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# TOXICITY BIOTESTS FOR DANGEROUS CHEMICALS (MONOLINURON AND TRICLORPHON) CONTROL TO EVALUATE THEIR TOXIC POTENTIAL

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**Abstract**. The main objective of the present work was to evaluate acute and chronic toxicity of 2 chemicals from pesticides class (monolinuron and trichlorphon), included in the List of Prior Dangerous Substances, on aquatic organisms – fish (*Cyprinus carpio* sp. – which is a commune species in Romanian fresh waters and able to be tested in aquatic toxicity laboratory tests). The study was performed in 3 experimental steps conducting to the following results: the medium lethal concentration values ($LC_{50}$) for the 2 chemicals established by performing acute toxicity tests in which evaluation criterion was the mortality of aquatic organisms, as a reply to noxious action of toxic; the values of the maximum allowable concentrations (experimental MATC) established through chronic toxicity tests monitoring evaluation criterion of influence of morpho-functional, physiological and biochemical state of contaminated aquatic organisms; and the toxic effects assessment on enzymatic antioxidant and hepatic systems (superoxide dismutase [SOD], glutathione peroxidase [GPx], catalase [CAT]), glutathione-S-transferase [GST], glucoso-6-phoshate dehydrogenase [G6PD], aspartate aminotransferase [GOT] and alanin aminotransferase [GPT]). Because ecotoxicological biotests represent a significant control parameter for determination of chemicals concentrations (possible existent in surface waters) with no toxic effect on aquatic organisms (fish), the obtained results can be used by chemicals manufactures/importers and authorities to evaluate the ecological risk and for elaboration of chemicals files in conformity with the European Regulation – REACH.

*Keywords*: monolinuron, trichlorphon, toxicity, risk, aquatic organisms.

## AIMS AND BACKGROUND

The present paper brings in discussion the problem of excessive use of the pesticides in agriculture. The pesticides are the chemicals/chemical mixtures which contain the active biological compounds against the pestiferous. Mostly, these chemicals have a great degradation potential of the environment, are toxic for the animals and humans by the big rate of bioaccumulation in the vegetables. From this considerations the production, packing, storage, transport and use of the pesticides are imposed by regulations.

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At European level, all the chemicals which are produced or imported in more than 1 t/year, inclusive the pesticides, are under Regulation No 1907/2006/CE (REACH) entered in force on the 1st of June 2007, which assures a high level of human health and environment protection, as well as the free circulation of the qualitative and environment friendly chemicals on the national and international market and the increase of innovation and competitivity[1]. At present, Romania as an European Union member has to respond to the all requests of REACH Regulation concerning the chemicals testing according to the OECD methods (Directive 67/548/EEC – Annex V) namely: toxicity tests on aquatic biological systems (fish, daphnia, green algae)[2].

Also, at national level exist two important frameworks acts: Governmental Decision No 1408/2008 concerning the classification, labelling and packing of the dangerous chemicals, and Governmental Decision No 351/21.04.2005 concerning the approval of the Program of disposal of the discharges, emissions and losses of the dangerous chemicals, designed to assure the environment health protection through the listing of dangerous and priority dangerous chemicals, the environment limits discharges establishment and the specific risk phrases award[3,4].

The purpose of the present paper was the assessment of the lethal acute and chronic toxicity of 2 chemicals from pesticides class – monolinuron and trichlorphon, specified in the 'List of dangerous priority chemicals' (G.D. No 351/21.04.2005) on aquatic organisms – fishes (*Cyprinus carpio* sp.)

For a complex ecotoxicity information supply for the studied pesticides were performed biochemical tests to assess the toxic effects on enzymatic antioxidant and hepatic systems (represented by superoxide dismutase [SOD], glutathione peroxidase [GPx], catalase [CAT]), glutathione-S-transferase [GST], glucoso-6-phoshate dehydrogenase [G6PD]) and hepatic enzyme – aspartate aminotransferase [GOT] and alanin aminotransferase [GPT]).

According to scientifical literature the organic-chlorinate pesticides (monolinuron) can affect the olfactory sense of fish[5]; delay the organogenesis, cause the immobility, heart dysfunctions and affect the enzymatic activity of glutathione-S-transferase in all the development stages[6]. The organic-phosphoric insecticides (trichlorphon) act on fish nervous centre, affecting the acetylcholine enzyme; not accumulate in fatty tissue but in the organism may be transformed in toxic metabolites; may give oxidative stress with direct effects and very devastating on the cell structure and function[7–9].

The antioxidant enzymes play a crucial role in cellular homeostasis maintaining, these being proposed as oxidative stress biomarkers of the toxic presence in organism[10].

## EXPERIMENTAL

The toxic potential of the dangerous chemicals was assessed by lethal acute toxicity tests for 96 h (the result was the chemical concentration that is lethal for 50% of the test fish – $LC_{50}$) and chronic toxicity studies for 60 days (the result was the maxim allowable concentration in the surface water – MATC). The toxicity tests technical characteristics applied for the ecotoxicity assessment of the 2 chemicals are specified in Table 1.

**Table 1**. Technical characteristics of toxicity tests[11–13]

| Technical characteristics | $LC_{50}$ OECD C01 / SR 13216-1994 | MATC INCD ECOIND Procedure |
|---|---|---|
| Type of method | static | semi-static |
| Time of experiment | 96 h | 60 days |
| Fish species/organisms number/length/weight average | *Cyprinus carpio* sp. 10 exemplars/test solution 5–7 cm, 10–15 g/exemplary | *Cyprinus carpio* sp. 20–30 exemplars/ test solution 12–14 cm, 25–30 g/exemplary |
| Testing vessels | minimum 10 l | about 100 l |
| Temperature, dissolved oxygen, pH, light | 18–25°C temperature, constant aeration minimum 4 mg $O_2$/l, pH in neutral range 6.5–8.5, 12–16-hour light (everyday, these parameters were monitored) | |
| Feeding | not food | 2% from the lot living weight day |
| Control test | all toxicity tests were carried out at the same time with a control test | |
| Toxicity criteria assessment | organisms death | growth rate ($G$), mortality rate ($Z$), biomass mean ($B$), production ($P$), used rate of the food ($K$) and biochemistry indicators – the hepatic transaminase activity – GOT and GPT (LiquiUV tests) |
| Estimation of toxicity values | the probity analysis method, based on the exponential regression model between the mortality (probity units) and the log concentration of the tested chemical | MATC estimated = $CL_{50} \times a$ (application factor 0.1)<br>$G = (\ln \overline{W}_f - \ln \overline{W}_i)/\Delta t$, $\overline{W}_i$, $\overline{W}_f$ – the initial and final fish mean weight; $\Delta t$ = experimental time (in days)<br>$Z = (\ln n_f - \ln n_i)/\Delta t$, $n_i$ and $n_f$ – initial and final number of the exemplars from a fish lot; $\Delta t$ = experimental period<br>$K = (P/C) \times 100$, $C$ – food consumption, $P$ – fish production = $\overline{B} \times G$, $G$ – instant growth rate, $\overline{B}$ – biomass mean, with the relation $\overline{B} = B_I (e^{G-Z} - 1)/(G - Z)$, for $G > Z$ |

The biochemistry methods performed in the laboratory for the chemicals effects assessment on the enzymatic system, were led according to scientifical literature and with the work protocols which accompanied the biochemical kits, as follows: the Lowry method for the determination of protein concentration; the Beer and Sizer method for catalytic activity dosage; MnSOD and CuZnSOD enzymatic dosage method – Calbiochem protocol; glutathione peroxidase enzymatic activity dosage – Calbiochem protocol; glutathion-S-transferase enzymatic activity dosage – Calbiochem protocol; the Lohr and Waller method for glutathion-6-phosphate transferase enzymatic activity dosage[14– 16].

The test chemicals were: monolinuron – IUPAC name: 3-(4-chlorophenyl)-1-methoxy-1-methylurea, CAS: 1746-81-2, molecular formula: $C_9H_{11}ClN_2O_2$, provided by Sigma-Aldrich, purity 99.9%, organochlorinated herbicide and trichlorphon – IUPAC name: 2,2,2-trichloro-1-hydroxyethyl phosphonate, CAS: 52-68-6, molecular formula: $C_4H_8Cl_3O_4P$, provided by Sigma-Aldrich, purity 97.8%, organophosphoric insecticide. In Figs 1 and 2 are represented the chemical structures of test pesticides.

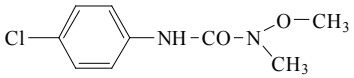


**Fig. 1**. Monolinuron

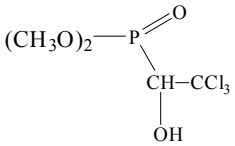


**Fig. 2**. Trichlorphon

The aquatic toxicity tests were performed with fishes from *Cyprinus carpio* sp., sensible to the toxic action of the chemicals taken into study. The fish were taken from selected lots of the S.C. Mario Piscicola S.A. Giurgiu, Romanian fishing farm, and acclimatised in laboratory conditions, in maintenance aquaria from aquatic Biobasis of INCD-ECOIND. The maintenance conditions of the fishes lots were done according to the OEDC C01 method – Acute toxicity assessment on aquatic organisms – fishes[11–13].

In Tables 2 and 3 are presented the stock and working solutions, mortality percentage and probity values used for establishment of the acute and chronic concentrations for the two pesticides. To prepare the stock solutions, test solutions and control test, the free chlorine potable water from distribution network was used as dilution water, by previous aeration for 24 h.

**Table 2**. Parameters used for determination of $LC_{50}$ values

| Studied chemicals | Theoretical conc. (mg/l) | Log conc. | Analytical conc. (mg/l) | | No fish | Mortality 96 h (%) | Probity values | Confidence interval | |
|---|---|---|---|---|---|---|---|---|---|
| | | | initial (0 h) | final (96 h) | | | | – | + |
| Monolinuron | 100 | 2 | 98.25 | 97.56 | 10 | 100 | 8.72 | 5.50 | – |
| | 50 | 1.69 | 49.02 | 48.29 | 10 | 40 | 4.75 | 3.83 | 5.64 |
| | 25 | 1.39 | 24.32 | 23.77 | 10 | 10 | 3.72 | 2.25 | 4.86 |
| Trichlorphon | 100 | 2 | 97.54 | 96.27 | 10 | 100 | 8.72 | 5.50 | – |
| | 50 | 1.69 | 48.23 | 47.09 | 10 | 30 | 4.48 | 3.50 | 5.39 |
| | 10 | 1 | 9.12 | 8.85 | 10 | 0 | 0 | – | 4.50 |

The analytic control of the pesticide concentrations was made through liquid phase chromatography (HPLC) method – according to SR EN ISO 11369/2004 for monolinuron and with INCD ECOIND Procedure (Company standard) – for trichlorphon solutions.

**Table 3**. Parameters considered for determination of MATC values

| Studied chemicals | Theoretical conc. stock solutions (mg/l) | Analytical conc. stock solutions (mg/l) | Theoretical conc. test solutions (mg/l) | Analytical conc. test solutions (mg/l) | | Mortality (% ) 60 days |
|---|---|---|---|---|---|---|
| | | | | initial (after replacement) | final (before replacement) | |
| Trichlorphon | 1000 | 994.58<br>984.45<br>978.02<br>989.34 | 3 | 2.01–2.63 | 1.04–1.65 | 0 |
| Monolinuron | 125 | 115.20<br>121.51<br>113.45<br>120.56 | 1.5 | 1.17–1.58 | 0.84–1.08 | 0 |

To obtain the protein extracts necessary for enzymatic dosage, from the chronically testing vessels were selected the living fishes which were sacrificed for the sampling of the representatives organs, respectively: liver, intestine, gills and muscle tissue.

After organs mechanic disintegration and extraction were performed enzymatic tests for protein concentrations, enzymatic antioxidant activity involved in oxidative stress and hepatic activity.

## RESULTS AND DISCUSSION

The experimental $LC_{50-96h}$ values obtained were: 60.26 mg/l for trichlorphon (confidence interval: 95%: 23.44 – 85.11 mg/l) and 57.54 mg/l for monolinuron (confidence interval: 95%: 30.90 – 128.82 mg/l).

To make a good estimation of studied pesticides acute lethal toxicity levels were used:

- American regulations EPA[17, 18], according to which chemicals are classified in 4 different toxicity classes based on $LC_{50}$ value, namely:

– highly toxic – $LC_{50} < 1$ mg/l;

– toxic – 1 mg/l $< LC_{50} \leq 10$ mg/l;

– harmful/hazardous for aquatic environment – 10 mg/l $< LC_{50} \leq 100$ mg/l;

– very low toxic, non-toxic – $LC_{50} > 100$ mg/l.

- The Romanian legislation, G.D. 1408/2008, according to which the classification criteria and the risk phrases of the chemicals/chemical products are established[4], based on acute lethal concentrations assessment on fishes/Daphnia/alga, respectively:

– very toxic for aquatic organisms, with possibility to raise adverse effects for long-term on aquatic environment (risk phrases R50 and R53): $LC_{50} \leq 1$mg/l;

– toxic for aquatic organisms, with possibility to raise adverse effects for long-term on aquatic environment (risk phrases R51 and R53): 1 mg/l $< LC_{50} \leq$ 10 mg/l;

– harmful for aquatic organisms, with possibility to raise adverse effects for long-term on aquatic environment (risk phrases R52 and R53): 10 mg/l $< LC_{50} \leq$ 100 mg/l.

The analysis of the experimental $LC_{50-96\,h}$ values, comparative with the above-mentioned criteria has showed that the trichlorphon and monolinuron are harmful for aquatic organisms, with possibility to raise adverse effects for long-term on aquatic environment (risk phrases R52 and R53).

Concerning the chronic toxicity tests ($MATC_{60\ days}$), comparative analysis of the biochemical and physiological indices assessed for test chemicals and control lots are presented in Table 4. The values obtained for the 2 chemicals are included in the control limits, which allows us to appreciate that the studied pesticides in the analytical concentrations 1.5 mg/l for trichlorphon and 1 mg/l for monolinuron, have no toxic effects comparative with the control test.

Also, we mention that the visual inspection of the tested organisms showed normal and active behaviour, with response at stimulus, with increased appetite, without the abnormal changes of the external organs. The visual inspection of internal organs did not show abnormal changes, the colour end volume being normal.

**Table 4**. Physiological and biochemical parameters in the chronic toxicity test

| Test chemicals/ conc. | *G* | | *P* | | *K* (%) | | *Z* | | Hepatic enzymes [U/mg protein] ×10$^{-3}$ | | | | Effect |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | T | M | T | M | T | M | T | M | GOT | | GPT | | |
| | | | | | | | | | T | M | T | M | |
| Trichlorphon ~1.5 mg/l | 0.003 | 0.003 | 2.3 | 2.19 | 0.66 | 0.62 | 0 | 0 | 2.51 | 2.66 | 1.20 | 1.08 | practically non-toxic |
| Monolinuron ~1 mg/l | 0.005 | | 3.66 | | 1.12 | | 0 | | 2.15 | | 0.96 | | |

M – control; T – test solutions; *G* – growth rate; *Z* – death rate; *P* – production; *K* – the coefficient food utilisation for growth; GOT – aspartate aminotransferase; GPT – alanine aminotransferase.

The enzymatic activity variations of the oxidative stress enzymes and hepatic transaminases based on the pesticide concentrations and target organ are presented schematically in Figs 1–6.

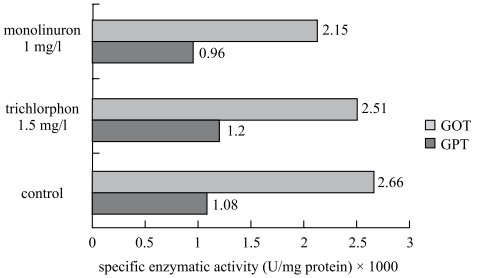


**Fig. 1**. Enzymatic activity of GOT and GPT

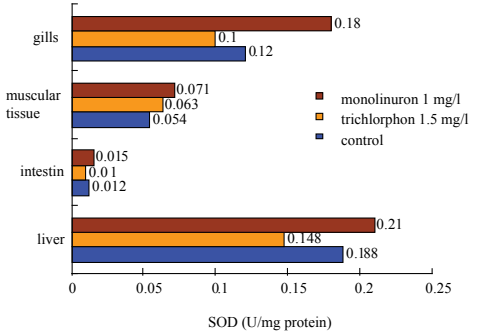


**Fig. 2**. Enzymatic activity of SOD

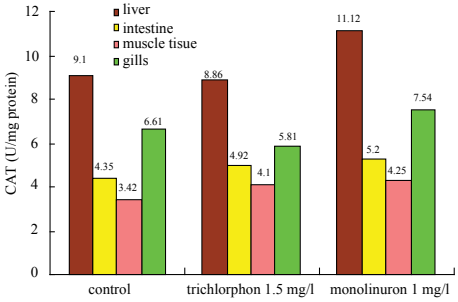


**Fig. 3**. Enzymatic activity of CAT

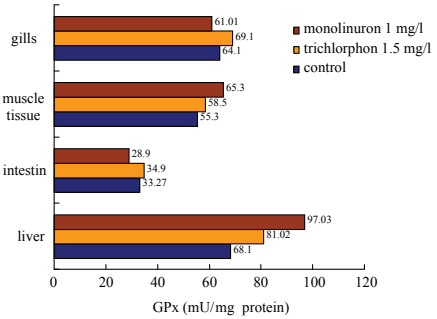


**Fig. 4**. Enzymatic activity of GPx

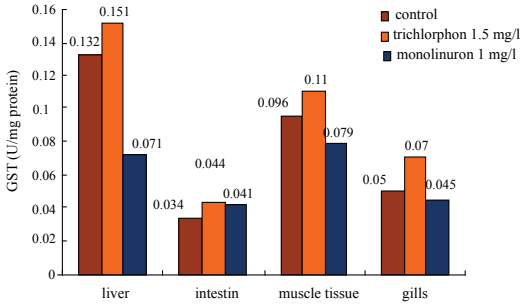


**Fig. 5**. Enzymatic activity of GST

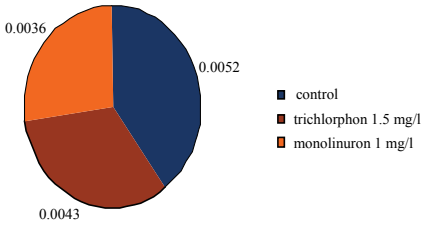


**Fig. 6**. Enzymatic activity of G6PD

From the experimental data obtained from biochemical enzymatic tests, chronic ecotoxicology and visual investigation of tested aquatic organisms, we found that:

- the trichlorphon has no toxic effects in analytical concentrations of 1.5 mg/l, was observed an easy change of antioxidant enzymatic activity at liver level, without behaviour repercussions and visual changes of external and internal organs. It was assessed that this type of pesticides can be metabolised easily because of their chemical structure which allowed the conjugation with glutathione and its excretion;
- the monolinuron with analytical concentrations of 1 mg/l showed toxic effects on antioxidant enzymatic system by change of the specific activity comparative with the control.

## CONCLUSIONS

According to experimental results of acute toxicity tests and with national and international legislation we consider that the $LC_{50-96\,h}$ values, 60.26 mg/l for trichlorphon and 57.54 mg/l for monolinuron are harmful for 50% of test aquatic organisms, with possibility to raise adverse effects for long-term on aquatic environment – especially for *Cyprinus carpio* sp.

Also we established the maximum allowable concentrations in surface water ($MATC_{60\,days}$) for about 1–1.5 mg/l for the 2 test pesticides, that have no toxic effects comparative with a control test.

The biochemical experiments on oxidative stress enzymes lead to the conclusion that 1.5 mg/l trichorphon induce an easy change in antioxidant enzymatic activity at liver level, without behaviour repercussions and visual changes of external and internal organs. On the contrary, the monolinuron showed toxic effects on antioxidant enzymatic system by change of the specific activity comparative with the control. For this reason it was appreciated that it can generate long-time toxic effects, even if 1 mg/l concentration has not generated mortalities during chronic toxicity test.

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