 1991; Guilhermino *et al.*, 1997). Molybdenum LC_{50} values determined in this study are comparable to 96-h LC_{50} for sodium molybdate dihydrate as reported by Sebenik *et al.* (1990) (3940 mg L^{-1}) and 48-h LC_{50} for "molybdate" as reported by Kálmán (1994) (3220 mg L^{-1}).

The results obtained from chronic tests revealed that both sodium dichromate and sodium molybdate significantly inhibited reproduction and growth of *D. magna* (Figs. 5 and 6). The effects of reproduction observed in chronic tests with chromium are in good agreement with some authors (Anger *et al.*, 1986). According to them, potassium dichromate concentrations higher than 0.1 mg L^{-1} significantly decrease offspring production and swimming ability of *D. magna* (EC_{50} 0.9 mg L^{-1}). Regarding the chronic tests with sodium molybdate, no comparable data could be found in the literature.

Inhibition of cholinesterases has been widely used as a biomarker for organophosphate and carbamate insecticides. The results obtained here agree with recent studies indicating that some metals (including chromium) may inhibit cholinesterases under both *in vivo* and *in vitro* conditions (Labrot *et al.*, 1996; Payne *et al.* 1996; Guilhermino *et al.*, 1998). At the concentrations tested in this study, chromium and molybdenum compounds did not inhibit AChE *in vitro*. However, a significant AChE inhibition *in vivo* was observed at concentrations under the corresponding LC_{50} values. These results suggest that the compounds are biotransformed in effective inhibitors inside the organism. Furthermore, these results also indicate that the *in vivo* AChE inhibition test is very sensitive, indicating significant effects at concentrations lower than those inducing death. The high sensitivity of the *in vivo* AChE test toward organophosphates reported by Guilhermino *et al.* (1996) was expected, considering the mechanism of action of these compounds. However, the high sensitivity of the *in vivo* AChE test relative to metals found in this study is quite surprising and supports the point of view that this test is advantageous for current ecotoxicity testing relative to conventional acute tests based on mortality.

The results obtained in AChE *in vitro* tests indicate that some precautions should be taken regarding the use of

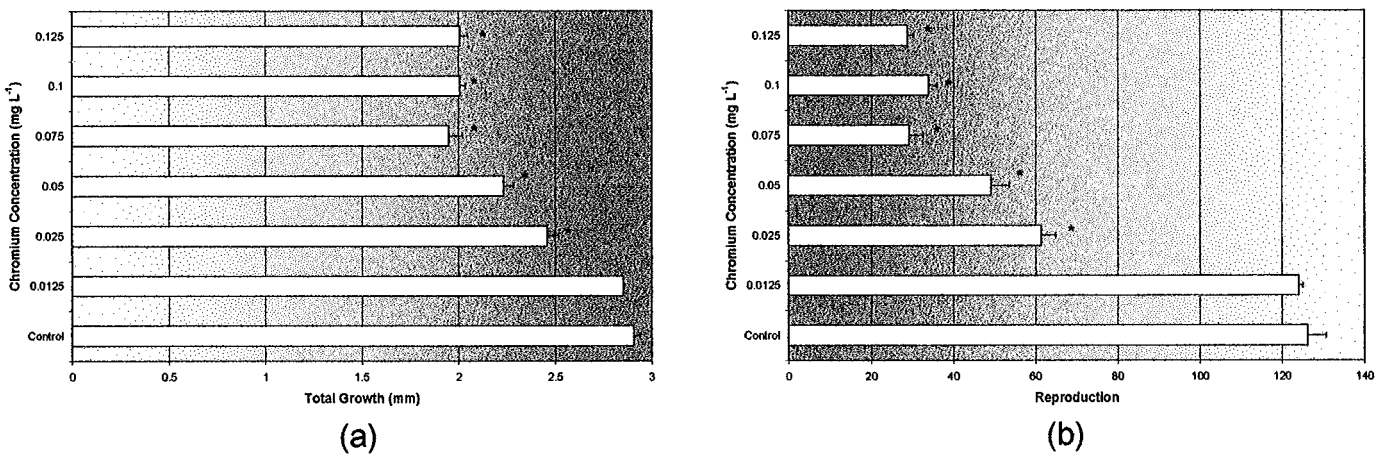

FIG. 5. Total growth (a) and total reproduction (b) for each concentration of chromium and for the control. Data are expressed as the mean ± SEM (* significantly different at $P \leq 0.05$).

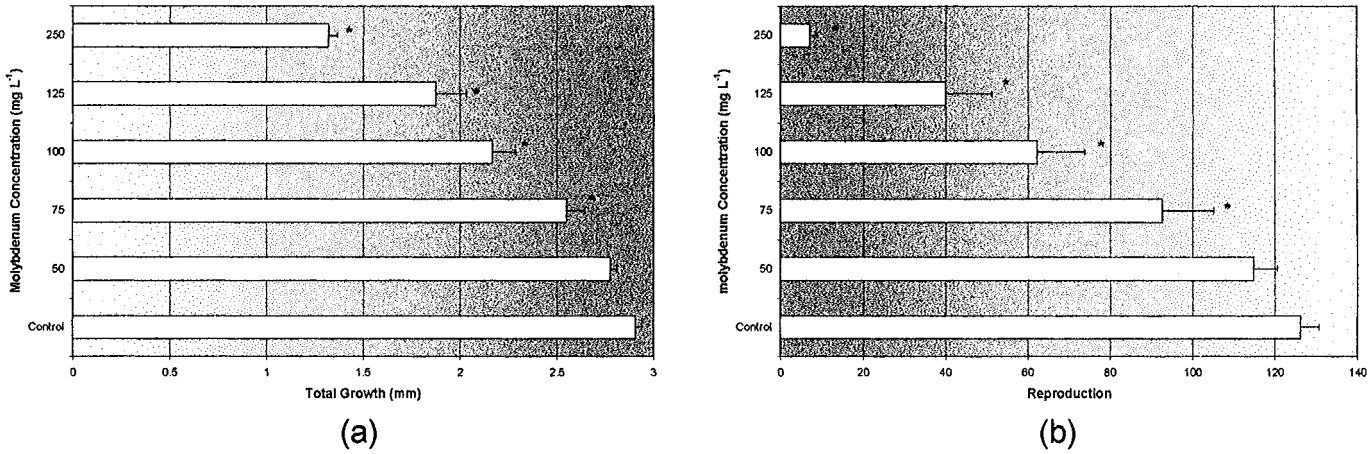

FIG. 6. Total growth (a) and total reproduction (b) for each concentration of molybdenum and for the control. Data are expressed as the mean ± SEM. (* significantly different at $P \leq 0.05$).

in vitro tests with colored compounds, which can lead to an over- or underestimation of the effects on enzyme activity.

5. CONCLUSION

Molybdenum and chromium compounds are included in the same list (List II) of European dangerous substances. The results of this study indicate great differences in toxicity between sodium dichromate and sodium molybdate to *D. magna* evaluated in acute, chronic, and acetylcholinesterase inhibition tests, with sodium molybdate being less toxic than sodium dichromate. Thus sodium molybdate seems to be preferable to sodium dichromate, at least for some industrial applications, since it is less toxic and demonstrates relatively good anticorrosive performance. Meanwhile, more studies are being done to complete the evaluation of toxicity of molybdenum compounds to several organisms.

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