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The Toxicity of Metal Salts and the Population Growth of the Ciliate *Colpoda cucculus*

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Legislation to regulate the release of newly produced chemicals in the environment requires routine testing to avoid possible side effects on non target organisms. Such tests have been reasonably well developed for the aquatic environment (Standard methods for the examination of water and wastewater, 1976). Screening procedures for the terrestrial environment are scarce and less advanced. A procedure in which colonization of protozoa in diluted percolate was used to determine the risk at hazardous waste sites has been developed recently (Pratt et al., 1988).

Toxicity of compounds may differ considerably between taxonomic groups. Freshwater protozoa are more sensitive to certain compounds than representatives from other taxonomic groups, and considerable differences within protozoa have also been observed. (Slooff et al., 1983, Bringmann & Kühn, 1980, Madoni et al., 1992). It may be hypothesized that the same is also true for soil protozoa. Protozoa occur in high numbers in the litter layers where they play an important role in the regulation of microorganisms and in the recycling of organic material (Clarholm, 1981, Foissner, 1987). The highest impact of air borne compounds can be expected in the upper soil layers. As protozoa are confined to soil pore water, effects of soluble compounds can be expected via uptake from the surrounding medium. In spite of their importance and their sensitivity, soil protozoa have, until now, been used in only a few toxicity tests.

Metals occur in different chemical forms in the soil ecosystem, whereas in test systems often only a few well soluble compounds are used. A number of cases have been described in which the chemical form of the metal added resulted in different toxicity values (Babich & Stotzky, 1978, Malecki et al., 1982, Maliszweska et al., 1985). In an experiment conducted by Loka Bharati et al. (1990) the addition of acetate resulted in a change of inhibitory concentrations of lead and mercury to stimulatory effects for microorganisms.

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The aim of the present study was to select a soil protozoan for toxicity studies, to estimate toxicity values of different metals, and to study the effect of anions used in the test system.

MATERIALS AND METHODS

An evaluation of literature data showed that in the majority of toxicity tests for the aquatic environment in which protozoa are used, ciliates are chosen as test species (Janssen, 1994). Ciliates contain the largest species among the protozoa, are more easy to isolate individually from suspension than amoebae and flagellates and are more easy to identify. Another important argument for selecting ciliates for toxicity tests is that they are often available as certified axene strain from the culture collections which provide certain advantages above non-axenic organisms (e.g. American Type Culture Collection, 1982, Thompson et al., 1988). Methods for aquatic toxicity tests with ciliates have been described by Persoone & Dive (1979).

We selected the ciliate *Colpoda cucullus* as test species as it is one of the most abundant soil ciliates and it belongs to a genus which is almost always confined to the soil ecosystem (Foissner, 1987, Sleigh, 1989, Foissner et al., 1991). Our test animals were isolated from a pine forest at Wekerom, The Netherlands. Ciliates are known to be relatively numerous in the litter layers (Sleigh, 1989), up to several thousands per gram litter were observed in our field site. The ciliates were identified using Foissner et al. (1991). Pine needles were mixed with tap water in a petri dish and put at room temperature for culturing. The ciliates were picked from the petri dish after a few days and were transferred to a litter extract medium using a micropipette. The cultures were kept for a number of weeks in the medium, which was refreshed twice every week to keep the culture active and to avoid encystment.

Litter extract medium was prepared from pine needles collected at a pine forest at Wekerom. The material was dried at 40 °C, and twigs and pine cones were removed. Twenty grams of dried material were mixed with 0.8 litre of demineralized water for 24 hours. The liquid was collected, mixed thoroughly and filtered several times to remove large organic particles. Then it was filtered twice through a 1.5 μm filter and centrifugated at 18,000 rpm to get a clear medium. The material was mixed again to obtain a homogeneous medium, poured into several bottles and sterilized at 120 °C. After three days the bottles were sterilized for a second time.

A large quantity of medium was prepared at the start of the experiment to prevent results being influenced by differences in medium quality. In a preliminary experiment, cultures in litter extract medium showed better population growth than populations in artificial medium such as Proteose Pepton Glucose. Litter extract medium was also thought to be more representative of the environmental conditions in the field. The pH of the medium was 6.8. Concentrations of copper and zinc were 1.3 and 0.9 μmol/L respectively. Concentrations of cadmium and lead were below detection limit, i.e. below 0.4 μmol/L and below 1.2 μmol/L respectively. Metals were analyzed by atomic absorption.

Toxicity tests were executed in microtiterplates containing 8x12 wells which were filled with 40 μl of litter extract medium. Two ciliates were inoculated from the original stock solution into each well using a micropipette. No food was added to the wells, assuming absorption of nutrients directly from the surrounding medium. Similar procedures are mentioned in the Standard Methods for the examination of water and waste water (1976) in which axenic strains of *Tetrahymena pyriformis* are cultured in sterile medium. The ciliates were cultured at 15 °C in a dark environment. To facilitate counting by eye, the ciliates were killed with 4 μl 0.5% formalin and stained with 4 μl neutral red. Counting was carried out using a stereomicroscope at 10-25 magnification. Population growth of *C. cucullus* was estimated by counting the number of protozoa after 1,2,3, and 7 days.

The toxicity of cadmium, copper, lead and zinc were tested using four different salts of each metal (Cl^-, CH_3COO^-, NO_3^-, SO_4^{2-}). To obtain a reliable dose-response curve six different concentrations of each metal were used, resulting in 96 different combinations. 0 mmol/L was used as lowest concentration for each treatment, the other five concentrations ranged from 0.005 to 0.080 mmol/L for copper, from 0.010 to 0.160 mmol/L for zinc, from 0.001 to 0.016 mmol/L for cadmium and from 0.002 to 0.032 mmol/L for lead. Ranges were chosen such that equal effects for all metals were expected at the highest concentration. Concentrations within each range differed by a factor two in all cases, ranges were chosen after carrying out a preliminary experiment. The anion concentrations within each metal treatment were kept constant by addition of the corresponding potassium salts to the lower metal concentrations.

Experiments were divided over two periods of four weeks. In each week four microtiterplates were used, and the four counting days were assigned to the plates by randomization. The main experiment used row two to seven of each microtiterplate. The available space was divided in three blocks of 24 wells. Because of practical restrictions on the number of salt solutions that could be prepared, it was decided to analyze only one metal each week (metals were randomized over the four weeks of each period). The 24 combinations of anion and concentration were arranged according to a randomized block design on each of the four plates of that week. In this way differences between the effect of metals became confounded with differences between weeks within a period, but differences between anions could be assessed with optimal precision. The randomization of the design was performed with the statistical program GENSTAT (Genstat 5 Committee, 1987). The effect of anions without any heavy metals added was tested by observing population growth of *C. cucullus* in litter extract medium in an additional experiment.

The ciliate counts transformed to $\log(n+1)$ were analyzed with GENSTAT (Genstat 5 Committee, 1987) with a multi-stratum analysis of variance in accordance with the experimental design. For the main experiment the main effects and interactions of metal, anion, concentration and counting day were assessed for significance, as well as the random variation between blocks on the plate, between plates, and between weeks. In a separate analysis, where the existence of blocks on the microtiterplates was ignored, all counts from wells without added heavy metal were analyzed in a similar way.

Differences between all treatments and interactions were tested by analysis of variance on the ln-transformed numbers of ciliates using GENSTAT. Differences between the potassium anion treatments were tested by the same method. Results from one metal but a different anion were lumped in one group for each concentration of each sampling day as no significant difference was found. Dose effect curves were estimated for the four metals on each of the days.

The effect of metal concentration on the growth of protozoa was estimated using the logistic model $y=c/(1+\exp(b^*(x-a)))$, in which a = the effective ln-dose (ln-EC_{50}), b the steepness parameter and c the uninhibited response. A Poisson distribution was assumed for the probability distribution of the response to a certain concentration (Finney, 1964). EC_{10} and EC_{50} values were calculated from the growth curves. These values have a better statistical background than the often used No-observed-effect-level (Hoekstra & Van Ewijk, 1993).

RESULTS AND DISCUSSION

In the treatments in which anions were added as potassium salt, small anion differences between counts of *C. cucullus* were observed ($p = 0.022$). Growth in the medium with potassium acetate was found to be slower than in the blank treatment and in media with other anion treatments (see Table 1). It is not clear to which phenomena this should be attributed, probably to the effect of pH which will be slightly lower in the acetate medium or to the fact that acetate is a metabolizable organic substrate.

In the tests in which anions were added as metal salts neither significant effects of any anion nor an interaction between anion-addition and other treatments were found. A slightly faster growth was observed in the treatments with $PbSO_4$, although growth was not significantly different from the other treatments. This could probably be attributed to the low solubility of $PbSO_4$. Measurements of the stock solution showed lower lead concentrations than in the other lead stock solutions. Malecki et al. (1982) found significant differences in the effects of metals added in different chemical forms on growth and reproduction of earthworms. The carbonate and oxide forms especially showed a reduced effect from which he concluded that solubility of the compound is one of the most important parameters determining toxicity. In our experiments differences in

Table 1. Geometric means of the numbers of ciliates in treatments after addition of different anions (as potassium salt) compared to a blank treatment.

no. days	Cl^-	SO_4^-	CH_3COO^-	NO_3^-	blank
1	3.67	3.61	3.27	3.77	3.58
2	6.55	7.02	5.55	7.14	6.54
3	13.14	13.32	11.57	12.87	12.71
7	23.36	21.69	21.04	23.31	22.34

solubility played a minor role, as most compounds were easily soluble and the concentrations used were relatively low.

The residual variation at the lowest stratum (wells within a block on a plate) is estimated with a standard error in the log-counts of 0.40, roughly corresponding to a coefficient of variation of 40% in the counts of equally treated wells. The variability between blocks on a plate is no larger than the variability within blocks (variance ratio 1.00, $p=0.48$). However, the variability of counts between plates is much larger than the variability within plates (variance ratio 18.2, $p<0.001$). Finally, there is also an indication that the variability between weeks might be larger than the variability within weeks (variance ratio 2.89, $p=0.08$).

The growth curves showed that reliable toxicity values could be obtained after about one week. Insufficient growth causes unreliable EC_{50} values during the first few days. This is illustrated by figure 1 which shows the population growth of *C. cucullus* at different copper concentrations during the sampling days.

The parameter estimations for the dose effect curves on different metals after 7 days are given in Table 2. The EC_{10} and EC_{50} values calculated from these dose effect curves are also given in Table 2 expressed in μmol/L. Cadmium is most toxic, lead and copper show intermediate toxicity and zinc is least toxic considering the EC_{50} values. The pattern in EC_{10} values is slightly different due to the shape of the dose effect curve, which lead to EC_{50} being more reliable than EC_{10} values. Dose effect curves for the different metals are given in Figure 2.

For comparison with other studies the EC_{10} and EC_{50} values are recalculated in mg/L. EC_{50} values are 0.97 mg/L, 1.37 mg/L, 0.22 mg/L and 2.07 mg/L for copper, zinc, cadmium and lead respectively. EC_{10} values are 0.33 mg copper/L, 0.29 mg zinc/L, 0.06 mg cadmium/L and 0.68 mg lead/L. Expressed in mg/L cadmium was most toxic, intermediate values were found for copper and zinc, lead was least toxic.

Table 2. Parameter estimations for the dose effect curves of the different metals on the population growth of *Colpoda cucullus* after 7 days, in which a = the effective ln-dose (EC_{50}), b the steepness parameter and c the uninhibited response. EC_{50} and EC_{10} values and the 95% confidence interval (in μmol/L) calculated from these dose effect curves are given in the last two columns.

	a	b	c	EC_{50} (μmol/L)	EC_{10} (μmol/L)
copper	-4.178	2.039	24.45	15.3 13.2-17.9	5.22 3.92-6.94
zinc	-3.859	1.408	24.20	21.1 15.8-28.2	4.43 2.58-7.60
cadmium	-6.134	1.543	28.56	2.17 1.68-2.80	0.522 0.326-0.834
lead	-4.606	1.991	19.99	9.99 8.63-11.6	3.31 2.45-4.48

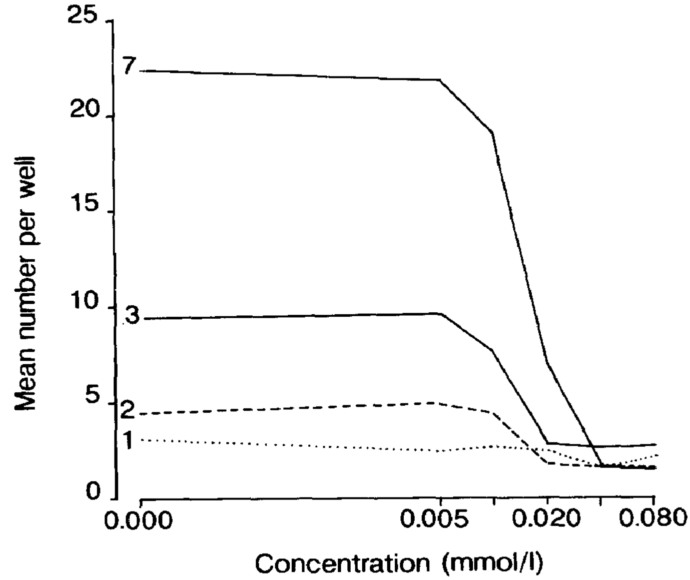

Figure 1. Population growth of *Colpoda cucullus* at different copper concentrations (in mmol/L) at day 1,2,3, and 7.

Toxicity tests with freshwater protozoa show a considerable range in threshold

Table 3. Toxicity values (in μmol/L) for some freshwater ciliates for different metals, derived from literature data. The number of species used, or the name of the species, and the endpoint used in these studies are given.

	Ruthven & Cairns (1973)	Madoni et al. (1992)	Carter & Cameron (1973)	Yamaguchi et al. (1973)	Dive & Leclerc (1977)	Bringmann & Kühn (1980)
no. of species	5	7	Tetrahymena	Tetrahymena	Colpidium	Entosiphon
endpoint	survival after 10 min.	24 hr. LC50	96 hr LC50	growth inhibition	no. of generations	cell multiplication
copper	0.38-21.3	0.016-0.331		1000	3.93	1.73
zinc	8.58-15,300	28.3-766	88.4	1000	383	
cadmium		1.60-12.5	7.47	4.00	1.42	0.098
lead	27-4830		183-1210		1.93	0.097

Note: For the first two studies ranges are given as different species were tested.

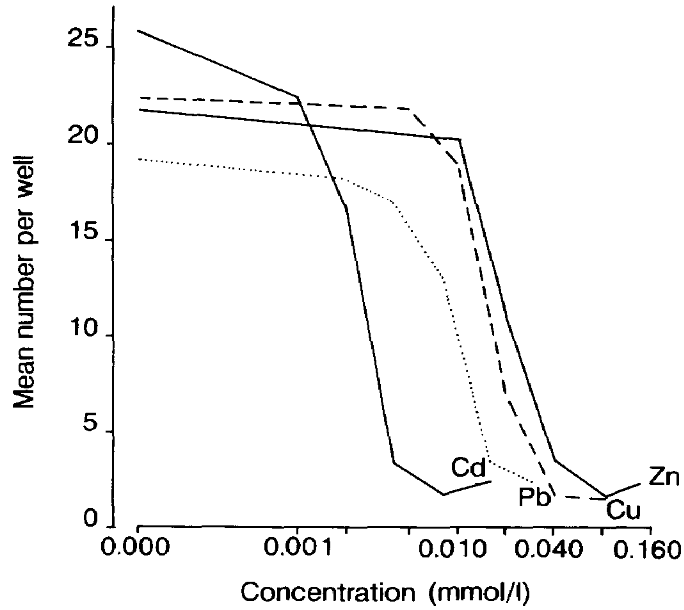

Figure 2. Dose effect curves for the effect of cadmium, lead, copper and zinc on the population growth of *C. cucullus* after 7 days. Concentrations of the metals are given in mmol/L.

values obtained for each metal (Table 3). Variation in toxicity data is relatively low for cadmium and zinc, but large for copper and lead. Some differences in threshold values obtained can be attributed to differences in experimental circumstances, species or endpoints, e.g. the high threshold values found by Ruthven & Cairns (1973) can be attributed to the fact that they measured survival after 10 minutes. Most of our results are in the same range as those found for the effects of heavy metals on the population growth of aquatic protozoa, although it appears that the threshold value for zinc is relatively low compared to those given by Yamaguchi (1973) and Dive & Leclerc (1977) (Table 3).

To make a comparison to soil organisms we have recalculated toxicity values of the effect of copper, zinc, cadmium and lead on the reproduction of the earthworm species *Eisenia foetida* Neuhauser et al., 1985) to concentrations in pore water. It is often assumed that toxicity of heavy metals for earthworms depend on the water soluble fraction (Van Gestel & Ma 1990). In order to recalculate the threshold values to threshold values in pore water we used K_d values given by Sheppard & Thibault (1990) for zinc, cadmium and lead in sandy and organic soils. The original threshold values and the values obtained after recalculation are given in table 4 and compared to the EC_{50} values estimated in this study. These values are in the same order of magnitude as our results with *C. cucullus* considering zinc and lead, our EC_{50} for cadmium is considerably lower than the values calculated through using the K_d values. The values used in Dutch legislation to indicate the necessity of sanitary measures are 2 to 20 fold lower than the values calculated in our toxicity tests (Table 4).

Table 4. EC_{50} values (μmol/L) calculated for *Colpoda cucullus* in this study compared to toxicity values of different metals for the earthworm *Eisenia foetida* in soil (μmol/kg) recalculated into threshold concentrations in pore water (μmol/L) and to threshold values for ground water as used in Dutch legislation.

	tox. value *E. foetida*	K_d	tox. value *E. foetida*	threshold value ground water	EC_{50} value *C. cucullus*
	μmol/kg		μmol/L	μmol/L	μmol/L
copper	10,100			3.14	15.3
zinc	10,100	200-1600	6.31-50.5	12.2	21.1
cadmium	16,300	80-800	20.3-204	0.09	2.17
lead	28,700	270-22,000	1.30-106	0.97	9.99

For the recalculation of metal concentrations in the soil to concentrations in pore water K_d values for sandy soil and organic soil were taken from Sheppard & Thibault (1990). Toxicity value in the soil was divided by K_d values to obtain toxicity values in the pore water. Threshold values for ground water are taken from Dutch regulation and indicate the necessity of sanitation measures (C-values)

Toxicity tests with protozoa have the advantage over tests with other soil inhabiting organisms in that they give insight into the amounts of metals which are immediately available due to solution in soil pore water and results can be obtained at relatively short term.

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