

Phytotoxicity of Chlorpyrifos to White Mustard (*Sinapis alba* L.) and Maize (*Zea mays* L.): Potential Indicators of Insecticide Presence in Water

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SUMMARY

Chlorpyrifos is a hazardous insecticide and important pollutant of the environment. The EU Directive 2008/105/EC lists it as one of the priority water pollutants. Its presence is mainly detected by chemical methods but, since biological tests have gained in importance in the last few years, this study aimed to assess the potentials of white mustard (*Sinapis alba* L.) and maize (*Zea mays* L.) as indicators of water pollution. The phytotoxic effects of chlorpyrifos (rates 0.05–10 µg a.i./l) were assessed based on physiological (germination energy and germination) and morphological traits (root and shoot length, fresh and dry weights) of the tested species. A slightly modified filter paper method was used and the results were processed by Duncan's multiple range test and Probit analysis (EC₅₀). According to the Directive, the maximal allowable concentration (MAC) of chlorpyrifos in water is 0.1 µg a.i./l. When applied at the MAC value, chlorpyrifos inhibited germination energy and germination (11.25%) of white mustard, as compared to the control (91.5; 93.5%), and its hypocotyls and epicotyls failed to form. At the rates 50% below the MAC, germination energy and germination (87.75; 88.25%) were significantly inhibited, as well as root and shoot growth of seedlings. Chlorpyrifos did not affect the germination energy and germination of maize, while all morphological traits were significantly reduced by chlorpyrifos at the MAC rate. The EC₅₀ of chlorpyrifos was 0.09 µg a.i./l for germination of white mustard and 3.21 µg a.i./l for maize.

Keywords: Chlorpyrifos; Water; Phytotoxicity; *Sinapis alba* L.; *Zea mays* L.

INTRODUCTION

Intensive use of pesticides in agriculture presents a serious environmental problem, especially to aquatic ecosystems because they can contaminate underground and surface waters by drifting, leaching and drainage from fields (Schulz, 1999, Cerejeira et al., 2003). Organophosphates, including chlorpyrifos, are the most hazardous water pollutants, which have already led to biotope pollution in some regions. Since 1965, chlorpyrifos has been in use throughout the world (Anonymous 1, 2012) and in Serbia for over 30 years (Mitić 1982, Janjić and Elezović 2010). According to Banks et al. (2005), chlorpyrifos is still one of the most frequently used insecticides in agriculture. Since 2001, EPA (Environmental Protection Agency) has banned its use in the USA due to negative effects of its long-term use. However, the market of chlorpyrifos has spread to underdeveloped and developing countries (Anonymous 1, 2012). In those regions, including Serbia, it is still used in agriculture as a soil and foliar insecticide and in communal hygiene (Sekulić and Jeličić 2013).

Chlorpyrifos is highly toxic for warm-blooded organisms, soil microflora and fauna and, as a result of long-term use and persistence, it can be found in underground and surface waters, thus presenting a potential risk for incorporation in food chains (Anonymous 2 1989, Chu et al. 2008). The European Water Framework Directive 2000/60/EC included chlorpyrifos on its Water Priority Pollutants list (33 pollutants) and its monitoring in water is conducted by a number of regulatory agencies. The European Union has limited the emission and loss of this insecticide by Decision No. 2451/2001/EC. The mentioned European directives referring to drinking water prescribe different methods and strategies for environmental pollution control. One of the first steps in prevention of ecosystem pollution is continuous monitoring of water quality and control of contaminant contents. It usually relies on chemical analysis, which identifies pollutants and determines their amounts in a medium, which are then compared to the maximal allowable concentrations (MAC) listed in relevant directives. According to Pascoe (1993), those methods have been improved regarding their sensitivity, precision and accuracy but they are not sufficient to assess the effects on living organisms and bioavailability. Therefore, it is necessary to involve biological tests, namely bioindicators, in risk assessment and water contamination detection. For an agricultural region such as ours (northern Serbia), the toxicological impact of contaminated water on crops is very important because pollutants affect plant production and may even reach food chains through irrigation. Therefore, the

most suitable methods for the assessment of toxic effects and detection of water contamination involve cultivated plants. Crop species have many advantages, such as short germination periods and fast seedling growth that make effects visible after relatively short periods of time. These methods are less time consuming, cheaper, and do not require expensive equipment, compared to chemical methods, and they are equally reliable and repeatable.

This study aimed to assess the biological potential of white mustard and maize for the detection of chlorpyrifos in water in terms of their future involvement as phytoindicators in water quality assessment.

MATERIAL AND METHODS

Test plants

The effect of chlorpyrifos was assessed based on physiological (germination energy and germination) and morphological traits (root and shoot length, fresh and dry weight of roots and shoots) of white mustard and maize. The test species were chosen as representatives of dicotyledon [white mustard (*Sinapis alba* L.), variety Torpedo] and monocotyledon [maize (*Zea mays* L.), variety NS 6030] species due to their morpho-anatomic differences.

Insecticide solutions

Chlorpyrifos (Pyrinex 48-EC) was prepared as a series of concentrations based on preliminary research: 0.05; 0.065; 0.075; 0.085; 0.095; 0.1; 0.5; 1; 2.5; 5 and 10 µg a.i./l for white mustard bioassay, and 0.05; 0.1; 0.5; 1; 2.5; 3; 3.5; 4; 4.5; 5 and 10 µg a. i. /l for maize. Distilled water was the control. According to Directive 2008/105/EC of the European Parliament and European Council on the environmental quality standards referring to water policy, the MAC for chlorpyrifos is 0.1 µg a. i. /l and the amount was used as referent data in our interpretation of results.

Experimental protocol

A standard filter paper method (ISTA Regulations book, 2011) with slight modifications was used. White mustard seeds (100 per replication) were placed in Petri dishes (Ø15 cm) on filter paper moistened with 10 ml of test solution. Maize seeds (50 per replication) were placed in plastic boxes (21x15cm) on pleated filter paper moistened with 25 ml of chlorpyrifos solution. The seeds were incubated in the dark at 25±2 °C for three days (white mustard) or four days (maize). After that period, germination energy was assessed and 10 seedlings per

replicate were placed on filter paper lanes (18x30cm) previously moistened with 30 ml of test solution of chlorpyrifos. The lanes were rolled up and put in PVC bags and into a thermostat together with Petri dishes and boxes. After seven days the following assessments were made: germination (%), length of seedling roots and shoots from rolls (cm) and fresh and dry weight (g) of roots and shoots. The experiment was set in four replicates.

Statistical analysis

Duncan's multiple range test was used for testing the significance of differences between treatments at a confidence interval of 95%. The effective concentration (EC_{50}) for germination was calculated using Probit analysis. All tests were performed in SPSS 17 software.

RESULTS

The evidence of germination and roots and shoots growth inhibition of white mustard caused by environmentally relevant concentrations of chlorpyrifos is given in the section Results - White mustard. The results given for maize in the section Results - Maize are also sufficiently documented and supported by experimental data.

White mustard. Chlorpyrifos applied at the MAC rate ($0.1\mu\text{g a. s. /l}$) significantly inhibited germination energy and germination (11.25%) of white mustard seeds, as compared to the control (91.3%, 93.5%, respectively) ($t=76.67^{**}$). When applied at the rate $0.05\mu\text{g/l}$, i.e. 50% less than the MAC, chlorpyrifos also significantly ($t=3.73^*$) inhibited germination energy (87.7%) and germination (88.3%) (Table 1), although the norm stipulated as minimal seed germination (75.0%) by the Regulation on the quality of seeds of agricultural plants (Official Gazette 58/2002) was fulfilled. However, since germination was below the mentioned norm in treatments with chlorpyrifos amounts higher than the MAC, the effect on morphological parameters of seedlings was assessed only for the amount of $0.05\mu\text{g/l}$.

Chlorpyrifos significantly inhibited root and shoot length of white mustard seedlings at the rate of $0.05\mu\text{g/l}$, while hypocotyls and epicotyls were not formed in treatments containing higher amounts ($0.065\text{-}10\mu\text{g a.i./l}$) (Figure 1). T-test determined significant differences between root and shoot lengths ($t=8.07^{**}$, 16.59^{**} , $p<0.01$, respectively) in the treatment with $0.05\mu\text{g/l}$, compared to the control (Tab 1). Fresh and dry weights of both roots and shoots were also significantly inhibited by the chlorpyrifos rate of $0.05\mu\text{g/l}$ ($t=7.14^{**}$; 6.46^{**} , 8.67^{**} and 1.88^{**} , $p>0.01$, respectively).

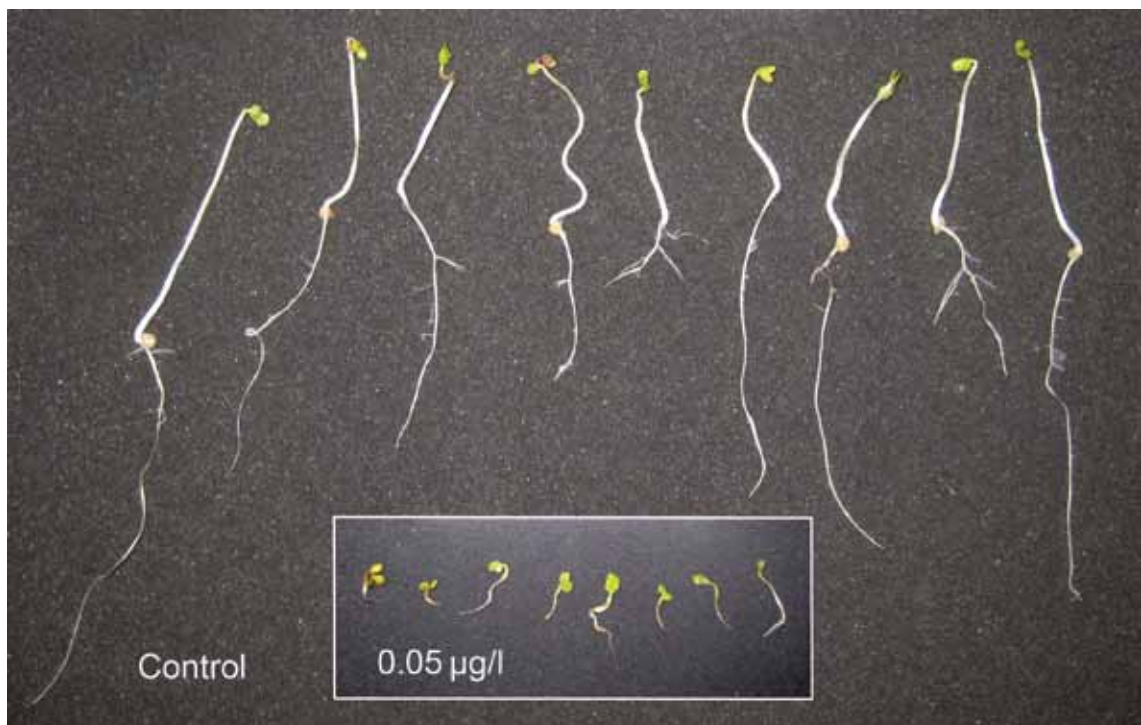


Figure 1. *S. alba* seedlings treated with $0.05\mu\text{g/l}$ chlorpyrifos and the control

Table 1. The effect of chlorpyrifos on physiological and morphological traits of white mustard (*S. alba* L.)

Parameters	Control	Treatment ($\mu\text{g as/l}$)										t value
		0.05	0.065	0.075	0.085	0.1	0.5	1.00	2,50	5	10	
GE	91.50 ± 1.23 a	87.80 ± 1.54 b	64.00 ± 2.43 c	53.20 ± 0.73 d	52.00 ± 0.87 d	11,25 ± 2.12 b	4,75 ± 1.67 c	4,75 ± 1.93 c	2,75 ± 2.32 d	0	0	3.73*
G	93.50 ± 0.98 a	88.20 ± 2.12 b	64.00 ± 2.43 c	53.20 ± 0.73 d	52.00 ± 0.87 d	11,25 ± 2.12 b	4,75 ± 1.67 c	4,75 ± 1.93 c	2,75 ± 2.32 d	0	0	3.46*
RL	5.37 ± 1.10 a	0.85 ± 0.21 b	0	0	0	0	0	0	0	0	0	8.07**
FR	0.03 ± 0.02 a	0.02 ± 0.01 b	0	0	0	0	0	0	0	0	0	7.14**
DR	0.01 ± 0.01 a	0.001 ± 0.01 b	0	0	0	0	0	0	0	0	0	6.46**
SL	4.65 ± 0.38 a	0.97 ± 0.25 b	0	0	0	0	0	0	0	0	0	16.59**
FS	0.19 ± 0.03 a	0.03 ± 0.02 b	0	0	0	0	0	0	0	0	0	8.67**
DS	0.011 ± 0.02 a	0.002 ± 0.01 b	0	0	0	0	0	0	0	0	0	1.88**
Root/shoot	1.16	0.91	0	0	0	0	0	0	0	0	0	0

GE - germination energy (%); G - germination (%); RL - root length (cm); FR - fresh root weight (g); DR - dry root weight (g); SL - shoot length (cm); FS - fresh shoot weight (g); DS - dry shoot weight (g)

Table 2. The effects of chlorpyrifos on physiological and morphological traits of maize (*Z. mays* L.)

Parameters	Control	Treatment ($\mu\text{g a.s./l}$)										F value	
		0.05	0.1	0.5	1	2.5	3.00	3.50	4.00	4.50	5		10
GE (%)	99.00 ± 0.58 a	96.50 ± 3.31 ab	95.00 ± 2.58 b	97.00 ± 1.15 ab	96.00 ± 1.00 ab	94.00 ± 3.86 b	57 ± 3.79 c	41.75 ± 10.41 d	37 ± 4.32 d	27 ± 3.65 c	0	0	2.25NS
G (%)	99.50 ± 0.58 a	98.00 ± 2.82 a	99.50 ± 1.00 a	98.00 ± 0.00 a	99.00 ± 1.54 a	95.00 ± 2.00 b	0	0	0	0	0	0	4.69**
RL (cm)	12.89 ± 0.95 a	5.85 ± 0.72 b	4.92 ± 0.25 c	1.92 ± 0.12 d	1.78 ± 1.18 d	0	0	0	0	0	0	0	268.5**
FR (g)	1.75 ± 0.45 a	0.76 ± 0.19 b	0.74 ± 0.13 b	0.1 ± 0.001 c	0.06 ± 0.18 c	0	0	0	0	0	0	0	21.71**
DR (g)	0.11 ± 0.09 ab	0.16 ± 0.03 a	0.14 ± 0.01 ab	0.09 ± 0.01 b	0.06 ± 0.00 b	0	0	0	0	0	0	0	2.93NS
SL (cm)	14.72 ± 2.23 a	4.49 ± 0.45 b	0.34 ± 0.13 c	0	0	0	0	0	0	0	0	0	118.46**
FS (g)	0.073 ± 0.01 a	0.007 ± 0.01 b	0.004 ± 0.04 ab	0	0	0	0	0	0	0	0	0	5.39**
DS (g)	0.066 ± 0.01 a	0.001 ± 0.04 b	0.002 ± 0.00 b	0	0	0	0	0	0	0	0	0	4.65**
Root/shoot	1	0.77	0.64	0.18	0	0	0	0	0	0	0	0	0

GE - germination energy (%); G - germination (%); RL - root length (cm); FR - fresh root weight (g); DR - dry root weight (g); SL - shoot length (cm); FS - fresh shoot weight (g); DS - dry shoot weight (g)

The EC_{50} for germination of white mustard seeds was $0.09\mu\text{g/l}$ of chlorpyrifos.

The results indicate a good potential of white mustard to detect chlorpyrifos presence in water even at amounts lower than the MAC (by 50%) defined in the mentioned EU directive, including both physiological and morphological traits.

Maize. Chlorpyrifos did not affect germination energy or germination of maize seeds (Table 2) even at rates that are 25-fold the EU-prescribed MAC ($2.5\mu\text{g/l}$). The highest percents were recorded in the control (99.0 and 99.5%, respectively), while the respective 96.5 and 98.0% were recorded in the treatment with $0.1\mu\text{g a.i./l}$ (MAC) without significant differences ($F=2.25_{ns}$, $p>0.05$) and the values were within the norms stipulated by the mentioned regulation (85.0%). Significant inhibition of germination energy and germination, as well as their total absence were registered in treatments with 5 and $10\mu\text{g/l}$ of chlorpyrifos (50 and 100-fold MAC).

Root and shoot length of maize seedlings were significantly inhibited by chlorpyrifos at $0.05\mu\text{g/l}$ rate (50% lower than the MAC), compared to the control ($F=268.5^{**}$, 118.6^{**} , $p>0.01$, respectively). Hypocotyls were not formed in treatments with $2.5\mu\text{g/l}$ and higher, and epicotyls in treatments with $0.5\mu\text{g/l}$ chlorpyrifos and higher. Fresh and dry weights of both roots and shoots were also significantly inhibited at the rate of $0.05\mu\text{g/l}$ and higher ($F=21.21^{**}$; 2.92^* , 5.39^{**} and 4.65^{**} , $p>0.01$, respectively).

The EC_{50} for maize seeds germination was $3.21\mu\text{g/l}$ of chlorpyrifos.

The results indicate that physiological traits of maize were not valid indicators of water contamination with chlorpyrifos. Therefore, morphological traits should be given advantage as they were significantly inhibited by the insecticide rates 50% below the MAC.

DISCUSSION

Biological assays have been used for several decades in risk assessment and detection of water contamination with chlorpyrifos, but they have mainly involved aquatic invertebrates (chironomid larvae, mosquitoes, dragon flies, prawns, shells and hydras), aquatic vertebrates such as fish and algae, and aquatic plants such as *Lemna minor* (Montagna and Collins 2007, Palma et al., 2008, Sperone et al. 2011, Rubach et al. 2012; Shafiq-ur-Rehman et al. 2012, Tongbai et al. 2012). Based on these facts, it is obvious that

the use of plants as indicators of contamination has been generally underestimated and rarely used in toxicological studies, compared to animal organisms (Moor and Kroege, 2010). However, the significance of research that involves phytoindicators should not be neglected because such data show the bioavailability of contaminants and enable risk assessment and creation of protocols for remediation of contaminated sites (O'Halloran 2006, Palma et al. 2008, Chapman 2010).

The results of this study contribute to a novel approach to contamination detection using phytoindicators. The test species, white mustard and maize, expressed different sensitivity levels to chlorpyrifos. This is consistent with the findings of Li et al. (2007) that tolerance levels of crops are species-dependant and vary under different stress intensities (concentrations and types of pollutants) and growth stages (germination, emergence, vegetative growth, etc.). Literature is rich in information referring to the phytotoxic and inhibitory effects of herbicides on germination, root and shoot growth (Boutin et al., 2004; OECD, 2003, White and Boutin, 2007), as well as the effects of seed-coating fungicides on germination (Klokočar-Šmit and Indić, 1991, Stevanović et al. 2009a, 2009b). However, very few reports can be found on the effects of insecticides, especially organophosphates, on these seed and plant traits. The effects of chlorpyrifos on cultivated plants have been examined by several authors. In the present study, chlorpyrifos caused phytotoxic effects on both tested plant species, manifesting as root and shoot growth reduction or total inhibition. The data are consistent with the findings of McEwen and Stephenson (1979), reporting on the toxicity of chlorpyrifos to lettuce. Kennedy (2002) reported phytotoxic effects of several different organophosphate insecticides on *Pennisetum glaucum* L., resulting in the inhibition of emergence and seedling growth. In that study, phorate was the most toxic insecticide that reduced growth rate to below 7%, and it was followed by aldicarb, chlorpyrifos and terbufos. Dubey and Fulekar (2011) conducted a research on the potential of the grass species *Cenchrus setigerus* Vahl. and *Pennisetum pedicellatum* L. to remediate soil contaminated with chlorpyrifos, cypermethrin and fenvalerate. Of all tested insecticides, chlorpyrifos (75 and 100 mg/kg of soil) caused the highest toxicity in terms of germination and seedling growth, which is consistent with the results of our study. According to Zhi-Yong et al. (2011), chlorpyrifos applied at the rates of 1.0 mg/l and 10.0 mg/l caused phytotoxic effects, i.e. significant

root growth inhibition and fresh root weight reduction in Chinese cabbage, *Brassica chinensis* L. Some literature data report the occurrence of pine needles necrosis and growth reduction (13%) after treatment with chlorpyrifos combined with the Savona insecticidal soap (Straw et al., 1996). In contrast to the results of this study, Wang et al. (2007) showed that chlorpyrifos had not affected the growth of wheat and oilseed rape seedlings even at high rates, indicating that those species were not good indicators of the presence of that insecticide in water.

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Fitotoksičnost hlorspirifosa za slačicu (*Sinapis alba* L.) i kukuruz (*Zea mays* L.): potencijalne indikatore prisustva insekticida u vodi

REZIME

Insekticid hlorspirifos prema Direktivi 2008/105/EC svrstan je među prioritne polutante vode i takođe značajan polutant životne sredine. Njegovo prisustvo se detektuje uglavnom hemijskim metodama, međutim biološki testovi sve više dobijaju na značaju u poslednjih nekoliko godina te je cilj ovog rada bila procena potencijala bele slačice (*Sinapis alba* L.) i kukuruza (*Zea mays* L.) kao bioindikatora kontaminacije vode. Fitotoksični efekti hlorspirifosa (količine 0,05-10 µg a.m./l vode) su procenjeni preko fizioloških (energija klijanja i klijavost) i morfoloških parametara (dužina korena i izdanka, sveža i suva masa korena i izdanka) ispitivanih vrsta. Korišćena je modifikovana metoda na filter hartiji. Podaci su obrađeni Dankanovim testom višestrukih poređenja i Probit analiza pri određivanju toksičnosti (EC₅₀). Prema pomenutoj Direktivi, maksimalno dozvoljena količina (MAC) hlorspirifosa u vodi je 0,1 µg a.m./l vode. Pri primeni hlorspirifosa u MAC količini, energija klijanja i klijavost semena bele slačice (11,25%) su bile značajno inhibirane u poređenju sa kontrolom (91,5; 93,5%) dok je formiranje hipokotila i epikotila izostalo. U količini 50% nižoj do MAC (0,05 µg a.m. /l), energija klijanja i klijavost (87,75; 88,25%) su u poređenju sa kontrolom bile značajno smanjene, kao i dužina korena i izdanka ponika. Hlorspirifos nije uticao na energiju klijanja i klijavost semena kukuruza, dok su morfološki parametri bili značajno smanjeni već pri primeni insekticida u količini od 0,1 µg a.m./l. Toksičnost hlorspirifosa, to jest EC₅₀ za klijavost semena bele slačice je iznosila 0,09 µg a.m./l, a za kukuruz 3,21 µg a.m./l vode.

Keywords: Hlorspirifos; voda; fitotoksičnost; *Sinapis alba* L.; *Zea mays* L.