Bull. Environ. Contam. Toxicol. (2003) 71:1158–1166

DOI: 10.1007/s00128-003-0228-5

Bulletin of Environmental Contamination and Toxicology

Single and Binary-Combined Toxicity of Methamidophos, Acetochlor and Copper Acting on Earthworms *Esisenia Foelide*

J. Liang, Q. Zhou

Key Laboratory of Terrestrial Ecological Process, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110016, People's Republic of China

Received: 31 August 2002/Accepted: 30 August 2003

Phaeozem (black soil) distributed in northeastern China, one of the three pieces of phaeozem regions in the world, is an important commodity grain base in China. In order to increase the yield of crops in agricultural areas, synthetic herbicides, insecticides and fungicides containing metals such as copper (Cu) and arsenic (As) were applied widely (Archer and Shogren 2001; Kikuchi et al. 2000). In particular, methamidophos and acetochlor are two of the most important agrochemicals with good sales and high dosage in the important agricultural area of China (Liang et al. 2001; Zhou and Huang 2001). Soil pollution by Cu is also becoming increased with the development of agricultural production in the phaeozem area (Zhou 1995; Zhou and Huang 2001). It has been indicated by our recent investigation that soil animals such as earthworms that are significant contributors to biomass and fertility of many temperate soils (Garca and Fragoso 2002; Liang et al. 2001) are becoming extinct in the area. Obviously, the security of soil ecosystems in the area has been threatened. However, relationships between death and extinction of earthworms in the area and the application of agrochemicals are still vague. Research on the problem has been not involved until now. Moreover, attention has been paid to soil ecotoxicology of earthworms (Johnson et al. 2002; Miyazaki et al. 2002). Single and binary-combined toxic effects of methamidophos, acetochlor, and Cu on earthworms were thus examined. The work involving in combined pollution (Zhou 1995; Cheng and Zhou 2002) may be meaningful to environmental, agricultural and food-chain security (Song et al. 2002).

MATERIALS AND METHODS

The tested soil was collected from a field that has been not planted for more than a decade in the Hailun Agro-Ecological Trial Station (47°26' N, 126°38' E), Hailun County, Heilongjiang Province, China, which located in the continental temperate monsoon zone, with a dry and cold winter and a warm and wet summer. Annual mean temperature is about 1.5°C. Annual precipitation averages range 500-600 mm, of which 70% occurs during May to September. The length of the annual non-frost period is around 30 days (Liang et al. 2001).

The form of the tested heavy metal, Cu, used in this experiment was $CuSO_4{\cdot}5H_2O$ (analytical grade). The tested organic pesticide, 40% of oil soluble concentration

Correspondence to: Q. Zhou

of methamidophos [$CH_3O(CH_3S)P(O)NH_2$], was obtained from the chemical factory of Zhejiang Technical University. The organic herbicide, 50% of oil soluble concentration of acetochlor ($C_{14}H_{20}ClNO_2$), was obtained from the Rize Pesticide Company in Dalian. The tested soil animal is earthworm *Esisenia foelide* that is a typical species used in toxicological experiments and widely distributed in soils of northeastern China. The average weight of the tested earthworms being no younger than three months is between 250-400 mg.

In the pre-experiment, MLC (the lowest concentrations of methamidophose, acetochlor and Cu which can induce the death of some tested earthworms) and LC_{100} (the lowest concentrations of the pollutants which can result in the death of all the tested earthworms) during single exposure of 2 weeks were determined. According to the results of the pre-experiment, the tested concentration of methamidophose added to relatively clean soil samples was 118.4, 151.2, 192.0, 244.0, 310.4 and 394.4 mg kg^{-1}, respectively. In the same way, the tested concentration of acetochlor was 164.0, 209.0, 265.0, 337.0, 428.0 and 545.0 mg kg^{-1}, and the tested concentration of Cu was 650.0, 825.0, 1000.0, 1175.0 and 1350.0 mg kg^{-1}, respectively.

Every 500 g air-dried soil sample was put to a polypropylene container filled with 125 ml of water and the above tested concentrations of methamidophos, acetochlor, Cu or the mixture of any two pollutants. The ratio of water to soil remained about 1:4 and regulated by adding deionized water. Then 16 washed earthworms were placed to soil in an above-mentioned container treated with the pollutants. All the treatments which were replicated 3 times to decrease experimental errors were kept in a culturing box (LRH-250-A, made in Guangdong, China) with the condition of the lightness of 400-800 lx and a constant temperature of 20±1°C. Every 2 days, dead earthworms were removed and the mortality of the earthworms exposed to the pollutants was calculated. After 6 and 14 days when earthworms were exposed to the pollutants, the weight of the earthworms was measured, respectively. The exposed experiment was finished after 14 days. The data from this experiment were statistically processed using the Microsoft EXCEL 2000, including calculation of average values and standard deviation, correlation and regression.

RESULTS AND DISCUSSION

The results indicated that methamidophos, acetochlor or Cu had a poisonous effect on the tested earthworms. There were earthworm weight and mortality changes with exposed concentrations of the pollutants (Table 1, Figures 1 and 2). It can be affirmed that we have to be faced with adverse ecological risk from environmental pollution of these chemicals once they are released into

Table 1. Mortality and body weight changes of earthworms when exposed to soil pollutants.

Pollutant	Concentration (mg kg^{-1})	6-day exposure		14-day exposure	
		Mortality (%)	Weight change (mg)	Mortality (%)	Weight change (mg)
Methamidophos	0	0	0.010	0	-0.060
	118.4	6.25	-0.061	62.50	-0.088
	151.2	18.75	-0.088	87.50	-0.092
	192.0	43.75	-0.094	100	
	244.0	56.25	-0.119	100	
	310.4	68.70	-0.065	100	
	394.4	87.50	-0.037	100	
Acetochlor	0	0	0.022	0	0.006
	164.0	0	-0.020	6.25	-0.053
	209.0	0	-0.075	43.75	-0.082
	265.0	12.50	-0.096	68.75	-0.073
	337.0	75.00	-0.027	100	
	428.0	100	/	100	
	545.0	100	/	100	
Copper	0	0	0.002	0	-0.014
	300.0	0	-0.014	0	-0.042
	650.0	0	-0.084	6.25	-0.116
	1000.0	18.75	-0.097	62.50	-0.105
	1350.0	75.00	-0.049	87.50	-0.112
	1700.0	93.75	-0.097	100	

ecosystems. According to Table 1, the weight change is a more sensitive factor than the mortality in indicating toxic effects of the pollutants. Perhaps, on one hand, when concentrations of the pollutants are so low that no earthworm dead, body weight can be lost; on the other hand, when the exposed time is too short to induce the death of earthworms, the weight change can be also taken place. The loss in body weight changing with time was related to the amount of pollutants added to the tested soil.

The mortality of earthworms under experimental conditions had an increasing trend with time (Figure 1). In Figure 1-methamidophos, the shape of each caved curve seemed similar, just like "S", which means that the death of earthworms took place slowly at the first 2-6 days of an exposure to methamidophos, then quickly, and then slowly. In Figure 1-acetochlor, the shape of the curves varied differently: when the concentration of acetochlor was at the concentration of 337

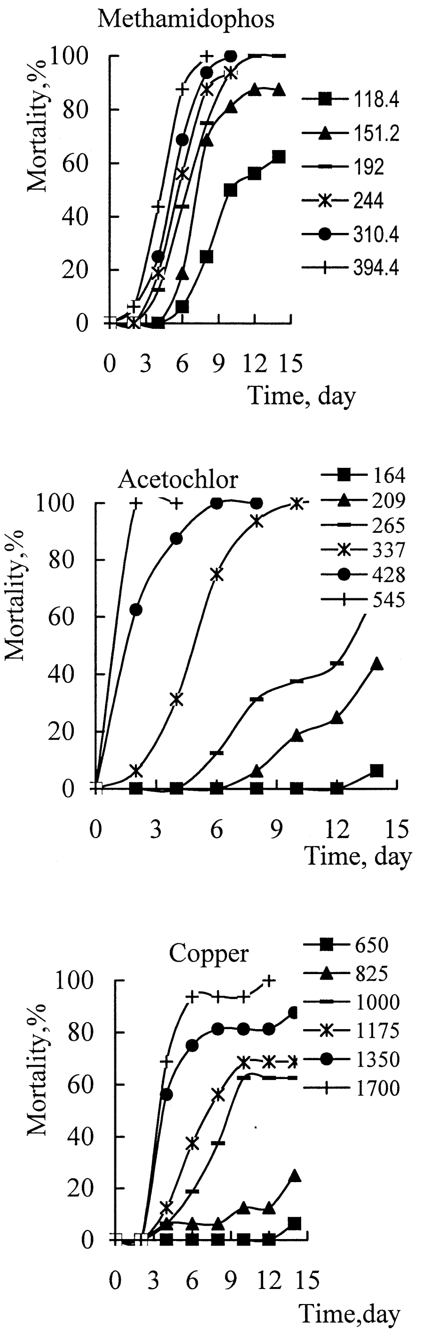

Figure 1. Time dynamics of toxic effects of earthworms exposed to methamidophos, acetochlor and copper.

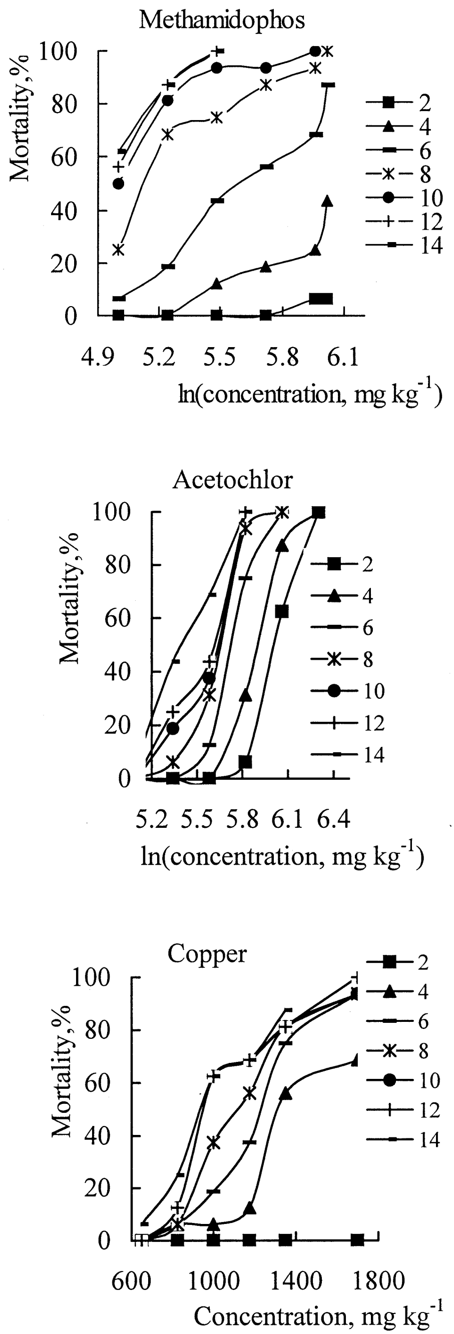

Figure 2. Relationships between mortality of earthworms and concentration of methamidophos, acetochlor and copper.

mg kg^{-1} or so, about accounting to the mean of MLC and LC_{100}, the caved curves were "S", just as described before; when it was lower than the concentration, the shape of the curves was concave, indicating that the death of earthworms under the concentrations took place slowly first, then quickly; when it was higher than the concentration, the shape of the curves was convex, which means that the death of earthworms under the concentrations took place quickly first, then slowly. In Figure 1-copper, the curves replied the treatment of any concentration of Cu showed "S", just like toxic effects of methamidophose.

The results also suggested that earthworm mortality increased with increasing concentration of one of the three pollutants (Figure 2). The changing trend in the mortality of earthworms exposed to methamidophos was a positive correlation with logarithmic value of concentration of methamidophos, namely, the death of earthworms under the experimental conditions was increased with the increase in the dose of methamidophos added to the soil. Similarly, there were positive linear relationships between the mortality of earthworms and ln (concentration of Cu). The mortality of earthworms was also increased with the increase in the concentration of acetochlor. The corresponding relationships can be expressed by regression equations in Table 2. In these equations, X is the concentration of a pollutant added to the tested soil, Y_1, Y_2, Y_3, Y_4, Y_5, Y_6 and Y_7 is the mortality of earthworms exposed to the polluted soil for 2, 4, 6, 8, 10, 12 and 14 days, respectively.

Apparently, relationships between the mortality of earthworms and the amount of methamidophos added to the soil was similar to that between the mortality of earthworms and the amount of Cu added to the soil, which can be described using a following equation:

$$Y = a\ln(X) + b \tag{1}$$

However, it is different from that between the mortality of earthworms and the amount of acetochlor added to the soil, which can be described using a following equation:

$$Y = a\ln^2(X) + b\ln(X) + c \tag{2}$$

It can be inferred according to equations 1 and 2 that toxic mechanisms of earthworms by methamidophos and by Cu may be similar one another, different from these by acetochlor.

Based on the regression equations in Table 2, half lethal dose (LD_{50}) under the conditions of different exposure time was calculated and listed in Table 2.

Table 2. Changes in the mortality of earthworms with concentrations of methamidophos, acetochlor and copper added to the soil.

	Time (day)	Regression equation (n=6)	R^2	Significance level p	LD_{50}
Methamidophos	2	$Y_1 = 6.04\ln(X)-33.6$	0.646	<0.025	228.6
	4	$Y_2 = 38.0\ln(X)-195.0$	0.857	<0.005	137.2
	6	$Y_3 = 74.4\ln(X)-367.6$	0.971	<0.005	112.6
	8	$Y_4 = 62.3\ln(X)-271.9$	0.865	<0.005	63.5
	10	$Y_5 = 46.9\ln(X)-173.1$	0.786	<0.005	32.5
	12	$Y_6 = 91.1\ln(X)-396.4$	0.942	<0.005	62.2
	14	$Y_7 = 78.1\ln(X)-326.0$	0.964	<0.005	52.3
Acetochlor	2	$Y_1=127.9\ln^2(X)-1375.3\ln(X)+3690.8$	0.968	<0.005	275.3
	4	$Y_2=89.1\ln^2(X)-921.6\ln(X)+2378.7$	0.937	<0.005	217.9
	6	$Y_3=124.0\ln^2(X)-1269.3\ln(X)+3245.0$	0.946	<0.005	194.9
	8	$Y_4=46.5\ln^2(X)-399.2\ln(X)+820.4$	0.918	<0.005	176.8
	10	$Y_5=189.9\ln^2(X)-1940.8\ln(X)+4961.1$	0.983	<0.005	
	12	$Y_6=135.6\ln^2(X)-1348.3\ln(X)+3350.7$	0.982	<0.005	154.6
	14	$Y_7=-27.1\ln^2(X)+423.8\ln(X)-1448.8$	0.996	<0.005	155.8
Copper	2	$Y_1 = 0$			
	4	$Y_2 = 75.2\ln(X)-499.3$	0.773	<0.005	767.9
	6	$Y_3 = 105.2\ln(X)-694.9$	0.907	<0.005	740.7
	8	$Y_4 = 108.9\ln(X)-713.4$	0.959	<0.005	701.2
	10	$Y_5 = 105.8\ln(X)-683.9$	0.923	<0.005	646.9
	12	$Y_6 = 110.7\ln(X)-717.4$	0.941	<0.005	655.0
	14	$Y_7 = 113.8\ln(X)-732.2$	0.969	<0.005	626.7

According to LD_{50}, it is clear that the toxicity of acetochlor acting on earthworms is weaker than that of methamidophos, but stronger than that of Cu under the condition of single-factor pollution. LD_{50} was decreased with the increase in an exposure time. In other words, even very small amount of a pollutant in soil may have a toxic effect on earthworms when having been exposed for a long period.

Tested concentration combinations of the three pollutants jointly acting on earthworms were determined and listed in Table 3 according to the above-mentioned single-factor effects of the pollutants on earthworms. Under the experimental conditions, binary-combined effects of the three pollutants were complicated. When the exposed concentration of Cu was 300 mg kg^{-1} (low Cu) and that of acetochlor was 164 mg kg^{-1} (low acetochlor), there was almost no dead earthworm after a 14-day exposure. When the exposed concentration of Cu was 1000 mg kg^{-1} (high Cu) and that of acetochlor was 265 mg kg^{-1} (high acetochlor), the mortality of earthworms was up to 65% or so after a 14-day exposure.

Table 3. Binary concentrations of methamidophos, acetochlor and copper.

Combination	Treatment	Concentration (mg kg^{-1})				
1	Methamidophos	118.4	151.2	192.0	244.0	310.4
	Low Cu	300.0	300.0	300.0	300.0	300.0
2	Methamidophos	118.4	151.2	192.0	244.0	310.4
	High Cu	1000.0	1000.0	1000.0	1000.0	1000.0
3	Acetochlor	164.0	209.0	265.0	337.0	428.0
	Low Cu	300.0	300.0	300.0	300.0	300.0
4	Acetochlor	164.0	209.0	265.0	337.0	428.0
	High Cu	1000.0	1000.0	1000.0	1000.	1000.0
5	Methamidophos	118.4	151.2	192.0	151.2	192.0
	Low acetochlor	164.0	164.0	164.0	164.0	164.0
6	Methamidophos	118.4	151.2	192.0	244.0	310.4
	High acetochlor	265.0	265.0	265.0	265.0	265.0

It is showed in Table 4 that toxic effects of methamidophos on earthworms under the condition of methamidophos-Cu combined pollution were strengthened compared with these of individual methamidophos on earthworms when the methamidophos was at low concentration (118.4 mg kg^{-1}) due to interaction of methamidophos and Cu. However, when methamidophos was at the concentration of $\geq$ 151.2 mg kg^{-1}, the toxicity of methamidophos under the condition of methamidophos-Cu combined pollution was weakened due to interaction of methamidophos and Cu. Whether the concentration of Cu was high or low, toxic effects of Cu under the condition of methmidophos-Cu combined pollution were strengthened compared with these of individual Cu on earthworms.

Toxicity of acetochlor acting on earthworms under the condition of acetochlor-Cu combined pollution was apparently less than that of individual acetochlor when the concentration of Cu was low (300 mg kg^{-1}) (Table 5). However, when the concentration of Cu was high (1000 mg kg^{-1}), joint toxicity of acetochlor was stronger than toxicity of individual acetochlor at the low concentration of $\leq$ 265 mg kg^{-1} but was weaker than single toxic effects of acetochlor at the high concentration of $\geq$ 337 mg kg^{-1}. Toxic effects of Cu under the condition of acetochlor-Cu combined pollution were stronger than these of individual Cu on earthworms at any exposed concentration of Cu. In particular, the higher exposed concentration of Cu was, the stronger the interactive toxic effects on earthworms they displayed.

Combined effects of acetochlor and methamidophos on the mortality of earthworms were significantly affected by changing concentrations of methamidophos and acetochlor added to the soil. Jointly toxic effects of

Table 4. Joint effect of methamidophos (Meth.) and Cu on earthworms after a 14-day exposure.

Cu mg kg^{-1}	Mortality %	Meth. mg kg^{-1}	Mortality %	Cu+Meth. mg kg^{-1}	Mortality %	Joint effect
300.0	0	118.4	0	300.0+118.4	12.5	Synergism
300.0	0	151.2	62.5	300.0+151.2	25	Addition
300.0	0	192.0	87.5	300.0+192.0	37.5	Addition
300.0	0	244.0	100	300.0+244.0	87.5	Addition
300.0	0	310.4	100	300.0+310.4	93.75	Addition
1000.0	62.5	118.4	0	1000.0+118.4	25	Addition
1000.0	62.5	151.2	62.5	1000.0+151.2	87.5	Addition
1000.0	62.5	192.0	87.5	1000.0+192.0	81.25	Addition
1000.0	62.5	244.0	100	1000.0+244.0	93.75	Addition
1000.0	62.5	310.4	100	1000.0+310.4	100	Addition

Table 5. Joint effect of acetochlor (Ace.) and Cu on earthworms after a 14-day exposure.

Cu mg kg^{-1}	Mortality %	Ace. mg kg^{-1}	Mortality %	Cu+Ace. mg kg^{-1}	Mortality %	Joint effect
300.0	0	164.0	6.25	300.0+164.0	0	Antagonism
300.0	0	209.0	43.75	300.0+209.0	6.25	Addition
300.0	0	265.0	68.75	300.0+265.0	18.75	Addition
300.0	0	337.0	100	300.0+337.0	62.5	Addition
300.0	0	428.0	100	300.0+428.0	100	Addition
1000.0	62.5	164.0	6.25	1000.0+164.0	81.25	Synergism
1000.0	62.5	209.0	43.75	1000.0+209.0	93.75	Addition
1000.0	62.5	265.0	68.75	1000.0+265.0	93.75	Addition
1000.0	62.5	337.0	100	1000.0+337.0	93.75	Addition
1000.0	62.5	428.0	100	1000.0+428.0	100	Addition

methamidophos and acetochlor were always promoted compared with these of individual acetochlor and methamidophos acting on earthworms. When low concentration of acetochlor combined with various concentrations of methamidophos, all the earthworms were died after a culture for 10 days, and when high concentration of acetochlor combined with various concentrations of methamidophos, all the earthworms were died after a culture for only 6 days. In other words, the interaction between acetochlor and methamidophos has synergic toxic effects on earthworms.

There were toxic effects of methamidophos, acetochlor and Cu on earthworms. The toxicity of a single pollutant is in the sequence methamidophos > acetochlor > Cu. Binary toxic effects of the three pollutants were complicated and depended more on their concentration combinations than individual concentration. This conforms to the generalized theory of joint effects put forward by Zhou (Zhou, 1995). Thus this theory appears to be very significant and will encourage the development of ecotoxicology.

Acknowledgments. The research was financially supported as an important direction project (No. KZCX2-SW-416) of the knowledge innovation engineering by Chinese Academy of Sciences and as an excellent young scientist award (No. 20225722) by the Natural Science Foundation of China. This work was presented at the First International Conference on Pollution Eco-Chemistry and Ecological Processes which was held in Shenyang, China, on August 26-31, 2002.

REFERENCES

Archer DW, Shogren JF (2001) Risk-indexed herbicide taxes to reduce ground and surface water pollution: an integrated ecological economics evaluation. Ecol Econ 38: 227-250

Cheng Y, Zhou QX (2002) Ecotoxicity of reactive X-3B red dye and cadmium acting on wheat (*Triticum aestivum*). J Environ Sci 14: 136-140

Garca JA, Fragoso C (2002) Growth, reproduction and activity of earthworms in degraded and amended tropical open mined soils: laboratory assays. Appl Soil Ecol 20: 43-56

Johnson DL, Jones KC, Langdon CJ, Piearce TG, Semple KT (2002) Temporal changes in earthworm availability and extractability of polycyclic aromatic hydrocarbons in soil. Soil Biol Biochem 34: 1363-1370

Kikuchi M, Sasaki Y, Wakabayashi M (2000) Screening of organophosphate insecticide pollution in water by using daphnia magna. Ecotoxicol Environ Saf 47: 239-245

Liang W, Zhang WM, Li WG (2001) Effect of chemical fertilizer on nematode community composition and diversity in the black soil region (in Chinese). Biodiver Sci 9: 237-240

Miyazaki A, Amano T, Saito H, Nakano Y (2002) Acute toxicity of chlorophenols to earthworms using a simple paper contact method and comparison with toxicities to fresh water organisms. Chemosphere 47: 65-69

Song YF, Zhou QX, Song XY, Sun TH (2002) Study on ecotoxicological diagnosis methods of soil-environmental contamination (in Chinese). Ecol Sci 21: 184-188

Zhou QX (1995) Ecology of combined pollution (in Chinese). China environmental Science Press, Beijing, China

Zhou QX, Huang GH (2001) Environmental biogeochemistry and global environmental changes (in Chinese). Science Press, Beijing, China