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ENVIRONMENTAL RISK ASSESSMENT OF SOME COPPER BASED FUNGICIDES ACCORDING TO THE REQUIREMENTS OF GOOD LABORATORY PRACTICE

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Abstract

The paper presents data demonstrating the functionality of biological systems reconstituted with aquatic organisms developed under Good Laboratory Practice testing facility within Research - Development Institute for Plant Protection Bucharest for environmental risk assessment of four fungicides based on copper, according to Good Laboratory Practice requirements. For risk assessment, according to GLP were made the following steps: Good Laboratory Practice test facility was established, we have ensured adequate space for growth, acclimatization and testing for each test species, it was installed a complex water production instalation needed to perform tests, it was achieved control system for checking environmental conditions and have developed specific operating procedures that have been accredited according to Good Laboratory Practice.The results showed that biological systems model of the Good Laboratory Practice test facility in Research - Development Institute for Plant Protection meet the requirements of Organisation for Economic Co-operation and Development Guidelines regarding GLP, and after testing copper-based fungicides in terms of acute toxicity Cyprinus carpio and to Daphnia magna revealed that three of them (copper oxychloride, copper hydroxide and copper sulphate) showed ecological efficiency, ie low toxicity. Metallic copper based fungicides showed a higher toxicity, resulting in fish toxicity symptoms: sleep, sudden immersion, faded, weakness, swimming in spiral, lack of balance, breathing slow and cumbersome, spasms and mortality.

Key words: *Ecotoxicology, biological system, Cyprinus carpio, Daphnia magna.*

INTRODUCTION

In accordance with European legislation, environmental risk assessment of plant protection products is an important step, aiming the approval and marketing of the products that meet certain criteria for toxicity to birds, aquatic organisms, bees, earthworms, soil microorganisms, useful insects etc. Organisation for Economic Co-operation and Development (OECD) established the „Principles of Good Laboratory Practice" that are applied to perform laboratory, greenhouses and fields tests, including toxicology tests for environmental risk assessment of plant protection products.

This paper presents data on the environmental risk assessment according to the principles of GLP to four fungicides based on copper, using reconstructed biological systems with aquatic organisms using GLP test facility within Research - Development Institute for Plant Protection, Bucharest.

MATERIALS AND METHODS

For risk assessment we used the following materials: copper-based fungicides (copper oxychloride, copper hydroxide, copper sulfate, copper metal) and biological materials (juvenile common carp - *Cyprinus carpio* and neonatal *Daphnia magna*).

The following methods were used:

- Organisation for Economic Co-operation and Development guide No. 203/17.07.1992 – Acute toxicity test on fishes;
- Organisation for Economic Co-operation and Development guide No. 202/17.07.1992 – Acute immobilizing test on daphnia;
- GD No. 490/ 16.05.2002;
- General and specific procedures developed under GLPtesting facility within Research - Development Institute for Plant Protection.

For the risk evaluation the acute toxicity of four copper-based fungicides were assesed using the static method, test duration being of 96 hours for fish and 48 hours for daphnia.

Test solutions have not been renewed during testing. The main indicators estimated in ecotoxicology tests with aquatic organisms are presented in Table 1.

Table 1. The main indicators estimated in the ecotoxicological tests with aquatic organisms

Indicator	Specification
Mean lethal concentration ($LC_{50\%}$)	calculated concentration of test substance which kills 50% of the number of fish at the end of the test period.
NOEC	the highest concentration to which no significant toxic effect is observed to the test organisms.
The medium inhibitory concentration ($EC_{50\%}$)	calculated concentration of test substance that induces immobilization * 50% of daphnia tested.
Morphological and behavioral changes	disturbances of breath, lack of response to tactile stimuli, loss of balance, sudden immersion, pigmentation, discoloration.

*Immobilization - daphnia are considered immobile when are not able to swim within 15 seconds after gentle agitation of the test vessel

For fish cumulative mortality was recorded at 24, 48, 72 and 96 hours and mean lethal concentration was calculated; the percentage of immobilization at 48 hours and the median inhibitory concentration were statistically calculated for daphnia. Temperature, pH and dissolved oxygen concentration in the test vessels were monitored daily.

RESULTS AND DISCUSSIONS

For the environmental risk assessment of copper-based fungicides according to Good Laboratory Practice, were made the following steps: Good Laboratory Practice test facility was established, appropriate space for growth, acclimatization and testing for each test species have been ensured, a complexe installation of water production for test performing was done, a control system for environmental conditions has been made and specific operating procedures have been developed which have been accredited GLP.

a) Environmental risk assessment to fish

The studies were conducted with juvenile common carp - Cyprinus carpio (*Teleostei*, *Cyprinidae)* (Linneaus 1758). The fish were purchased from accredited fish farms. The reception of the batch of fish was carried out in accordance with specific procedures, with health being registered mortalities and aclimatization parameters in the reconstituted water. For this study young fish around the same age and size were used. Prior to the start of testing the weight and length of a total of 10 fish was determined, the average body length was 6.3 cm and weight of 2.7 g, according to GLP technical procedures. (Table 2).

Table 2. The weight and length of test fish

Nr. copy	Weight (g)	Length (cm)
1	3.0	6.5
2	2.8	8.0
3	2.8	5.5
4	2.7	6.0
5	2.6	4.5
6	2.6	6.0
7	2.8	5.5
8	2.5	6.5
9	2.8	8.0
10	3.0	6.5
Mean	2.7	6.3

After the acclimatization period in the laboratory under similar conditions to those during testing, fish were exposed to copper-based fungicides in the following conditions:
- test method: static;
- duration: 96 hours;
- number of fish: 7 for each concentration tested, including control tanck;
- water volume: 20 l/aquarium;
- test water: reconstituted water;
- light 16 h light and 8 hours dark;
- concentration of dissolved oxygen: 63-69;
- temperature: 21ºC constant during the test;
- water pH: 7.7-7.9;
- aeration: without aeration;
- feeding: no food.

Fish were observed after the first 2-4 hours from the begining of the test and then, at every 24 hours. Fish were considered dead if not reaching caudal peduncle produced no visible reaction and were not breathing movements. Dead fish were removed when they were observed and recorded mortality.

Copper-based fungicides tested under identical conditions have exerted different toxic effects on fish, the results are presented in Table 3.

Table 3. The acute toxicity of the copper-based fungicides on fish

Fungicides	$LC_{50\%}$ mg a.i./l (96 h)	NOEC mg a.i./l (96 h)	Modification of functional and anatomical indicators
Copper hydroxide 53.8%	1.0	0.50	loss of balance
Copper sulphate 22.5%	0.60	0.20	asphyxiation, loss of balance
Copper oxychloride 50%	0.20	0.10	asphyxia, spasms, eliminating massive intestinal contents
Copper metal 50%	0.015	0.0025	inactivity, sudden sinking, bluish coloration, weakness, lack of balance, skin formation of mucus, heavy and slow breath

Therefore the fungicide based on copper hydroxide showed a low toxicity to fish $CL_{50\%}$=1.0 mg a.i./l and NOEC=0.5 mg a.i./l. It was found that copper fungicide conditioning as metal, lead to an increase in acute toxicity, the median lethal concentration being 0.015 mg a.i./l and 0.0025 mg a.i./l for the concentration with no effect (Table 4).

Table 4. The acute toxicity of 50% metallic copper fungicide on juvenile carp

Concentration mg a.i./l	No. tfish	Cumulative mortality at:			
		24 h	48 h	72 h	96 h
0.025	7	0	0	0	0
0.05	7	0	0	0	1
0.1	7	0	0	1	1
0.2	7	0	1	1	2
0.4	7	1	1	2	2
0.8	7	2	5	-	-
Control	7	0	0	0	0

NOEC (concentration with no effect) = 0.025 mg a.i./l (96 h)
$LC_{50\%}$ (96 h) = 0.15 mg a.i./l
Correlation coefficient R = 0.9991

Exposure to high concentrations of metallic copper for juvenile common carp induces respiratory disturbances, lack of response to tactile stimulation, loss of balance and spinal deformities (Table 5).
At concentrations above 0.1 mg a.i./l, the body surface and particularly surface of the gills were covered with a film of blue organic mucus resulting in the precipitation of copper with organic and colloidal substances from water.

Table 5. Symptoms of toxicity observed on juvenile common carp maintained in sublethal solution of metallic copper

Concen-tration mg a.i./l	Modification of functional and anatomical indicators:			
	24 h	48 h	72 h	96 h
0.025	-	-	-	-
0.05	-	a,b	a,b	a,b,c,m
0.1	a,b,c	a,b,c	a,b,c,d,m	a,b,c,d,m
0.2	a,b,c,d, m	a,b,c,d, e,m	a,b,c,d, e,f,m	a,b,c,d, e,f,m
0.4	a,b,c,d,e, g,m	a,b,c,d,e, f,g,m	a,b,c,d,e, f,g,h,m	a,b,c,d,e, f,g,h,m
0.8	m	m	m	m

Definition of symbols:
a = inactive
b = sudden immersion
c = color blue
d = weakness
e = imbalance
f = the film formation of mucus
g = slow breathing
h = shortness of breath
m = mortality

Therefore, insoluble metal-protein compounds that harm gill tissue resulted. Hypoxia occurs when gill ventilation was insufficient to cover the oxygen. Death occurred by suffocation. Copper is part of the heavy metals, which in sufficient quantities is toxic to fish. Using copper salts on a larger scale in combating algae can seriously affect aquatic life due to the high toxicity of Cu. The appearance of mucus on the gills is a sure indication of poisoning fish with copper ions. Fungicide based on copper sulphate showed a moderate toxicity to fish, median lethal concentration $CL_{50\%}$ (96 h) was 0.60 mg a.i. /l. At concentrations above 0.1 mg a.i./l one fish have clear symptoms of asphyxiation. Copper oxychloride has not shown signs of high toxicity towards fish $CL_{50\%}$ (96 h) = 0.20 mg a.i./l. However, at concentrations exceeding 1 mg a.i./l, fish suffered, become excited, have spasms and showed massive removal of the intestinal content.

b) Environmental risk assessment to daphnia

For the environmental risk assessment of copper-based fungicides to daphnia, the bio-logical system according to specific procedures developed in the laboratory was completed first:
- selection of test species;
- increasing the test species;

- ensuring the conditions for reproduction;
- new born separation;
- verification of test species sensitivity.

The study was conducted on the crustacean *Daphnia magna* Straus (*Cladocera*, *Crustacea*). Daphnia were grown and multiplied in the GLP test facility according to technical procedures. Lot of Daphnia has been verified with a reference substance to show if under laboratory conditions, the sensitivity to the presence of toxic Daphnia has not changed significantly and if that growth parameters like temperature, pH and oxygen concentration meet the requirements of the test method. At the beginning of the test daphnids were younger than 24 hours, and to reduce variability they came from the first generation of offspring. Daphnia came from a healthy batch which showed no signs of stress such as high mortality, presence of males, delays in producing the first generation, faded copies, etc. acclimatization before testing. The batch has been maintained in culture conditions light, temperature and medium) similar to those used during testing.

Daphnia were exposed to copper-based fungicides in the following conditions:
- duration: 48 h;
- number: 20 for each concentration, split in 4 lots, each with 5 daphnia;
- the volume of water: 2 ml test solution for each dafnia;
- test water: reconstituted water;
- photoperiod:16 h light and 8 hours dark;
- temperature: 24ºC constant during the test;
- aeration: without aeration;
- feeding: no food;
- concentration of dissolved oxygen: 6.0-6.5;
- water pH: 7.6 to 7.8;
- test vessels: covered.

Daphnids were examined at 24 and 48 hours, and was registered the numbers of immobile daphnia conform with the procedure of test. Were considered immobile, daphnia that were not able to swim within 15 seconds after gentle agitation of the test vessel.

Copper-based fungicides tested under identical conditions have exerted different toxic effects on the swimming ability of *Daphnia* and led to different percentages of mortality (Table 6). The fungicide based on copper hydroxide showed a moderate toxicity to daphnia $EC_{50\%}$ (48 h) = 0.50 mg a.i./l and NOEC (48 h) = 0.040 mg a.i./l. When the fungicide was conditioned as metallic copper, the toxicity increased, the mean inhibitory concentration being 0.01 mg/l and 0.003 mg/l to no effect concentration, resulting in massive immobilization of *Daphnia*. Symptoms of toxicity induced by sublethal concentrations of metallic copper on the crustacean *Daphnia magna* were agitation and loss of balance. Fungicides based on copper sulphate and copper oxychloride, showed a moderate toxicity on daphnia, but at concentrations above 0.1 mg/l daphnia have suffered and have shown clear symptoms of immobilization.

Table 6. The acute toxicity of the copper-based fungicides on daphmia

Fungicides	$CE_{50\%}$ mg a.i/l	NOEC mg a.i/l	Modification of functional and anatomical indicators
Copper hydroxide 53.8%	0.50	0.040	-
Copper sulphate 22.5%	0.01	0.020	-
Copper oxychloride 50%	0.10	0.010	-
Copper metal 50%	0.01	0.003	agitation loss of balance

CONCLUSIONS

Fungicides based on copper hydroxide, copper sulphate and copper oxychloride have shown ecological efficiency or low toxicity to fish and daphnia.

Metallic copper based fungicides showed high toxicity to fish and daphnia main symptom recorded being mortality.

Symptoms of toxicity induced by sublethal concentrations of copper metal were: inactivity, sudden immersion, faded, weakness, swimming in spiral, lack of balance, breathing slow and cumbersome, spasms and mortality.

Symptoms of poisoning to fish kept in lethal solution of copper based fungicides were atypical, similar to that described in the literature. The phases of the progressive installation of poisoning by pesticides are phase of excitation the phase of partial loss of balance

and the final stage of total loss of balance. Death occurs at different times depending on the concentrations of pesticide solutions.

Biological model systems with fish and daphnia, conducted under GLP test facility in Research - Development Institute for Plant Protection have proven effectiveness and validity of the environmental risk assessment of plant protection products according to Good Laboratory Practice Principles.

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