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Investigation of acute toxicity of cadmium-arsenic mixtures to *Daphnia magna* with toxic units approach

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Abstract: The acute toxicities of cadmium chloride ($CdCl_2.H_2O$), arsenic trioxide (AS_2O_3), and their mixtures to *Daphnia magna* were investigated by employing the statistical method of aquatic toxicity. The data obtained were statistically evaluated with probit analysis method (SPSS 17.0). The 24 h LC_{50} of Cd and As to *Daphnia magna* was estimated to be 44 µg L^{-1} for Cd, and 509 µg L^{-1} for As, respectively. With Behrens-Karber method the 24 h LC_{50} of these metals was found to be 46 µg L^{-1} and 509 µg L^{-1}, respectively. The 2 methods were in good agreement. The results suggested that *D. magna* is more sensitive to the 90% Cd + 10% As mixture than either metal alone. Toxic units (TUs) and additional index (AI) showed that all mixtures were synergistic.

Key words: **Key words:** Acute toxicity, toxic unit, cadmium, arsenic, mixture, LC_{50}

Kadmiyum-arsenik karışımlarının *Daphnia magna* üzerine akut toksik etkilerinin toksik birim yaklaşımı ile incelenmesi

Özet: Bu çalışmada kadmiyum klorür ($CdCl_2.H_2O$), arsenik oksit (AS_2O_3) ve karışımlarının *Daphnia magna* üzerine akut toksik etkisi sucul toksikolojinin statik metodu kullanılarak araştırılmıştır. İstatistiksel olarak Probit analiz metoduna (SPSS 17.0) göre hesaplanan 24 saatlik LC_{50} değeri kadmiyum için 44 µg L^{-1}, arsenik için 509 µg L^{-1} bulunmuştur. Behrens Karber metodu ile letal dozlar sırasıyla 46 µg L^{-1} ve 509 µg L^{-1} olarak hesaplanmıştır. Bu iki metod sonucu birbirini destekler nitelikte bulunmuştur. Sonuçlar *D. magna*' nın tek başına kullanılan metallerin etkisine kıyasla %90 Cd + %10 As karşımına karşı daha hassas olduğunu desteklemiştir. Toksik Birim ve Additional İndeks tüm karışımların birbiri üzerinde sinerjistik olduğunu göstermiştir.

Anahtar sözcükler: Akut toksisite, toksik birim, kadmiyum, arsenik, karışım, LC_{50}

Introduction

Of late, the pollution of the aquatic environment by heavy metals has become a worldwide problem. Most heavy metals exhibit toxic effects on organisms. Heavy metals have not only the ability to bioaccumulate within aquatic ecosystems and in organisms; their structures are also resistant to environmental conditions (Klassen et al., 1986; MacFarlane and Burchettt, 2000; Miller et al., 2002; Censi et al., 2006).

Cadmium chloride (Cd) is a highly toxic heavy metal at very low exposure levels and produces acute and chronic effects on the health of both the

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environment and organisms (Nordic Council of Ministers Cadmium Review, 2003).

Arsenic trioxide (As) and its compounds are ubiquitous in nature (WHO, 2000). Anthropogenic activities result in extensive contamination of water with As. In natural water As is transformed into its various forms by some microorganisms, and this affects the toxicity of As to biota (He et al., 2009).

Zooplankton is one of the groups most sensitive to toxic chemicals, and these organisms occupy the central position in the lentic food chain; therefore, they are most commonly used in ecotoxicological tests (Hanazato, 1998). The cladocerans are an important link in the aquatic food chain. Daphnids are aquatic organisms also frequently used in toxicological studies worldwide. The use of *Daphnia* spp. as toxicity indicator organisms is well documented in the literature because of their fast growth rate, high reproductive rates, and short life cycles (Adema, 1978; Harmon et al., 2003; Cooman et al., 2005; Altındağ et al., 2008).

The development of industry and agriculture has resulted in an increase in the pollutants released into aquatic receiving systems, and the complex pollution of aquatic organisms has become increasingly prevalent. Toxicity in natural ecosystems typically does not occur from a single toxicant. It results from exposure to a mixture of toxicants, and, therefore, mixture toxicity has been a subject of ecotoxicological interest for several decades. For instance, Meng et al. (2008) studied the toxicity of 5 heavy metals on the *D. magna*; Ferreira et al. (2008) investigated the toxicity prediction of the binary combinations of cadmium and carbendazim on *D. magna*; and Vandenbrouck et al. (2009) worked on the nickel and binary metal mixture responses in *D. magna*.

Generally, in ecotoxicological studies the combined effects of heavy metals are analyzed with a toxic units (TUs) approach, which is used to test the response addition model for the chemical mixtures (Pape-Lindstrom and Lydy, 1997; Van der Geest et al., 2000; Meng et al., 2008).

The aim of the present study was to examine the effects of acute single and different rate of mixture of 2 common trace metal contaminants, Cd and As, on the cladoceran *D. magna*. Mixture rate effects were investigated with TU and additional index (AI).

Materials and methods

Culturing conditions

Deionized water from a Millipore Milli-Q ultrapure (Milli-Di, France) water system was used throughout the study, with the exception of the daphnid culture. The test organism *D. magna*, obtained from the Kepez Aquaculture Research Institute (Antalya, Turkey), was introduced into 30 L aquariums with dechlorinated tap water, which served as holding tanks. Cultures and all experiments were run in an environmental chamber. The water temperature, dissolved oxygen, pH, and electrical conductivity were measured regularly in the laboratory; the temperature was 20.2 ± 1.3 °C and the photoperiod was 12:12 h light:dark. Dissolved oxygen levels and electrical conductivity in the holding tanks were 6 mg L^{-1} and 250 µS cm^{-1}, respectively. Daphnids were maintained and fed *Scenedesmus* sp. daily, according to the animals' age: 3 × 106, 5 × 106, and 6 × 106 cells/day per individual aged 0-7 days, 8-15 days, and older than 15 days, respectively. *D. magna* was cultured according to the procedures outlined in ISO–6341 (ISO, 1996).

Experimental procedure

All reagents were of analytical grade, and all laboratory glassware was soaked in 10% HNO_3 for at least 48 h and rinsed 3 times with distilled water prior to use.

A stock solution of 1 g L^{-1} Cd was prepared using $CdCl_2.H_2O$ 1 g L^{-1}. As was prepared using As_2O_3 and acidified (0.2%, HNO_3) with no pH variations in test media. In all controls, respective amounts of acidified water (0.2%) were added. Materials in contact with the culture and test media were soaked for 24 h in 5% HNO_3 and rinsed with deionized water.

Acute 24 h toxicity tests for Cd (0.03, 0.04, 0.05, 0.07, 0.1 mg L^{-1}), As (0.35, 0.50, 0.70, 0.80, 1.00 mg L^{-1}), and their mixtures (10% As + 90% Cd: 0.01, 0.02, 0.03, 0.04, 0.05 mg L^{-1}; 50% As + 50% Cd: 0.04, 0.05, 0.06, 0.07, 0.08 mg L^{-1}; 90% As + 10% Cd: 0.10, 0.15, 0.20, 0.25, 0.30 mg L^{-1}) were performed 3 times with control groups under static non-renewal conditions in 100 mL of reconstituted water in 250 mL Erlenmeyer flasks. This concentration was chosen from first experiments according to the ISO 6341 standard protocol (ISO, 1996). Each treatment was performed on 20 animals placed in individual test chambers in general accordance with the methods described by ISO 6341 standard protocol (ISO, 1996).

Reconstituted water was used as dilution water ($CaCl_2.H_2O$ 290 mg L^{-1}, $MgSO_4.7H_2O$ 120 mg L^{-1}, $NaHCO_3$ 65 mg L^{-1}, KCl 6 mg L^{-1} with a pH of 7.1 ± 0.7). Toxicity tests were performed according to ISO 6341 standard protocol (ISO, 1996).

A total of 20 neonates (age <24 h) obtained from the original culture were exposed to different concentrations of Cd, As, and Cd + As mixtures (90:10; 50:50; 10:90 v/v). There was no feeding during the test. The containers were slightly aerated without disturbing the daphnids with air bubbles. The toxicity was expressed by the median lethal concentration, that is, the dose required to kill half of the daphnid members during a 24 h period of LC_{50} exposure. After 24 h the live *D. magna* were counted, and after gently shaking the glass containers the ones that did not move were considered dead.

Statistical analysis

Exposure to the different concentrations was carried out in triplicate. LC_{50} (median lethal concentration) values were calculated using the regression line obtained by plotting the concentration against the death percentage on a probit scale, and the results were evaluated with probit analysis (SPSS 17.0). The data were also evaluated according to mathematical terms—the degree of median lethal concentration toxicity of metals—using the Behrens-Karber method (Klassen, 1991).

$$LC_{50} = LC_{100} - (ab + \ldots + ab) / n$$

where LC_{50} and LC_{100} stand for the lethal concentration for 50% and 100% of the *D. magna*. In the above formula a, b, and n are the difference between 2 consecutive doses; the arithmetic mean of the deaths that occurred due to 2 consecutive doses and the total number of *D. magna* used, respectively.

In this study, the effect of acute single and different mixtures of Cd and As on the cladoceran *D. magna* was evaluated. Mixture rate effects of TUs (Dener and Sinnige, 1988) and AI (Marking, 1977) were investigated. Toxic interactions were characterized by calculating toxic units based on the LC_{50} or EC_{50} estimates from bioassays with mixtures and single metals (Dener and Sinnige, 1988). TUs were derived by dividing the LC_{50} or EC_{50} estimate from the mixture by the corresponding estimate from the individual metal test. The degree of joint toxicity developed for different mixtures of metals was investigated using AI.

Results and discussion

Single metal toxicity: Table 1 shows the half-lethal concentrations for 24 h of Cd and As alone. The LC_{50} for As was found to be 509 µg L^{-1}, whereas for Cd it was 44 µg L^{-1}. Cd showed more toxicity than As to *D. magna*. Cd is defined as a priority hazardous sample for water policy by the European Community (European Commission, 2001). Cd is also more mobile in the soil and is easily absorbed by several organisms (Nordic Council of Ministers Cadmium Review, 2003). Cd plays a crucial role in some processes that lead to the disruption of cellular homeostasis, such as oxidative stress (Pinto et al., 2003), DNA damage (Badisa et al., 2007), acidification of the cytoplasm, and membrane depolarization (Conner and Schimid, 2003). Therefore, achieving a higher LC_{50} value for Cd than for As was an expected result.

In addition, the results were evaluated with the Behrens-Karber method, and the results were similar to those achieved with probit analysis. No deaths or behavioral changes were observed in the control group during the experiment. Moreover, the theoretical spontaneous response in the control group was zero.

The dosage-mortality curves for Cd (Figure 1) and As (Figure 2) were calculated with probit analysis. Cd and As showed different trends characterized by an increase in the daphnids after 24 h of exposure.

This study revealed similar results as did Forget et al. (1998), Ward and Robinson (2005), and Pestana et al. (2007) (Table 2).

Table 1. Acute 24 h toxicity of Cd and As alone.

Heavy metals	Cd	As
LC_{50} probit results (µg L^{-1})	44 (25-57)	509 (319-618)
LC_{50} Behrens-Karber results (µg L^{-1})	46	509

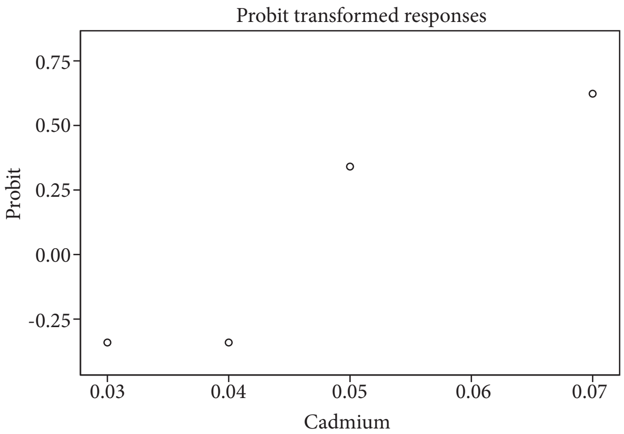

Figure 1. The dosage-mortality curve for *D. magna* exposed to Cd for 24 h.

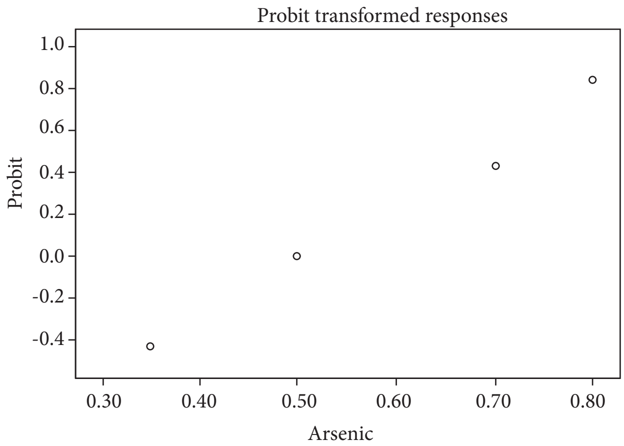

Figure 2. The dosage-mortality curve for *D. magna* exposed to As for 24 h.

Table 2. LC_{50} values of same examples the literature.

Species	Heavy metal	Exposure time	LC_{50} value	
Micropterus salmoides	As	28 days	42.1 mg L^{-1}	Birge et al. (1978)
Oncorhynchus mykiss	As	8 days	0.54 mg L^{-1}	Birge et al. (1978)
Channa punctatus	As	31 days	7 mg L^{-1}	Shukla et al. (1987)
Paratya compressa improvisa	Cd	48 h	77.5 µg L^{-1}	Hatakeyama and Sugaya (1989)
Tigriopus brevicornis	Cd	96 h	47.9 (45.8-50) µg L^{-1}	Forget et al. (1998)
T. brevicornis	As	96 h	27.5 (25.4-29.6) µg L^{-1}	Forget et al. (1998)
D. magna	As	48 h	2.5 (2.4-2.7) mg L^{-1} EC_{50}	Tisler and Zagorc-Konean (2002)
Oncorhynchus mykiss	As	96 h	15.3 (12.9-17.3) mg L^{-1}	Tisler and Zagorc-Konean (2002)
Ctenopharyngodon idellus	Cd		9.42 mg L^{-1}	Yorulmazlar and Gül (2003)
Tinca tinca	Cd	96 h	6.5 ppm	Shah and Altındağ (2005)
D. magna	Cd	48 h	26-120 µg L^{-1}	Ward and Robinson (2005)
D. magna	Cd	48 h	101 µg L^{-1}	Shaw et al. (2006)
Atyaephyra desmarestii	Cd	96 h	51.82 (38.59-74.71) µg L^{-1}	Pestana et al. (2007)
Echinogammarus meridionalis	Cd	96 h	44.15 (33.76-63.39) µg L^{-1}	Pestana et al. (2007)
D. magna	Cd	48 h	79.05 µg L^{-1}	Ferreira et al. (2008)
D. magna	Cd	24 h EC_{50}	35.54 µg L^{-1}	Ferreira et al. (2008)

Binary metal toxicity: By exposing both species and using endpoint tests, the same combinations of metals (As, Cd, Cr, Cu, Hg, Pb, etc.) showed different interactive effects (Spehar and Fiandt, 1986). Moreover, exposing the same species to different combinations of metals showed different effects. In this study different combinations of binary mixtures were examined.

According to the 24 h LC_{50} values of Cd and As binary mixtures, the order of toxicity was: 90% Cd + 10% As > Cd > 50% Cd + 50% As > 10% Cd + 90% As > As. The results suggested that *D. magna* is most sensitive to the 90% Cd + 10% As mixture.

When metals mix they become more toxic against biological systems than when they are present as individual compounds (Faust et al., 1994; Sharma et al., 1999). However, the results of this study showed that mixture rates are important, because Cd alone is more toxic than 50% Cd + 50% As mixture to *D. magna*.

In this study, mixtures of these compounds were examined with individual compounds because heavy metals do not exist alone in nature. Mixture rates are important, but the results of this study showed that it was quite unreasonable to make assumptions with a single toxicity experiment; antagonistic, additive, or synergistic effects on organisms are the result of the mixture (Paustenbach, 2000).

The dosage-mortality curves for 90% Cd + 10% As (Figure 3), 50% Cd + 50% As (Figure 4), and 10% Cd + 90% As (Figure 5) were calculated with probit analysis. The results were then evaluated with Behrens-Karber method, and results were similar to those achieved with probit analysis. After 24 h of exposure, binary mixtures showed different trends characterized by an increase in the daphnids.

Toxic interactions were described by calculating toxic units based on the LC_{50} or EC_{50} estimates from bioassays with mixtures and single metals (Spehar and Fiandt, 1986). We used TUs and AI to understand the effect of components in the mixture, whether synergistic or antagonistic to *D. magna* (Table 3).

Values of TUs are explained on a relative basis; the apparent LC_{50mix} value of one component in a mixture is divided by the LC_{50} of the component as a pure

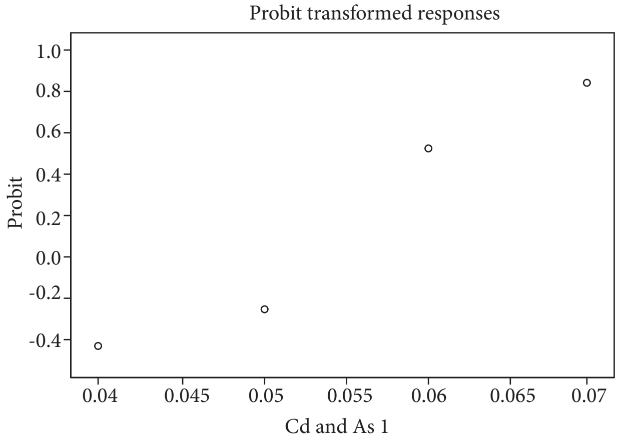

Figure 3. The dosage-mortality curve for *D. magna* exposed to 90% Cd and 10% As for 24 h.

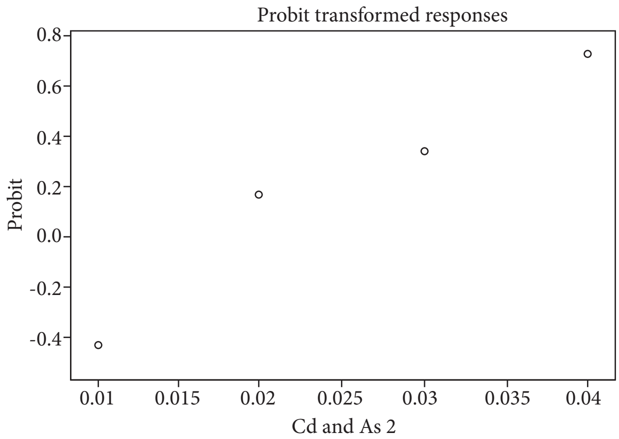

Figure 4. The dosage-mortality curve for *D. magna* exposed to 50% Cd and 50% As for 24 h.

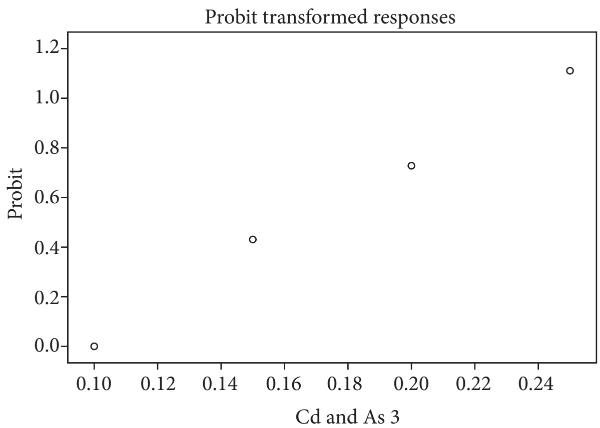

Figure 5. The dosage-mortality curve for *D. magna* exposed to 10% Cd and 90% As for 24 h.

Table 3. Acute 24 h toxicity of binary mixtures of Cd and As.

Heavy metals	90% Cd + 10% As	50% Cd + 50% As	10% Cd + 90% As
LC_{50} probit results ($\mu g\ L^{-1}$)	19 (−5-29)	51 (38-59)	107 (71-129)
LC_{50} Behrens-Karber results ($\mu g\ L^{-1}$)	22	48	122.5

substance, determined with tested organisms from the same group (Dener and Sinnige, 1988). The TUs can be described using the following equation:

$$TU_i = LC_{50mix,i} / LC_{50i}$$

where TU_i is the toxic unit of the component i in the mixture, $LC_{50mix,i}$ is the concentration of the component i in the mixture, and LC_{50i} is the half-lethal concentration of chemical i when it acts independently on the organisms.

In the mixture, M is the sum of the TU_i of each component (Sprague and Ramsay, 1965).

M can be expressed using the following equation:

$$M = TU_s = \Sigma TU_i$$

When M is equal to 1.0 simple addition is indicated; when M is less than 1.0 the action is more than additive (synergism), and when it is greater than 1.0 the action is less than additive (antagonism) (Spehar and Fiandt, 1986). According to our study results, M is 0.00040 for 90% Cd + 10% As, 0.00102 for 50% Cd + 50% As and 0.00214 for 10% Cd + 90% As. All TU values were found to be less than 1.0, implying that the action is synergism.

Heavy metals do not exist alone in nature. This study and other recent studies show that when heavy metals exist together they have a synergistic effect. This synergism depends on the species and heavy metals involved. For instance, Hagopian-Schlekat et al. (2001) obtained mixtures of Cd, Cu, Ni, Zn, and Pb synergistic on *Amphiascus tenuiremis.* Meng et al. (2008) investigated the toxicity of Hg-Cd-Cu-Pb-Cr, Hg-Cd-Cu-Pb, Hg-Cd-Cu, Hg-Cd, and Cu-Cd mixtures. The mixture experiments indicated that toxicity for all mixtures was synergistic to *D. magna*. For the more toxic Hg-Cd-Cu-Pb-Cr and Hg-Cd mixtures, the Cu ion strongly reduced the combined toxicity of the Hg-Cd mixture, and the Pb ion had a slight effect on the toxicity of the Hg-Cd-Cu mixture. The Cr ion was least toxic to *D. magna*, but its existence increased the joint toxicity of the Hg-Cd-Cu-Pb mixture more than 6 times. Cooper et al. (2009) obtained acute toxicity of Cu, Zn, and Pb on *Ceriodaphnia dubia* and *Daphnia carinata.* They indicated the mixture of Cu-Pb on *C. dubia* and *D. carinata*, the mixture of Cu-Zn on *D. carinata*, and the mixture of Cu-Pb-Zn on *C. dubia* is synergistic.

In mathematical terms, the degree of joint toxicity developed for different mixtures of metals was evaluated using the AI method, from which the times of toxicity enhancement could be calculated.

AI is defined by Marking (1977) as follows:

$AI = M - 1.0$ when $M = 1$

$AI = 1/M - 1.0$ when $M < 1$

$AI = M \times (-1) + 1.0$ when $M > 1$

When AI equals zero the mixture toxicity is simple addition; when AI is greater than zero, it reveals synergism; when AI is less than zero, it indicates antagonism. In this study, as shown in Table 4, AI is 2499 for 90% Cd + 10% As, 979.4 for 50% Cd + 50% As, and 466.3 for 10% Cd + 90% As. The toxic effects for all AI were greater than zero. The combined effects of all mixtures were found to be synergistic as TUs. Moreover, 90% Cd + 10% As showed the greatest synergistic effect.

Some metal elements are essential for living organisms; however, when the amount of these metals increases beyond their critical level they

Table 4. Toxicity results of heavy metals to *D. magna* in single and mixture experiments.

Mixtures	Cd ($\mu g\ L^{-1}$)	As ($\mu g\ L^{-1}$)	TUs for each mixture (M)	TUi for each component in the mixture	AI for mixture
LC_{50mix} in (90% Cd 10% As)	0.00880	0.1020	0.00040	0.00020	2499
LC_{50mix} in (50% Cd 50% As)	0.02244	0.2600	0.00102	0.00051	979.4
LC_{50mix} in (10% Cd 90% As)	0.04710	0.5450	0.00214	0.00107	466.3

become harmful to aquatic organisms. Cd was found to be more toxic than As. The mixture of Cd and As was found to be more toxic than Cd alone, and Cd and As were identified as having a synergistic effect on each other.

Acute toxicity effects of Cd and As have been widely described for *D. magna*. Cd and As are important constituents of industrial wastewater and anthropogenic waste discharged into freshwater and seas. As a result of this study, we can deduce that it is crucial to control the use of heavy metals such as Cd and As. *D. magna* is an important test species for studying the effects of toxic substances, and utilizing a mixture of heavy metals, rather than using them individually, produces more accurate results.

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