Quantitative analyses of relationships between ecotoxicological effects and combined pollution

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Received September 9, 2003

Abstract The responses of wheat *Triticum aestivum*, rice *Oryza sativa*, earthworms *Eisenia foetida*, and prawns *Penaeus japonicus* to combined acetochlor-Cu, Cd-Zn were studied in hydroponic and soil-culturing systems using the methods of ecotoxicology. In particular, systematically quantitative analyses were documented by field experiments. Results showed that ecotoxicological effects under the combined pollution were not only related to chemical properties of pollutants but also dependent on the concentration level of pollutants, in particular on the combination of concentrations of pollutants in ecosystems. Additionally, species of organisms, especially the type of ecosystem, determined the influences. To some extent, biological tissue targets attacked by pollutants were an important factor.

Keywords: combined pollution, ecotoxicological effect, pollutant, biological toxicity, quantitative analysis.

DOI: 10.1360/03yc0042

In 1939, Bliss first defined the toxicity of poisons applied jointly by "antagonism, synergism, addition and dependent action". He thought that this kind of joint effects were only subject to their natural chemical properties[1]. With the increasing categories and quantities of contaminants released into the environment since the 1970s[2,3], effects of combined contamination and the interactions between pollutants have been increasingly seen as a source of concern[4—7]. Besides the regularly tested animals such as rats, rabbits, guinea pigs, frogs and dogs used in the early toxicological studies, aqueous organisms including fish and algae, some crops, rice and wheat for example, even soil animals and soil microbes have been used to investigate the ecotoxicity of pollutants. Indexes of toxic effect evaluation, the extent of toxicity or IC_{50}, and the effects on the production and toxin-accumulation in bodies have been extensively studied[7—10]. Many studies have helped develop the widely accepted conclusion that the ecotoxicological effects of combined contamination were determined by not only the chemical properties of pollutants or toxic elements, but also by the concentration level[11,12]. However, a few observations have recently revealed that the concept must be improved and corrected because of significant limitations.

1 Materials and methods

1.1 *In-vivo* soil culture toxicity test

In this part, wheat *Triticum aestivum* was selected as the living organism for the toxicity test. The tested pollutants included a herbicide in common use, 50% of oil soluble concentration of acetochlor ($C_{14}H_{20}$-$ClNO_2$) which was provided by the Ruize Pesticide Limited Co. Dalian, and $CuSO_4 \cdot 5H_2O$ (analytic grade), bought from the Third Reagent Factory Shenyang. Based on the previous combined pollution experiments[7], the dose of acetochlor exposed to wheat was 0, 0.1, 0.3, 0.6, 0.9, 1.2, and 1.5 mg/kg, and that of copper (Cu) was 30, 90, and 150 mg/kg.

After being mixed with the above various doses of Cu and acetochlor, the air-dried soil samples collected from a field in the Hailun Station of Agricultural Ecology, Heilongjiang Province were put in pots overnight and irrigated with 200 mL of tap water. All treatments were replicated 4 times, and each replicate consisted of 4 kg soil and 5 seedlings of wheat. They were moistened using 200 mL of tap water every 3 days. Once mature, fruits were harvested, washed, air-dried and weighed. Roots and fronds were separately harvested, washed, air-dried and ground by machine. The ground samples were then analyzed for the total concentration of Cu using the atomic absorption spectrophotometer after a digestion in concentrated HNO_3-$HClO_4$ solution.

1.2 *In-vivo* water culture toxicity test

Rice *Oryza sativa* was selected for the hydroponic experiments. The tested pollutants were $ZnSO_4 \cdot 7H_2O$ and $CdCl_2 \cdot (5/2)H_2O$. On the basis of the previous experimental results[13], the concentration of Cd exposed to rice plants was 0, 1, 1.5, 3.0, and 6.0 mg/L, and that of Zn was 100, 200, and 300 mg/L.

Four kilograms of air-dried black soils were completely mixed with 1.5 liters of tap water and allowed to stand for 5 min. After being mixed with the above various concentrations of Zn and Cd, 1 liter of suspension liquid was put in a plastic bucket. Each bucket consisted of 5 seedlings of rice fixed with sponge and plastic ropes. All treatments were replicated 4 times. 400 mL tap water was added every 3 days. Once mature, fruits were harvested, washed, air-dried and weighed. Roots and fronds were separately harvested, washed, air-dried and ground with a machine. The ground samples were then analyzed for the total concentration of zinc using the atomic absorption spectrophotometer after a digestion in concentrated HNO_3-$HClO_4$ solution.

1.3 *In-vitro* terrestrial ecotoxicity test

The earthworm *Eisenia foetida*, the tested organism living in soil, was used as an international standard for ecotoxicological experiments. Before the formal experiments, healthy and mature *E. foetida* with weight ranging from 300 to 500 mg and with a similar size of band were precultivated to determine the MLD and LD_{100}, namely the minimum and 100% lethal dose after a 48-h exposure. Within the range between the MLD and LD_{100}, the dose of acetochlor was set to 0, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mg/kg, and that of Cu was 30, 60, 90, 120, and 150 mg/kg for the single pollution experiments. Based on the single factor investigations, two levels of copper (30 mg/kg and 90 mg/kg) were mixed with the above doses of acetochlor.

The *in-vitro* experiment for toxicity followed the below procedures: *E. foetida* were washed and put to beakers with water to merge half bodies after precultivation. The beakers were then sealed with a thin plastic film with numerous tiny holes punctured by a needle, and finally cultivated in a biochemical incubator at 20 ± 1℃ overnight to clean the intestines. After precultivation, 15 *E. foetida* were washed, toweled off with filter paper, and put in a Petri dish with 10 mL of acetochlor and $CuSO_4$ at the designed level with 5 pieces of filter paper laid at the bottom. In order to prevent the worms from escaping, the Petri dishes were sealed with gauze; they were then placed in a dark incubator at 20 ± 1℃ for 48 h. All the treatments were replicated 3 times. The toxicological symptoms and actions were recorded periodically. If the tail was insensitive to mechanical incentives, *E. foetida* was considered dead. The number of dead *E. foetida* was recorded after 24-h and 48-h exposure for the statistic analyses.

1.4 *In-vitro* aquatic ecotoxicity test

The tested prawn *Penaeus japonicus* was collected from the Zhoushan seawater, Zhejiang Province. Before the formal experiments, healthy and mature *P. japonicus* with a similar size and weight ranging from 5 to 6 g were precultivated to determine the concentration range under single contamination. The dose of acetochlor exposed to the prawns was 0, 0.04, 0.08, 0.12, 0.16, 0.20, and 0.24 mg/kg, and that of Cu was 30, 45, 60, 75, and 90 mg/kg. Based on the single factor investigations, two levels of copper (30 mg/kg and 90 mg/kg) were mixed with the above doses of acetochlor.

The *in-vitro* experiment for toxicity followed the below procedures: *P. japonicus* were washed with seawater and put in 1 liter beakers with 600 mL seawater and the above various doses of pollutants. 10 *P. japonicus* were put in one beaker and aerated vigorously. All the treatments were replicated 3 times. The toxicological symptoms and actions were recorded periodically. If the tail was insensitive to mechanical incentives, *P. japonicus* was considered dead. The number of dead *P. japonicus* was recorded after 24-h and 48-h exposure for the statistic analyses.

1.5 DGGE fingerprinting analysis

Tested phaeozem (black soil) samples for DGGE analysis were also collected from the Hailun Station of Agricultural Ecology, Heilongjiang Province. The background concentrations of acetochlor and Cu in the clean soil from a clean field without any pesticide application for more than a decade were 0.8 and 21.3 mg/kg, respectively. Concentrations of acetochlor and Cu in the polluted soil from a polluted field to which acetochlor and germicide containing Cu have been heavily applied for many years were 91.7 and 83.2 mg/kg, respectively. Tested pollutants were the same as those in sections 1.1, 1.3 and 1.4. Fresh clean soil samples were equably mixed and ground to pass through a sieve with 0.2 mm openings. Acetochlor and Cu were added to plastic bottles filled with 150 g of ground soil samples. According to the pre-experimental results, the concentration of acetochlor added to the soils was 0, 100, and 250 mg/kg, and that of Cu was 0, 100 and 300 mg/kg. Having been sealed, the bottles were stored in a constant-temperature incubating box at 20℃. In other words, soil microorganisms in the bottles were exposed to single-factor and combined pollution. All the treatments were replicated 3 times. Total soil-microbial DNA was extracted based on the method suggested by Martin-Laurent et al.[13]. The purification of extracted DNA was carried out according to the instructions for DNA gel reclamation in a silver-bead reagent box (made in Shanghai).

Nest-PCR cloning based on the Thermo Hybaid PCR express cloning vector was used. Reaction I (50 μL) was: 5 μL of 10 × Buffer, 4 μL of 2.5 mmol/L dNTP, 4 μL of 10 pmol/μL primers F8-27f, 4 μL of 10 pmol/μL primers 1541-1522r, 1 μL templet DNA, 0.1 μL of 5 U/μL Ex Taq enzyme (Dalian Bao Bio. Co.), 31.9 μL hyperpure water. There were 16 cycles in the reaction: 95℃ (5 min), 1 cycle; 95℃ (1 min), 62℃ (1 min), and 72℃ (3 min), 15 cycles. Reaction II was basically the same as reaction I, but the primers were P338f-GC and P518r, model DNA was 3 μL of products from reaction I, and 29.9 μL hyperpure water. There were 36 cycles in reaction II: 94℃ (1 min), 60℃ (30 s), and 72℃ (2 min), 10 cycles; 94℃ (30 s), 60—55℃ (30 s) (0.5℃ was decreased at a cycle), and 72℃ (2 min), 10 cycles; 94℃ (30 s), 55℃ (30 s), and 72℃ (2 min), 15 cycles; 72℃ was extended to 8 min after 35 cycles.

Two groups of PCR amplified primers were used. The first primers (the general bacterium primers): the forward primer P8-27F (5′AGAGTTTGATCMTGGCTCAG3′, M is A or C) and the reverse primer P1541-1522R (5′AAGGAGGTGATCCAGCCGCA 3′)[14]; the second primers (primers for analysis of bacterium communities): the forward primer P338f (5′ACTCCTACGGGAGGCAGCAG3′) and the reverse primer P518r (5′ATTACCGCGGCTGCTGG3′). In order to avoid conglutination of amplified segments, the GC clamp (5′CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG3′)[15] was linked with the forward primers.

PCR products were analyzed on denaturing gradient gel electrophoresis (DGGE) gels by using the Dcode system electrophoresis apparatus (from the Bio-Rad Co.) method based on the protocol of Muyzer and co-workers[16]. After the DGGE analysis, silver staining of DNA was undertaken based on the modified method suggested by Bassam and Caetano-Anollés (1991)[17]. DGGE fingerprinting analysis and bands were identified using the gel imaging system (the Bio-Rad Co.).

1.6 *In-situ* quantificational analysis

In order to determine the effects of combined pollution on Cd and Zn accumulation in crops, potato (*Solanum tuberosum*), Chinese cabbage (*Brassica Pekinensis Rupr*), and wild amaranth (*Amaranthus retroflexus*) were collected from the Zhangshi Sewage-Irrigation area, and the Qingchengzi Lead-Zinc mining region. Those from the uncontaminated Shilihe area were used as the control.

1.7 Statistical analysis

The data from the above experiments were statistically analyzed using Microsoft Excel. The statistical analyses included relationships between a decrease in crop yield and the amount of pollutants in the tissues of crops and exposed doses of pollutants. When the correlation coefficient r is a positive value, a positive correlation relationship can be shown; if r is a negative value, a negative correlation relationship can be shown. When the significance level $p < 0.01$, there is a significant correlation relationship; when $p < 0.005$, the correlation relationship is very significant.

2 Results

2.1 Dominant action of pollutant concentrations and their combinations

Many related results have shown that crop production responded to agricultural pollution by inhibition, less productivity or serious “zero” yield[7,18]. This study had the consistent results, in that the yield of wheat (*Triticum aestivum*) was decreased to different extents under the stress of combined acetochlor and Cu. With a low concentration of copper (30 mg/kg), the significant positive linear correlation relationship can be expressed by the following equation:

$$Y_1 = 14.41X_{cae} + 122.66$$

$$(r = 0.524, n = 28, p < 0.005), \quad (1)$$

where Y_1 is the yield of wheat, X_{cae} is the concentration of the added acetochlor (mg/kg). It is evident that when copper concentrations were low, the interaction between these two pollutants inhibited the toxicity of acetochlor, and low Cu could stimulate the growth and development of wheat; the yield, therefore, showed an increasing trend. When copper was at the medium level (90 mg/kg), the correlation relationship can be expressed as follows:

$$Y_1 = -26.86X^2_{ace} + 23.68X_{ace} + 116.87$$

$$(r = -0.610, n = 28, p < 0.005). \quad (2)$$

The parabola with a down open indicated that the relationship was very complicated, with the antagonistic action being followed by synergetic effects. When the dose of copper became high (150 mg/kg), the negative correlation between the concentration of acetochlor and the yield was indicated by the following equation:

$$Y_1 = -31.61X_{ace} + 122.14$$

$$(r = -0.497, n = 28, p < 0.005). \quad (3)$$

In these three sets of experiments, ecotoxicological effects differed from each other as a result of the difference in the copper concentrations even though acetochlor was added at the same level, 0, 0.1, 0.3, 0.6, 0.9, 1.2, and 1.5 mg/kg. In other words, in the case of effects on the productivity of wheat, concentration combination relationships of the two pollutants added to soils should be a determinant factor.

Similarly, effects of heavy metals such as Cd and Zn on output of rice were largely determined by their combination of concentrations (table 1). With a constant concentration of zinc, increasing the dose of Cd increased the output of rice, which significantly showed a positive correlation relationship. With zinc concentrations of 200 and 300 mg/kg, the output of rice decreased with the increasing concentration of Cd, namely a significantly negative relationship.

It was also indicated by the DGGE fingerprinting analysis of partial 16S rDNA gene segments from the contaminated soil that ecotoxicological effects at the molecular levels under combined pollution to a large

Table 1 Relationships between added Cd-Zn concentration combinations and the yield of rice

Added Zn/mg • kg^{-1}	Yield curve (n = 20)	Correlation coefficient r	Correlativity	Significance level p
100	$Y_2 = 315.82 + 2.80X_{Cd}$	0.522	Positive	0.01
200	$Y_2 = 334.03 - 5.51X_{Cd}$	0.782	Negative	< 0.005
300	$Y_2 = 323.58 - 7.79X_{Cd}$	0.677	Negative	< 0.005

degree were directly dependent on concentration combinations of pollutants added to the soil. When the exposed concentrations of combined acetochlor and Cu were 250 and 300 mg/kg, respectively, 3 lucent specific bands appeared (fig. 1). In other words, there were possible mechanisms that the high concentration combination of the two pollutants could induce the gene mutation of soil microorganisms due to their strong acute toxicity, and/or specific microorganisms (e.g. endurable and degradable bacteria) which can be represented by the 3 specific bands were greatly increased and enriched under the stress of combined acetochlor and Cu at high concentrations[13,17]. When the exposed concentrations of combined acetochlor and Cu were only 100 and 100 mg/kg, 3 specific bands did not appear. Moreover, there was no band under the stress of single acetochlor or Cu. However, 3 brightness-decreasing specific bands appeared in 16S rDNA gene segments from the contaminated soil due to the long-term application of acetochlor and germicides containing Cu. Thus, the low-dose and long-term exposure of combined acetochlor and Cu could result in the appearance of 3 specific bands due to a possible heritable mutation of microorganisms in order to accommodate this kind of environmental change. We expect that these interesting relevant mechanisms will be further explored in due course.

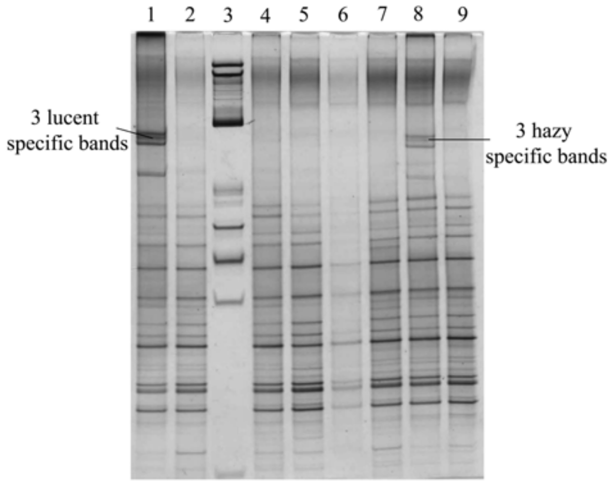

Fig. 1. DGGE analysis and imaging of 16S rDNA gene segments from contaminated soils. Lane 1, Treated by combined acetochlor (250 mg/kg) and Cu (300 mg/kg); lane 2, treated by acetochlor (100 mg/kg) and Cu (100 mg/kg); lane 3, ϕX174; lane 4, treated by single acetochlor (100 mg/kg); lane 5, treated by single acetochlor (250 mg/kg); lane 6, treated by single Cu (100 mg/kg); lane 7, treated by single Cu (300 mg/kg); lane 8, contaminated soil by agricultural activity; lane 9, clean soil.

2.2 Directive effects of living organism species and ecosystem types

Exposed to single contaminants, acetochlor and Cu, *Eisenia foetida* showed 0.307 and 118.70 mg/kg LC_{50}. The LC_{50} of combined acetochlor and Cu reached 0.401 mg/kg after 48 h exposure. This demonstrated that combined contamination at a low level had a weak toxicity, namely antagonistic effects; when the Cu concentration was 90 mg/kg, 48 h-exposure killed all the *E. foetida*. A conclusion can be reached that combined acetochlor and Cu at a high level promoted the toxicity, namely synergetic effects.

In contrast to *E. foetida*, *Penaeus japonicus* was more sensitive to single acetochlor contamination, which was evident through the lower LC_{50}, 0.128 mg/L. Once copper was added, the toxicity to *P. japonicus* was promoted no matter whether copper concentration was low (30 mg/kg) or high (90 mg/kg). Further analyses, however, showed that acetochlor combined with low doses of copper strengthened the effects on *P. japonicus*, whereas acetochlor combined with high copper content affected the toxicity by adding their own single effects, namely additive effects. Therefore, in addition to the above-mentioned factors, the type of organisms also played an important role in their responses to poisonous compounds after interaction between pollutants in the environment, the so-called ecotoxicological effects of combined pollution.

The absorption and accumulation of Cd and Zn by potato (*Solanum tuberosum*), Chinese cabbage

(*Brassica Pekinensis Rupr*), and wild amaranth (*Amaranthus retroflexus*) sampled from the Zhangshi Sewage-Irrigation area, the Qingchengzi Lead-Zinc Mining region, and the Shilihe agricultural area of Shenyang were analyzed (table 2). The former two regions were respectively polluted by irrigation with Cd-waste water and mining activities. Compared with those from uncontaminated Shilihe area, these three crops sampled from contaminated districts accumulated cadmium to an obvious extent. In particular, in the case of *Brassica Pekinensis Rupr* there was a four-fold increase. As for zinc absorption, three kinds of plants from polluted areas demonstrated different trends with a significant increase in *Solanum tuberosum* and a noticeable decrease in *Brassica Pekinensis Rupr*, and *Amaranthus retroflexus.* Similarly, in the same control system, all crops from the Qingchengzi Lead-Zinc Mining region had a greater increase of 4.6—5.6 times in cadmium accumulations. With zinc uptake, *Solanum tuberosum* increased its extraction remarkably, whereas *Brassica Pekinensis Rupr* decreased it to some extent and *Amaranthus retroflexus* displayed a striking reduction, but a less severe slope in comparasion with that from the Zhangshi area. It can be concluded that combined cadmium and zinc mutually enhanced the toxicity to *Solanum tuberosum*, resulting from some accelerating absorption mechanism between them. Cadmium can inhibit Zn uptake in *Brassica Pekinensis Rupr*, and caused antagonistic action in *Amaranthus retroflexu*.

2.3 Impacts on living tissue targets attacked by pollutants

Under the stress of the combined pollution of acetochlor and Cu, there existed a substantial difference in Cu absorption and accumulation between root systems and fronds of wheat. It was shown that at a low concentration of copper, 30 mg/kg, there was a significant positive correlation relationship between the action of the root absorbing copper and the dose of acetochlor, according to the following equation:

$$Q_{Cu} = 6.22C_{ace} + 21.62$$
$$(r = 0.648, n = 28, p < 0.005), \quad (4)$$

where Q_{Cu} is the amount of copper taken up by the root, C_{ace} is the concentration added (mg/kg). It indicated that acetochlor can promote the biological absorption of copper by the underground parts, namely synergetic effects. Contrarily, the negative correlation relationship between the quantity of copper extracted in fronds and the added concentration of acetochlor in the soil can be expressed as follows:

$$Q_{Cu} = -2.48C_{ace} + 8.57$$
$$(r = -0.853, n = 28, p < 0.005). \quad (5)$$

In other words, acetochlor inhibited the Cu uptake by the so-called antagonistic effects.

Similarly, when copper was at the high level, 150 mg/kg, there was no noticeable correlation between the amount of Cu accumulated by roots and the added dose of acetochlor, with r being only 0.049. That is to say that the dose of acetochlor had no effect on the Cu uptake. Fronds, however, can accumulate increasing copper with an increment of acetochlor concentration, giving the regression equation:

$$Q_{Cu} = 8.64C_{ace} + 20.57$$
$$(r = 0.736, n = 28, p < 0.005). \quad (6)$$

Therefore, it can be concluded that at the high level, the added acetochlor can promote Cu accumulation in the fronds, namely showing synergetic effects.

Table 2 Cd-Zn accumulation by crops in different ecosystems

Type of ecosystem	Potato (*Solanum tuberosum*) (n = 50)		Chinese cabbage (*Brassica Pekinensis Rupr*) (n = 50)		Wild amaranth (*Amaranthus retroflexus*) (n = 50)	
	Cd/μg • kg^{-1}	Zn/mg • kg^{-1}	Cd/μg • kg^{-1}	Zn/mg • kg^{-1}	Cd/μg • kg^{-1}	Zn/mg • kg^{-1}
Zhangshi domain	223.5	38.4	393.2	28.2	429.6	8.2
Qingchengzi region	271.6	45.9	471.3	36.0	682.1	14.6
Shilihe area	59.1	14.2	98.9	41.8	121.9	55.3

Exposed to the combined pollution of Cd and Zn, rice differentially absorbed Zn in different tissues as a result of the interaction between Zn and Cd. When the concentration of Zn was 300 mg/kg, increasing the dose of Cd increased the absorption of Zn in rice roots. The positive correlation relationship can be expressed by the following equation:

$$Q_{\mathrm{Zn}} = 3.79C_{\mathrm{Cd}} + 150.00$$
$$(r = 0.522,\ n = 28,\ p = 0.01). \quad (7)$$

This suggested that the capability of extracting Zn by the root can be enhanced by Cd. Instead, the negative correlation relationship between the amount of accumulated Zn in fronds and the level of added Cd can be expressed as follows:

$$Q_{\mathrm{Zn}} = -1.89C_{\mathrm{Cd}} + 60.71$$
$$(r = -0.585,\ n = 28,\ p < 0.005). \quad (8)$$

It was obvious that the interaction between Cd and Zn showed an antagonistic action.

3 Discussion

Under stress from a single pollutant, harmful effects of the pollutant on organisms were basically determined by its physical and chemical properties[18], especially the dose of pollutant affecting organisms[5,11]. Similarly, the concentration level played an essential role in the toxicological influences under the combined pollution of varieties of contaminants[7]. However, there has been a dispute about which factor, the concentrations themselves or the concentration combinations of pollutants, was key in this role. Results in this study supported the latter supposition. In other words, based on the yield and microbiological community changes, ecotoxicological effects of combined contamination were dependent on concentration combination relationships of pollutants in ecosystems despite other important factors, e.g. natural characteristics and single concentration levels. Also, experiments on the uptake by wheat of toxic elements validated this conclusion: acetochlor combined with a low dose of copper decreased Cu accumulation; at the high level of Cu, the interaction between acetochlor and copper showed a synergetic action. The toxicity of combined acetochlor and Cu to *E. foetida* further proved the critical role of the concentration combination of pollutants by the demonstrated result that a high level of copper lowered the poisonousness of acetochlor, whereas a low level promoted the toxicity of acetochlor. Harmful impacts of combined acetochlor and copper on *P. japonicus* confirmed the conclusion when considering aqueous organisms.

Additionally, although exposed to the same pollution degree, different kinds of organisms responded to the interaction between pollutants with different trends. For example, under the stress of combined copper and acetochlor, the low dose of Cu can alleviate the toxicity of acetochlor to *E. foetida*. Contrarily, the low dose of Cu magnified the toxicity of acetochlor to *P. japonicus*. This kind of difference, of course, should contribute to interactions between *in vivo* constituents of organisms and pollutants. For this reason various ecotoxicological effects can be observed even if the organisms were exposed to the same circumstances of stress, hence bioaccumulation was influenced to a different extent despite of identical levels and types of contamination. Sometimes, in the same species, the different populations even showed different toxicological effects under the same kind of pollution[19].

Previous studies[2,18,20] showed that many heavy metals, such as lead, copper and cadmium, tended to concentrate in the livers and kidneys of animals, and concentrated more strongly in the roots of plants than in the overground parts of plants. The mechanism underlying this intriguing distinction has remained unknown. This study, however, documented that heavy metals in different tissues of organisms can interact not only with the metals themselves but with the constituents of organisms. This result, although with differences between interactions, further demonstrated the different ecotoxicological effects, including the distinct capability of accumulating metals.

Acknowledgements This work was supported financially by the National Science Fund grant (Grant No. 20225722) for Distinguished Young Scholars, Key Basic Research Project (Grant No. G1999011808) of the National Development Planning, and Knowledge Innovation project (Grant No. KZCX2-SW-416) of the Chinese Academy of Sciences.

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