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Toxicity of the organophosphorous insecticide chlormephos to the earthworm *Eisenia andrei* and the terrestrial isopod *Porcellio scaber*

Strupenost organofosfatnega pesticida klormefosa za deževnike *Eisenia andrei* in kopenske enakonožce *Porcellio scaber*

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Abstract: In the present study we determined the toxicity of chlormephos to two common soil organisms – earthworms (*Eisenia andrei*) and isopods (*Porcellio scaber*) using Lufa 2.2 soil. The LC_{50} values for the effects on earthworm and isopod survival were 58 and 76 mg/kg dry soil, respectively. Mass change of earthworms and behaviour of isopods were more sensitive end points as survival. Based on earthworm body mass changes, NOEC and LOEC values were 1 and 3 mg/kg dry soil, respectively. The earthworms did not reproduce even at the lowest chlormephos concentration (LOEC < 1 mg/kg dry soil). Isopods significantly avoided burrowing in soil treated with ≥40 mg/kg dry soil. Compared with some other organophosphate insecticides, chlormephos was highly toxic to beneficial terrestrial invertebrates.

Keywords: ecotoxicity; organophosphates; chlormephos; soil exposure; soil invertebrates

Izvleček: Toksičnost klormefosa smo določili na deževnikih (*Eisenia andrei*) in rakih enakonožcih (*Porcellio scaber*). Obe vrsti sta pogosto uporabljeni v tovrstnih študijah. Živali smo izpostavili klormefosu preko standardizirane zemlje Lufa 2.2. Vrednost LC_{50} za preživetje deževnikov je bila 58 mg/kg suhe zemlje in 76 mg/kg suhe zemlje za rake enakonožce. Ugotovili smo, da so sprememba telesne teže in razmnoževanja pri deževnikih ter vedenjski odziv rakov enakonožcev bolj občutljivi parametri kot preživetje. Na podlagi sprememb telesne teže pri deževnikih, so bile določene vrednosti NOEC in LOEC, in sicer 1 in 3 mg/kg suhe zemlje. Deževniki se niso razmnoževali niti pri najnižji koncentraciji klormefosa (LOEC< 1 mg/kg suhe zemlje). Raki enakonožci so se značilno manj zakopavali v zemljo, v kateri je bilo ≥40 mg/kg suhe zemlje (LOEC). V primerjavi z nekaterimi drugimi organofosfatnimi insekticidi, je bil klormefos izjemno toksičen za testirane kopenske nevretenčarje.

Ključne besede: ekotoksičnost; organofosfati; klormefos; izpostavitev preko zemlje; kopenski nevretenčarji

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Introduction

Organophosphates (OPs) are a large class of chemicals. Since World War II, several thousand OPs have been synthesized for various purposes. A lot of these OPs are still in use, mainly as pesticides in agriculture, but some are used as flame retardants and parasiticides in veterinary medicine (Gupta 2007). OP insecticides are highly effective in pest control, and relatively non persistent in the environment but potentially toxic also to non-target species, including vertebrates (Gupta 2007). OP insecticides cause acute toxicity by inhibiting acetylcholinesterase (AChE) through phosphorylation, in both invertebrate and vertebrate organisms (Gupta 2007).

Symptoms of high level exposure to OPs include muscle twitching, hyperactivity, paralysis, loss of equilibrium and eventually death (Fulton & Key 2001, Sandahl & al. 2005), whereas low-level exposure has been implicated in various behavioural and physiological impairments (Bayley 1995, Engenheiro & al. 2005).

Chlormephos (*S*-chloromethyl *O,O*-diethyl phosphorodithioate) is an OP insecticide that was introduced on the market in 1973 to control soil-dwelling pests like **cockchafer, click beetles, mole cricket,** wireworms, millipedes and other pests (Footprint 2006, Lynch 1978). It has been sold mostly under the trade name Dotan (Aventis, France). The World Health Organization (2004) classified chlormephos as extremely hazardous due to its high toxicity to mammals. In rats, acute lethal dose (LD_{50}) of chlormephos after oral administration was 7 mg/kg body weight (BW). It has been withdrawn from the market in all member states of the European Union not later than 2006 (**EU 2006**). Despite its prohibition in Europe, possibility of its abuse exists as it still might be in use in some less developed countries.

Although chlormephos was in use in Europe for more than 30 years only scarce data are available on its ecotoxicity and fate in the environment. Chlormephos is highly toxic to some aquatic crustaceans (*Echinogammarus tibaldi*, LC_{50} = 0.04 mg/L) (Pantani & al. 1997) and fish (*Rasbora heteromorpha*, acute LD_{50} = 2.5 mg/L) (Footprint 2006), but generally less toxic to birds (*Coturnix japonica*, acute LD50 = 260 mg/kg) (Footprint 2006). No data are available on the toxicity to non-target soil organisms.

The aim of this study was to determine the toxicity of chlormephos to some soil non-target invertebrates (earthworms and isopods) upon exposure to contaminated soil. Earthworms are standardised test organisms in toxicity testing and isopods are a model test species, commonly used to study the mode of action as well as for risk assessment. Both animals have an important ecological role as decomposers of organic material.

Besides, both species are convenient for analyzing the response to contaminants at different levels of biological organisation. In earthworms, we followed survival, mass gain/loss and reproduction in relation to the degree of soil contamination with chlormephos. In isopods, mortality and behavioural response to contaminated soil was observed. The effects of chlormephos were compared with those of some other OP insecticides.

Materials and Methods

Test species

In the experiments we used the earthworm species *Eisenia andrei* (Oligochaeta: Annelida, Lumbricidae) originating from a laboratory culture at the Veterinary Faculty, University of Ljubljana. Animals were kept in a climate chamber at 20±1 °C with a 12/12 h photo period and 80% relative humidity (RH). Plastic containers were filled with a bedding of potting soil and peat, adjusted to pH 6. The cultures were regularly fed with ground dried horse faeces. Sexually mature animals with clearly visible clitellum and weighing between 200 and 300 mg were used in the experiment.

Porcellio scaber, Latr. (Isopoda, Crustacea), originated from an unpolluted environment in the vicinity of Ljubljana, Slovenia. Animals were kept in a climate chamber at 20±1 °C with a 16/8 h photo period, caged in glass containers with moist sand and peat on the bottom.

They were fed with leaves from various trees (mainly hazel), with periodical addition of commercial food designed for experimental animals (Altromin 1324, Germany), fresh vegetable and apples. All tests were performed on animals of both sexes, having body masses of 18–30 mg.

Soil preparation

Analytical grade chlormephos in liquid form was obtained from Riedel de Haën (45386 3045X), Germany. Its purity was 99.2%.

The tests were performed using Lufa 2.2, a standardized natural soil having 3.7% organic matter, 6.8% clay and a pH (1 M KCl) of approx. 6.0. The test substance was introduced into the soils using acetone as a solvent.

A small portion of the soil (approx. 25c) was spiked with the acetone solution (25 ml acetone per 30 g soil), thoroughly mixed and incubated over night in a fume cupboard. After evaporation of the acetone, the remainder of the soil was added, carefully mixed and the moisture content was adjusted to 40–50% of the Water Holding Capacity (WHC). The control soil was treated with acetone in the same way.

Experimental design

Tests were performed with earthworms and isopods, lasting 28 days. Chlormephos concentrations in the Lufa 2.2 soil were 0-1-3-9-15-30-45-60-100-200 mg/kg dry soil for earthworms and 0-10-40-60-100-200 mg/kg dry soil for isopods.

The clormephos concentrations in soil were checked randomly at the beginning of the test and did not differ for more than 15% from nominal concentrations.

The deviation in concentrations due to degradation or volatisation during the experiments was not checked.

Earthworm tests

Glass jars (0.8 L) were filled with 500–600 g moist soil. Ten adult pre-weighed earthworms were randomly introduced into the test containers. There were 4 replicates per test concentration and a control. Incubation took place in a climate chamber at 21 °C, with 80% RH and a 12/12 h light/dark cycle. A small amount of finely ground dried horse manure was added for food once a week and by weighing water loss was compensated where necessary. After 28 days, test containers were emptied and surviving adults were counted and weighed.

Soil was returned into the test containers to allow for hatching of the cocoons for an additional 28 days (OECD 2004). To count the number of juveniles produced, the test containers were placed in a water bath at 60 °C; after approx. 15 minutes, the juvenile earthworms appeared on the soil surface and were gently transferred to a separate jar and counted manually.

Isopod tests

Glass jars (100 ml) were filled with approx. 30 g moist soil, and three adult isopods were introduced. There were six replicates for each treatment, including the control. Twice a week, we observed the presence of isopods on the surface of the soil and statistically evaluated the obtained results.

As test jars were filled with three organisms, their presence was evaluated in shares – if there was one organism present that meant 0.33, if two 0.66 and if three 1.00. Isopods received food pellets, consisting of maple leaves (50%), ground commercial rabbit food (40%) and potato powder (10%), on the soil surface (Hornung & al. 1998).

Test jars were covered with perforated aluminium foil and placed on trays in a climate chamber at 21 °C, with 75% RH and a 16/8 h light/dark cycle. Additional food was given when needed but at least once a week. Moisture content was checked weekly by weighing the containers and replenishing the water loss with deionised water. The animals were extracted from the tested substrates after 28 days of exposure period by emptying the test jars, hand sorting the test substrates and counting the surviving animals.

Data analysis

LC_{50}, the concentration causing a 50% reduction in survival, was estimated applying the trimmed Spearman-Karber method (Hamilton & al. 1978). EC_{50} and EC_{10}, the concentrations causing 50 and 10% reduction, respectively in the number of juveniles produced, were estimated using a logistic model (Haanstra & al. 1985). Calculations were done using the software package Excel for Windows (Microsoft, USA, 2002).

No observed effect concentrations (NOECs) and lowest observed effect concentrations (LOEC) were estimated using T-test and ANOVA followed by the Dunnett's test (pair-wise comparison of means with the control) in the TOXSTAT® software package (Gully & al. 1991). All LC_{50}, ECx and NOEC values are based on nominal concentrations in the Lufa 2.2 test soil.

Results

Test with earthworms

Mortality and abnormalities

After 28 days of exposure, survival in the control group was 100%, whereas at 200 mg/kg there were no survivals. Significant increase in mortality was observed at chlormephos concentrations ≥45 mg/kg dry soil (Tab. 1). The LC_{50} value was 58 mg/kg dry soil (confidence interval: 50–67).

At chlormephos concentrations of ≥45 mg/kg organisms were swelled and red coloured, but at higher concentrations, their bodies softened and became covered with a large amount of yellow liquid.

Body mass change

Earthworms lost mass during the 28 days of exposure in all groups, apart from the control and the lowest chlormephos concentration (Fig. 1). The average mass gain in the control group was 10%, while at 1 mg/kg chlormephos mass gain was 4.7%.

The increase in mass loss was dose-related and was most prominent at 60 mg/kg dry soil (56.6%). It was noted during the experiment that food, offered in upper parts of jars, remained uneaten as earthworms mainly stayed in the bottom of the test jars.NOEC and LOEC for the effect of

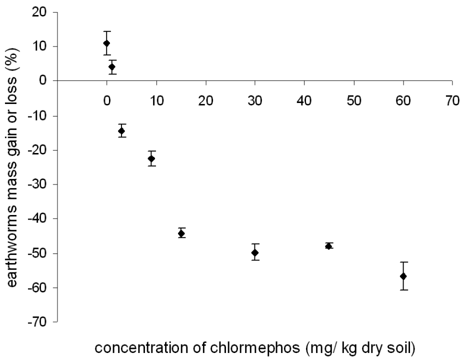

Fig. 1: Mass change (gain or loss) in earthworms (*Eisenia andrei*) exposed for 28 days to different concentrations of chlormephos in Lufa 2.2 soil (AVR±SE).

Slika 1: Sprememba teže deževnikov, ki so bili 28 dni izpostavljeni različnim koncentracijam klormefosa v zemlji (POVP±SN).

Table 1: Mortality rate of earthworms (*Eisenia andrei*) and isopods (*Porcellio scaber*) after 28 days of exposure to chlormephos in Lufa 2.2 soil, expressed in percentage of the number of individuals exposed.

Tabela 1: Stopnja smrtnosti deževnikov (*Eisenia andrei*) in enakonožcev (*Porcellio scaber*) po 28 dneh izpostavitve klormefosu, izraženo v odstotkih.

Eisenia andrei	
Chlormephos (mg/kg dry soil)	Mortality %
0	0
1	0
3	2.5
9	0
15	7.5
30	7.5
45	37.5
60	42.5
100	95
200	100

Porcellio scaber	
Chlormephos (mg/kg dry soil)	Mortality %
0	0
10	5
40	22
60	16.5
100	89
200	100

chlormephos on earthworm mass loss were 1 mg/kg and 3 mg/kg dry soil, respectively.

Reproduction

After 56 days, in the control groups the number of juveniles was 44 ± 11 (AVR±SD), while already at the lowest test concentration (1 mg/kg) no juveniles were found. Therefore no EC_{50} value could be established.

Test with isopods

Mortality

After 28 days of exposure, the survival in control groups was 100%, while at 200 mg/kg all isopods died (Tab. 1).

Significant increase in mortality was observed at chlormephos concentrations of ≥40 mg/kg dry soil. The LC_{50} was 75 mg/kg dry soil (confidence interval: 67–84).

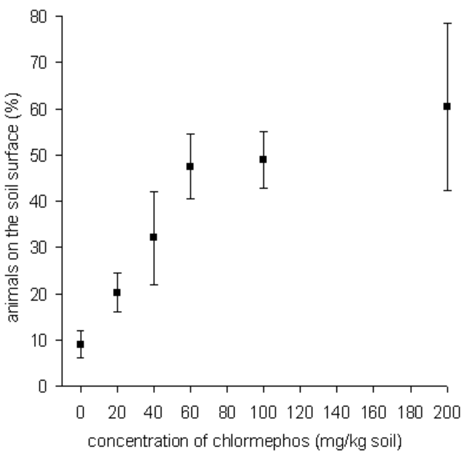

Fig. 2: Influence of chlormephos on the behavioural response of isopods (*Porcellio scaber*) during 28 days of exposure in soil. Shown are the average percentages (AVR±SE) of animals detected on the soil surface as a function of exposure concentration in Lufa 2.2 soil.

Slika 2: Vpliv klormefosa na vedenje enakonožcev (*Porcellio scaber*) med 28-dnevno izpostavitvijo onesnaženi zemlji. Prikazani so povprečni deleži (POVP±SN) živali, ki smo jih opazili na površini zemlje, v odvisnosti od koncentracije klormefosa v Lufa 2.2 zemlji.

Behavioural response

During observations, control animals were found mostly hidden in the soil (Fig. 2). Animals exposed to soil with chlormephos concentrations of ≥40 mg/kg dry soil were more frequently observed on the soil surface (t-test, $p<0.05$). LOEC for behaviour is therefore 40 mg/kg dry soil.

Discussion

Organophosphates are still quite often used in agriculture as pesticides. Ecotoxicological studies related to OP's and using earthworms and isopods as model organisms exist (Yasmin & D'Souza 2007, Drobne &. al. 2008) but in comparison to the numerous studies on aquatic organisms (Hanazato 1998, Barry 1999, Dutta & Maxwell 2003, Sherrarda & al. 2004) they are not that abundant..

This study revealed high sensitivity of both tested organisms to chlormephos. The NOEC for effects on earthworm body mass gain/loss was 1 mg/kg soil, while no reproduction occurred at this concentration.

The exposed earthworms probably lost weight due to their feeding and locomotor dysfunctions, as was noticed previously for ethyl-parathion in experiments with *Aporrectodea caliginosa* (Olvera-Velona & al. 2008).

The authors reported affected burrowing behaviour of *A. caliginosa* already at concentration 0.07 mg/kg dry soil. Behavioural response was recorded also in the presented experiment with *Porcellio scaber*. Isopods exposed to chlormephos were observed on the soil surface more frequently compared to control animals found mostly burrowed in the soil. The burrowing activity was probably affected by a locomotor dysfunction previously reported by Engeheiro & al. (2005) but it can also be explained by avoidance behaviour response that is known for these animals (Loureiro & al. 2005, Zidar & al. 2005).

Terrestrial isopods are able to avoid soil (Loureiro & al. 2005) or food (Zidar & al. 2005) contaminated with pesticides or metals if they have an alternative. Loureiro & al. (2005) found behavioural response parameters to be equally or even more sensitive than other sublethal parameters like growth or reproduction.

Table 2: Literature data on the toxicity for earthworms and isopods of different organophosphorous insecticides.
Tabela 2: Podatki iz literature o toksičnosti nekaterih organofosfatnih insekticidov za deževnike in enakonožce.

Test substance OP	Test organism(s)	Time (Days)	Test substrate	End-Point	Concentration (mg/kg dry soil)	Reference(s)
Chlorfenvinphos	*Eisenia fetida*	14	Sandy loam (pH 7.2)	LC_{50} NOEC (growth) LOEC (growth) LOEC (behaviour)	204 123 234 62	Weyman (1997)
Dichlorvos	*Eisenia fetida*	14	Sandy loam (pH 6.9)	LC_{50} NOEC (growth)	80.9 12.3	Vial (1991)
Methamidophos	*Eisenia fetida*	18	Natural soil	LC_{50}	29.5	Qi-xing & al. (2006)
Diazinon	*Lumbricus terrestris*	21	Natural soil *	LC_{50} (different soil)	32, 233, 59	Lanno & al. (1997)
Ethyl-parathion	*Aporrectodea caliginosa*	14	Natural soil **	LC_{50} (different soil) LOEC (growth)	30, 24, 11, 32 7 (all soils)	Olvera-Velona & al. (2008)
Malathion	*Drawida willsi* (juvenile, adult)	4 15	Sandy loam (pH 6.8)	LC_{50} LOEC (growth)	15.1 (juv.); 18.8 (adult) 2.2	Panda & Sahu (1999)
Chlormephos	***Eisenia andrei***	**28** **56**	**Lufa 2.2**	**LC_{50}** **NOEC (growth)** **LOEC (growth)** **NOEC (reproduction)**	**58** **1** **3** **<1**	**This study**
Dimethoate	Porcellio scaber	18	Lufa 2.2	LC_{50}	34 (juvenile)	Fischer & al. (1997)
Diazinon	*Porcellionides pruinosus*	35	Sand	LC_{50}	3.03	Vink & al. (1995)
Dimethoate	*Porcellionides pruinosus*	2	Lufa 2.2	NOEC (behaviour)$ LOEC (behaviour)$	10 40	Loureiro & al. (2005)
Dimethoate	*Porcellio dilatatus*	2 10	Silt loam	LOEC (behaviour)# LOEC (behaviour)##	5 10	Engenheiro & al. (2005)
Chlormephos	***Porcellio scaber***	**28**	**Lufa 2.2**	**LC_{50}** **NOEC (behaviour)** **LOEC (behaviour)**	**75** **20** **40**	**This study**

* three different natural soils (Broxton Clay, Fox Sand, Guelph Loam); ** natural soil from three different locations in Mexico and one from France (Vertisol 1, Vertisol 2, Andosol, Calcosol); & avoidance; # pathlength, active time; ## stops per path

Earthworms (Mosleh & al. 2003), isopods (Zidar & al. 2004) and probably also some other invertebrates can avoid accumulation of toxic substance in their body by reducing or even stopping food intake. This leads to diminished intake of necessary nutrients, resulting in starvation and upon long-term exposures to death of the organisms. This shows how important it is to also determine sublethal effects, with an emphasis on physiological parameters, besides the "classical" LC_{50} determination.

Reproduction of adult earthworms was completely inhibited already at 1 mg/kg dry soil. Besides direct OP poisoning, this might also be caused by lack of energy due to starvation in adult organisms or due to body abnormalities registered.

Namely, bodies of earthworms showed some typical changes for organophosphate exposure, observed also by other authors (Qi-xing & al. 2006, Venkateswara & al. 2003).

In Table 2, data on the toxicity of different OP insecticides for earthworms and isopods are listed. Toxicity depends on the test species, soil type used and duration of exposure, which makes the comparison more difficult. For earthworm survival and growth, chlormephos was as toxic as malathion, parathion and methamidophos, but more toxic than dichlorvos and chlorphenvinpros. All these insecticides are already prohibited in the EU (EU 2008) but (like dichlorvos and parathion for example) may still be in use in some parts of Africa and Asia (FLO 2007). Chlormephos is less toxic to isopods compared to diazinon, which was withdrawn from the European market recently (EU 2008) and compared to dimethoate, which is still in use. Isopods seem less succeptible to chlormephos than earthworms, probably due to the avoidance response mentioned above.

Conclusions

- Chlormephos is highly toxic to earthworms, especially affecting their growth and reproduction, with an LC_{50} of 58 mg/kg dry soil, and NOEC growth of 1 mg/kg dry soil, and no reproduction at the NOEC growth.
- Isopods were less sensivitive, with an LC_{50} of 76 mg/kg dry soil and burrowing behaviour significantly reduced at ≥40 mg/kg dry soil.

Povzetek

Uporaba toksičnih organofosfornih snovi se v zadnjem času zmanjšuje, vendar je njihova uporaba v kmetijstvu še vedno obsežna. Slednja lahko predstavlja resen ekološki problem, saj pogosta uporaba organofosfornih snovi negativno učinkuje na neciljne talne organizme, ki so sestavni člen prehranjevalnih verig. Glede na znane lastnosti organofosfornih snovi – visoka reaktivnost, hitro delovanje in toksičnost, smo za modelno substanco izbrali klormefos, ki v Evropi ni več v uporabi, vendar za katerega v literaturi ni veliko podatkov. V raziskovalnem delu smo spremljali učinke klormefosa na deževnike (*Eisenia andrei*) in rake enakonožce (*Porcellio scaber*), ki so bili 28 dni izpostavljeni kontaminirani standardizirani zemlji Lufa 2.2.

Določili smo koncentracijo klormefosa, ki povzroči smrt polovice izpostavljenih živali (LC_{50}). Ta je bila za deževnike 58 mg/kg suhe zemlje, pri rakih enakonožcih pa 76 mg/kg suhe zemlje. Spremljali smo tudi subletalne učinke, ki so občutljivejši pokazatelji toksičnosti snovi, zlasti ob izpostavitvi nižjim koncentracijam.

Na osnovi spremembe v masi deževnikov smo ugotovili, da je 1 mg/kg suhe zemlje koncentracija klormefosa brez opaznega učinka (NOEC), 3 mg/kg suhe zemlje pa koncentracija z opaznim učinkom na maso živali (LOEC). Pri izpostavljenih deževnikih smo opazili tudi telesne spremembe kot so nabrekanje, izžemanje, rumenkasti izločki ipd. (LOEC = 45 mg/kg suhe zemlje) ter prizadet proces reprodukcije (LOEC < 1 mg/kg suhe zemlje). Pri rakih enakonožcih smo poleg smrtnosti spremljali tudi vzorce obnašanja in ugotovili, da so se organizmi pri višjih koncentracijah zadrževali na površini in niso bili zakopani v zemlji kakor kontrolne živali.

Najnižja koncentracija z opaznim učinkom na vedenje živali je bila 40 mg/kg suhe zemlje.

Testiranje je pokazalo visoko toksičnost klormefosa za uporabljena testna organizma, pri čemer so bili enakonožci manj občutljivi na prisotnost klormefosa v zemlji od deževnikov, saj je pri enakonožcih bolj izražena sposobnost izogibanja onesnaženi hrani. Toksičnost klormefosa je primerljiva z nekaterimi sorodnimi organofosfornimi insekticidi, pri čemer so nekateri že umaknjeni s trga, nekateri celo bolj strupeni za talne organizme pa so še v uporabi (npr. dimetoat). Pridobljeni podatki lahko služijo tudi za izdelavo natančne ocene tveganja za okolje v primeru nenadzorovane rabe klormefosa.

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