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Assessment of Pesticide Toxicity Using The Freshwater Amoeba *Rosculus ithacus* in vitro

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Free-living amoebae are widely distributed in the aquatic environment with increasing importance in hygiene, medical and ecological relationships to man. Only few data are available concerning the behavior of this group of protozoa toward the accidental chemical changes in the aquatic environment. The inhibitory of 8 pesticides (methomyl, dimethoate, malathion, dicuran, cypermethrin, carbendazim, fenitrothion and butachlor) was estimated using the isolated and purified freshwater amoebae *Rosculus ithacus*. Toxicity experiments were carried out using short-term static relative sensitivity toxicity tests. Test organisms (*Rosculus ithacus*) were separately exposed to different concentrations (0.001, 0.004, 0.007, 0.01, 0.1 and 1 mg/l) from each of the selected 8 pesticides for 1, 10, 24, 48, 72 and 96 h. The mean inhibitory (IC50) concentrations of tested pesticides for *Rosculus ithacus* ranged from 0.0020 to 0.0064 mg/l. Concerning the inhibitory effect, for *Rosculus ithacus* butachlor was highly significantly more than malathion > carbendazim > methomyl > dimethoate > cypermethrin > dicuran > fenitrothion.

Keywords: Toxicity, pesticides, freshwater amoebae, *Rosculus ithacus*.

INTRODUCTION

Pesticides are commonly encountered singly and as mixtures in drinking water, rivers, lakes and other aquatic bodies (Allsop et al., 1993). Surface water may be polluted by organic pesticides, either directly by application in water and runoff from the agricultural drift and/or indirectly from the discharge of industrial wastewater (Allen, 1995). The toxicity of pesticide-contaminated effluent depends on the amounts and types of the individual pesticide present. However, even for pure compounds the concentration-toxicity relationships are complex (Faust et al., 1994).

The contamination of the aquatic environment by organic pollutants and heavy metals is greatly concerned because of the presence of these residues in varying quantities in different compartments of the aquatic ecosystem (Leita et al., 1995).

The biological indicators of pollution are organisms being used more frequently for monitoring the aquatic contamination. The water hyacinth (Eichhornia) is a common aquatic plant successfully used as an indicator of heavy metal pollution where the uptake of heavy metals in this plant is stronger in roots than in the floating shoots (Gonzalez et al., 1989). Fishes are affected by adequate concentrations of chemical pollutants through the cumulative effect on their various organs. Also, Daphnia (a crustacean invertebrate) is a multicellular animal used for the estimation of unfavorable physico-chemical environmental variables (Isabelle et al., 2000).

Free-living amoebae (FLA) are unicellular inhabitants of the aquatic habitats and moist soil. They are common and important organisms of ecological communities within different substrates and biofilms. The role of the gymnamoebae as a more widely distributed protozoan group and their meaning for microbial communities of soil, for example in nutrient cyclisation, is presently still under discussion (Anderson, 2000).

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Naked lobose amoebae (Phylum Rhizopoda, Class Loboses, and Sub-class Gymnamoebia) are the most common type of amoebae found in soils, freshwater and marine water habitats (Rogerson and Patterson, 2000). The rapid rate of propagation, small size and sensitivity to minor surrounding environmental changes are the characteristics which merit the preliminary use of freshwater amoebae as a biological indicator. So the present work was directed towards the isolation, identification, purification and maintenance of one of the predominant strains of freshwater amoebae to be used as test organisms for the preliminary assessment of toxic effects of some pesticides.

MATERIALS AND METHODS

Preparation of the Freshwater Amoeba

Rosculus ithacus amoebae have been concentrated and isolated from the collected Nile water samples in a previous work by Al-Herrawy and Hikal (2005). In brief, the isolated freshwater amoebae were identified morphologically according to Page (1988). The identified strains of amoebae were cultured monoxenically on non-nutrient (NN) agar plates previously seeded with 100 µl *Escherichia coli* (*E. coli*) that were used as a source of food for the growth of free-living amoebae according to the method of Al-Herrawy et al. (2014).

Preparation of the Pesticides Used

Eight organic compounds in the form of 8 insecticides, acaricides, fungicides and herbicides are already used for the agricultural purposes in Egypt. Physical and chemical properties of the tested pesticides were presented in Table 1.

Methomyl, dimethoate, malathion, dicuran and butachlor were dissolved in distilled water. Cypermethrin was dissolved in chloroform. Carbendazim and fenitrothion were dissolved in acetone and dichloromethane, respectively. Stock solutions of the selected pesticides were prepared on the bases of the concentration of the active ingredient in the raw material. The prepared stock solutions were calculated and adjusted to give a final concentration of the toxicant equivalent to 1 mg/ml.

Toxicity Test Procedures

A short-term static relative sensitivity toxicity test was used in the present work according to duration, method of adding test solutions and purpose of the experiments (American Public Health Association, 1998). The isolated test organisms (freshwater amoebae) were separately exposed to duplicate containers of each experimental concentration used. A control sample including amoebae alone was presented with each experiment. From the control amoebae sample, the percentage of positivity (inhibition) was calculated. Stock solutions of the 8 selected toxicants were separately used for the preparation of desired different concentrations. Three basic preliminary concentrations (1, 0.1 and 0.01 mg/l) were prepared and tested for each toxicant. According to the obtained results from the three preliminary tested concentrations, other ascending (3, 5, 7 and 9 mg/l) or descending (0.007, 0.004 and 0.001 mg/l) concentrations were prepared and used.

Determination of median inhibitory concentrations (IC50): 103 amoebae isolate of *Rosculus ithacus* were equally distributed into Petri dishes each containing one of the previously prepared concentrations of each pesticide and incubated at 30ºC for different contact times (1, 10, 24, 48, 72 and 96 h). The mean of three replicates of each pesticide concentration was calculated. A Petri dish containing amoebae only with distilled water was used as a control. After 96 h of exposure, treated amoebae were examined microscopically to detect pesticide toxicity through loss of movement, rounding and encystations. When stained with 1% vital stains (e.g. trepan blue), living amoebae didn't take stains, while the dead ones stained with blue color. Inhibition values were estimated as a result of loss of movement, rounding and encystation, but not death of amoebae.

Statistical Analysis

The obtained data were subjected to analysis of variance (ANOVA) according to Snedcor and Cochran (1990). Least significant differences (LSD) were used to compare between the means of treatments according to Waller and Duncan (11969) at probabilities 5% and 1%. Data were statistically analyzed using "MSTATC" computer program V. 2.1 (1985). The IC50 values were calculated by "SPSS" computer program.

RESULTS

The morphological and physiological characterization of cultured freshwater amoebae revealed the isolation and purification of Rosculus ithacus amoebae.

The toxic effects of 8 different pesticides were tested towards the isolated amoebae strains using a wide range of pesticide concentrations (0.001, 0.004, 0.007, 0.01, 0.1 and 1 mg/l) and different contact times (1, 10, 24, 48, 72 and 96 h). The calculated values of median inhibitory concentrations (IC50) were recorded in Table 2.

Results in table 2 showed that butachlor was highly significantly more than malathion > carbendazim > methomyl > dimethoate > cypermethrin > dicuran > fenitrothion.

DISCUSSION

In recent years, there has been growing concern about the toxic effects of chemical substances in the aquatic environment. Many countries, including Egypt, are facing serious ecological and toxicological problems resulting from the discharge of complex effluents and toxic chemical substances into watersheds (Codina et al., 1993).

Toxicity tests are desirable in water quality evaluations because the chemical and physical tests alone aren't sufficient to assess potential effects on the aquatic biota (Grothe et al., 1996). Different species of aquatic organisms aren't equally susceptible to the same toxic substances. Also, organisms aren't equally susceptible throughout their life cycles. Even the previous exposure to toxicants can alter susceptibility. In addition, organisms of the same species can respond differently to the same level of a toxicant from time to time, even when all other variables are held constant.

The prime considerations in selecting test organisms are based on: a) sensitivity to the factors under consideration, b) geographical distribution, abundance and availability within a practical size range throughout the year, c) recreational, economic and ecological importance as well as relevance to the purpose of the study, d) a biotic requirements approaching the conditions normally found at the study site, e) availability of culture methods for rearing them in the laboratory with a knowledge of their physiological and nutritional requirements and f) general physical condition and freedom from parasites and disease (Nilsson, 1989; American Public Health Association, 1998).

Table 1. Physical and chemical properties of the tested organic compounds.

Compounds	Mol. wt.	Molecular formula	Mode of action	Chemical class
Methomyl	162.2	C5H10N2O2S	Systemic insecticide	Oxime carbamate
Dimethoate	229.3	C5H12NO3PS2	Systemic insecticide and acaricide	Organophosphorus
Malathion	330.3	C10H19O6PS2	Non-systemic insecticide and acaricide	Organophosphorus
Dicuran	212.7	C10H13ClN2O	Selective herbicide	Urea
Cypermethrin	416.3	C22H19Cl2NO3	Non-systemic insecticide	Pyrethroid
Carbendazim	191.2	C9H9N3O2	Systemic fungicide	Benzimidazole
Fenitrothion	277.2	C9H12NO5PS	Non-systemic insecticide	Organophosphorus
Butachlor	311.9	C17H26ClNO2	Selective systemic herbicide	Chloroacetanilide

Table 2. Median inhibitory concentrations (1 and 10 hr-IC50) of tested pesticides on Rosculus ithacus

Pesticides	IC 50 (mg/l)		
	Rosculus ithacus		
	1hr	10hr	Mean
Methomyl	0.006830	0.006030	0.006430
Dimethoate	0.007080	0.005337	0.006209
Malathion	0.005320	0.002950	0.004135
Dicuran	0.007090	0.004970	0.006030
Cypermethrin	0.006060	0.003050	0.004555
Carbendazim	0.004360	0.002300	0.003330
Fenitrothion	0.006890	0.004090	0.005490
Butachlor	0.003230	0.000790	0.002010
	Time (A)	**Pest. (B)**	**(AB)**
LSD 5%	0.00093	0.00186	NS
LSD 1%	0.00112	0.00224	NS

* NS = non-significant

The toxicological effects of various chemical substances were extensively studied in fish, although they may be time-consuming, labor-intensive and costly (Madoni, 2000). Other aquatic biota such as algae (Parent and Campbell, 1994), Daphnia (Havas, 1985), the stone fly Perla marginata (Guerold et al., 1995), ciliates (Nilsson, 1989; Miyoshi et al., 2003) and bacteria (Richards et al., 2002; Viamajala et al., 2004) are used in less extent as test organisms.

In the present work, Rosculus ithacus amoebae were used as test organisms for the first time (to our knowledge). On the other hand, almost all the previously used freshwater amoebae in other studies were potentially pathogenic, such as Acanthamoeba castellanii (Krawczynska et al., 1989; Buck and Rosenthal, 1996), Naegleria fowleri (Cassells et al., 1995) and Hartmannella vermiformis (Rohr et al., 2000). Again the previously mentioned freshwater amoebae were exposed to lethal doses of toxicants to kill them and not to explore their sensitivity and applicability as test organisms. To our knowledge only scarce data were published concerning the use of freshwater amoebae as bioindicators.

The aquatic environments receive direct or indirect pesticide inputs, inevitably exposing organisms to these pesticides. In addition to toxicity effects elicited by pesticides, micro-organisms also have the capability to accumulate, detoxify or metabolize pesticides to some extent (Ahlgren et al., 1990). Toxicity data involving micro-organisms and pesticides are limited. Most studies have focused on microbial degradation of pesticides rather than impacts on natural microbial populations (Lal and Lal, 1988). Moreover, studies of pesticide effects on soil microbes are far more common than studies of those in aquatic environments (Miles and Pfueffer, 1997). Pesticides can be classified, according to their mechanisms of action, into 4 major classes. For each class a number of related groups of pesticides are included. The first class includes organophosphates that act as inhibitors of the nervous system through acetyl cholinesterase. The second class includes organochlorines that act also as inhibitors of nervous system but through GABA receptors. Members of the third class, herbicides, provoke their toxic effects through photosynthesis and biosynthesis exhibit multiple inhibiting actions (phosphorylation, protein synthesis and biosynthesis) and respiratory system inhibition (mitochondrial ATPase) (De Lorenzo et al., 2001).

In the present work, the tested 8 pesticides were chosen so as to cover a wide range of chemical groups that were usually used for synthesis and production of pesticides. Such groups were organophosphorous (dimethoate, malathion and fenitrothion), oxime carbamate (methomyl), urea (dicuran), pyrethroid (cypermethrin), benzimidazole (carbendazim) and chloroacetanilide (butachlor). The mode of actions of these selected pesticides upon target organisms varied from systemic insecticide (methomyl and dimethoate) to non-systemic insecticide (malathion, cypermethrin and fenitrothion), selective herbicide (dicuran and butachlor) and systemic fungicide (carbendazim) (Tomlin, 1994). Moreover, the tested pesticides in the present work were manufactured and consequently applied for usage in agricultural purposes in Egypt. The published data concerning toxicity of these tested pesticides to non-target aquatic micro-organisms, especially protozoa, are scarce. Concerning inhibitory of Rosculus ithacus in the present study, it was shown that butachlor was highly significant more than malathion > carbendazim > methomyl > dimethoate > cypermethrin > dicuran > fenitrothion.

The response of the ciliated protozoan Paramecium caudatum to 6 organophosphorous insecticides were studied by (Rajini et al. 1989). Their result agreed with us in that malathion was the least toxic to protozoa. In another study by Lal and Lal (1988), malathion was shown to have a partial inhibitory effect on growth of the blue green algae Chlorogloea fritchii and growth was permanently suppressed at 200 mg/l.

In a study of the effect of pesticides on the populations of bacteria, actinomycetes, fungi and protozoa, Ekundayo (2003) found that agrosan (phenyl mercuric acetate) was the highest toxic one at 5 µg/g soil. It totally eliminated all protozoa, inhibited bacterial density from 4,600,000 to 22 cells/g and reduced the fungal population from 34,000 to 60 cells/g. In general, protozoa and fungi were susceptible to fungicides than bacteria and actinomycetes.

On studying the effects of biocides on soil protozoa, Foissner (1997) found that insecticides were usually more toxic than herbicides while fungicides had rather varied effects and most of them didn’t influence soil protozoa critically.

The effects of organophosphorous insecticide fenitrothion on 12 freshwater algae were studied by Kent and Weinberger (1991). They found that 10 mg/l fenitrothion significantly reduced growth rate in all tested species. In a study conducted by Mohapatra and Mohanty (1992) it was found that the 10-d LC50 for Chlorella vulgaris algae was as high as 51 mg/l dimethoate.

In recent years, aquatic toxicity testing has been applied to a variety of different regulatory and scientific purposes, including toxicity testing of municipal and industrial effluents as part of monitoring/permit compliance (Weber, 1993; Lewis et al., 1994; Grothe et al., 1996), the derivation of national and site-specific water quality criteria for individual chemicals (U.S. Environmental Protection Agency, 1994), product safety evaluations (U.S. Environmental Protection Agency, 1985), chemical persistence studies (Weber, 1993), testing sediments and studies included in toxicity reduction evaluation (TRE) programs to identify constituents causing toxicity in effluents (U.S. Environmental Protection Agency, 1991). These diverse applications have broadened the utility of toxicity testing and made more important the judicious interpretation of their results.

In conclusion, freshwater amoebae Rosculus ithacus were used as test organisms for the first time. Toxicity tests must be employed with a variety of test organisms to provide data that can be used to indicate toxicant concentrations likely to be harmful to freshwater ecosystems. Further investigations are needed to determine the interactions of different toxicants to each other and in the field.

CONCLUSION

Rosculus ithacus could be used as sensitive and convenient early warning bioindicators for the detection of toxicity of waters polluted with heavy metals and/or pesticides.

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