Bull. Environm. Contam. Toxicol. 25, 169-175 (1980)

Toxicity Tests of Aquatic Pollutants Using *Chilomonas paramecium* Ehrenberg (Flagellata) Populations

Robert A. Honig, Matthew J. McGinniss[1], Arthur L. Buikema, Jr., and John Cairns, Jr.

Biology Department and University Center for Environmental Studies, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061

Protozoans have several attributes which make them attractive for use in toxicity tests (CAIRNS 1974): they are easily handled and require relatively little space; they multiply rapidly, making observation of effects over several generations possible in a short time period; and they are in very close contact with their environment since they are unicellular and would be expected to respond to stresses with less of a time lag than other organisms. Furthermore, sublethal effects on metabolism and reproduction may result in lowered growth rates. Consequently, growth rate inhibition should be more sensitive in detecting effects of stress than the more common acute lethality tests (GRAY & VENTILLA 1973).

Protozoans have been used widely as medical and pharmcological tools (e.g., assays for antibiotics). However, studies of the effects of pollutants on protozoans have appeared only recently in the literature. The majority of these studies have utilized ciliates as test organisms (e.g., CARTER & CAMERON 1973, GRAY & VENTILLA 1973, SUDO & AIBA 1973). Other studies include research on amoeba (PRESCOTT et al. 1977) and protozoan communities (e.g., CAIRNS & DICKSON 1970, RUTHVEN & CAIRNS 1973, CAIRNS & PLAFKIN 1975). CAIRNS et al. (1972) and CAIRNS (1974) have reviewed the pollution ecology of protozoans.

The purpose of this work was to evaluate the suitability of the common achlorophyllous cryptomonad Chilomonas paramecium as a test organism for the assessment of toxicity. In addition, the influence of acclimation temperature on the sensitivity to pollutants was studied.

MATERIALS AND METHODS

Pure cultures of C. paramecium were developed from a mixed protozoan culture obtained from Carolina Biological Supply Co., Burlington, N.C. This mixed culture was inoculated into an organic medium developed for C. paramecium (PROVASOLI 1958) and allowed to grow for a week. The medium consisted of : tryptone (DIFCO)(0.1

[1]Present Address: Metcalf and Eddy, Inc.
50 Staniford Street
Boston, Massachusetts 02114

0007-4861/80/0025-0169 $01.40

w/v%), yeast extract (DIFCO) (0.2 w/v%), and sodium acetate (0.1 w/v%). Glass distilled water was added to make the required volume, and pH was adjusted to 6.3 ± 0.1. The medium was suction filtered through Whatman No. 2 filter paper and autoclaved at 15 psi for 15 min.

Pure cultures were obtained by making several sterile transfers into flasks containing the medium and 26 m/L each of streptomycin sulfate and penicillin-g (Sigma Chemical Co., St. Louis, Mo.). When C. paramecium was the only protozoan species evident and bacterial contamination was not apparent, the use of antibiotics was discontinued because low levels of bacterial contamination did not affect electronic cell counts (see below). Stock cultures were maintained in 100 mm x 16 mm glass culture tubes, with the polypropylene caps, containing 5 mL of media. The cultures were maintained under 1000-2000 lumens/m^2 light intensity, a 12L:12D photoperiod, and 10, 20, and 30 ± 2°C. Subcultures were made at 1-3 day intervals at 30°C, 3-5 day intervals at 20°C, and 5-10 day intervals at 10°C.

Toxicity tests with C. paramecium involved determination of log-phase growth rate constants over a range of toxicant concentrations at 10, 20, and 30°C. At each temperature, duplicate culture tubes were used for each toxicant concentration, and four replicates were used for controls. The toxicants were: chlorine ($Ca(OCl)_2 \cdot 3H_2O$), chromium ($K_2Cr_2O_7$), copper ($CuSO_4 \cdot 5H_2O$), zinc ($ZnSO_4 \cdot 7H_2O$), and phenol. For tests with chromium, copper, and zinc, 1 mL of metal solution (or distilled water for controls) was added to culture tubes containing 5 mL of medium. The culture tubes were then autoclaved and, after cooling to the appropriate temperature, inoculated with 0.2 mL of a well-mixed log-phase C. paramecium culture. Concentrations for chromium, copper, and zinc were determined in situ following the tests by atomic absorption (AMERICAN PUBLIC HEALTH ASSOCIATION [APHA] 1976); atomic absorption assays were not performed on the 10°C tests, so these concentrations were based on introduced amounts. A good agreement existed between introduced concentrations and concentrations based on atomic absorption analyses. Background levels of metals in control tubes ran up to 0.48 mg/L Cu and 0.174 mg/L Zn and were below the level of detection for chromium.

Autoclaving was not possible with chlorine and phenol due to their volatility. Therefore, 1 mL of these solutions was added to culture tubes using sterile techniques and were then inoculated with 0.2 mL of C. paramecium culture. All phenol and chlorine concentrations were based on introduced amounts. Phenol stock solutions were prepared on the day of use. A chlorine stock solution, pH adjusted to 7.2 to achieve complete solution, was

stored in the dark at 5-6°C. Chlorine concentrations in the stock solution were determined by amperometric titration. Due to the high protein and low ammonia concentrations in the medium, a large percentage of chlorine in the culture tubes was undoubtedly combined with amino groups (MURPHY et al. 1975).

Concentrations of C. paramecium were monitored with an electronic particle counter (Electrozone/Celloscope Model 112 LTH, Particle Data Inc., Elmhurst, Ill.) using a 95 μ orifice. The settings of the particle counter were adjusted to obtain empirical counts which correlated well with visual counts made with a Sedgwick-Rafter counting cell (FRANKEL 1965). The problem of bacterial interference with electronic cell counts was minimal. An aliquot was taken from each culture tube (after mixing with a vortex mixer) and diluted in an isotonic electrolyte solution. Three replicate counts were made for each tube. Cell concentrations were determined immediately after inoculation (time= t_0). Final cell counts (time= t_1) were made at 19-25 h for the 30°C assays, 44-48 h for the 20°C assays, and 98-163 h for the 10°C assays.

The average daily growth rate constant (K) was calculated using the following:

$$K = \frac{\ln(N_1/N_0)}{t_1 - t_0} \times 24$$

where N_1 = final cell concentration (at time= t_1),

N_0 = initial cell concentration (at time= t_0), and

$(t_1 - t_0)$ = elapsed time in h (GUILLARD 1973).

For cultures which exhibited growth rates near zero, electronic cell counts were supplemented with microscopic examination to determine if any cells were living.

RESULTS AND DISCUSSION

Ranges of average daily growth rate constants (day^{-1}) in the control tubes were 0.19-0.35 at 10°C, 1.05-1.45 at 20°C, and 1.99-2.99 at 30°C. Estimated median inhibitory levels (the concentration required to reduce growth rate to 50% of that of controls [SUDO & AIBA 1973]) for the five toxicants at three temperatures are presented in Table 1. These results indicate that zinc and chlorine toxicity to Chilomonas decreased with increasing temperature. No definite temperature effects on copper, chromium, and phenol toxicity were observed except possibly that the organism was more tolerant to phenol and chromium at 20°C. Of the metals studied, chromium was the most toxic.

TABLE 1. Estimated median inhibitory levels of selected compounds on Chilomonas paramecium populations. Concentrations are expressed as mg/L as the appropriate ions or compounds.

Compound	Temperature (°C)		
	10	20	30
Copper	23	>23	3 - 35
Chromium	1.1	3.0	1.8
Zinc	>5	16	40
Chlorine	0.3	0.3	0.75
Phenol	68	95	79

Very little literature for achlorophyllous protozoans exists to which these data can be compared (Table 2). In acute lethality tests (RUTHVEN & CAIRNS 1973), C. paramecium was more sensitive to copper than chromium and zinc (Table 2), whereas in our growth tests chromium was more toxic than zinc and copper. In both studies, phenol was less toxic than the metals. The growth rates of the three protozoans studied by SUDO & AIBA (1973) were more sensitive to copper than to chromium which was contrary to our findings (Table 2). Tetrahymena appears to be more sensitive to zinc than Chilomonas (CARTER & CAMERON 1973). However, our own preliminary observations indicated that T. pyriformis could tolerate 500 mg/L of copper, chromium, or zinc. Studies by KONOPIKOVA (1973) found that T. pyriformis was "highly resistant" to chromium and cyanide. Reasons for these apparent discrepancies in organism sensitivity may be: species differences, differences in culture and test medium, acclimation history, and length of the experiment.

The above method using C. paramecium in toxicity tests has several advantages which were discussed above. Additionally, tests with Chilomonas can be completed in less than the 96 h (except at 10°C) proposed by APHA (1976). The use of growth rate inhibition, which is very appropriate for organisms with such short generation times, should also provide a more sensitive assay than acute mortality tests.

A disadvantage of using protozoans to monitor toxicity concerns the concentrated growth medium required to obtain good laboratory growth and the medium constituents. Complexation by organic chelators in the medium may reduce the toxicity of metals, e.g. of chromium, copper, and zinc. STEEMAN-NIELSEN & KAMP-NIELSEN (1970) noted in studies with the alga Chlorella that for every

TABLE 2. Results of selected aquatic toxicology studies using protozoans.

Species Temp.	Toxicant	Conc. (mg/L)	Effect	Reference
Tetrahymena pyriformis (23-25°C)	Zn^{++}	2.70	Lethal threshold conc.	CARTER & CAMERON 1973
Chilomonas paramecium (19-25°C)	Cr^{+6} Cu^{++} Zn^{++} Phenol	1000 0.056 >10 1500	Lowest conc. at which all organisms died within 10 min	RUTHVEN & CAIRNS 1973
Chilomonas paramecium (19-25°C)	Cr^{+6} Cu^{++} Zn^{++} Phenol	>18 0.024 3.2 560	Highest conc. at which some organisms survived after 3 h	RUTHVEN & CAIRNS 1973
Vorticella microstoma (20°C)	Cr^{+6} Cu^{++}	0.53 0.25	Median inhibitory level	SUDO & AIBA 1973
Colpidium campylum (20°C)	Cr^{+6} Cu^{++}	12.9 0.32	Median inhibitory level	SUDO & AIBA 1973
Opercularia sp. (20°C)	Cr^{+6} Cu^{++}	20.2 0.27	Median inhibitory level	SUDO & AIBA 1973
Cristigera sp. (marine, 16°C)	Zn^{++}	0.125	8.3% growth rate reduction	GRAY & VENTILLA 1973

mg of protein hydrolyzate (e.g., tryptone and yeast extract) in the medium approximately 0.01 mg of copper may be bound; they observed that this binding was most pronounced during the first 24 h. SUNDA & LEWIS (1978) have also noted that copper toxicity to the unicellular green alga Monochrysis lutheri decreased because of binding by natural organic ligands. Various researchers have attempted to circumvent this problem by using dilute media (e.g., HANNAN & PATOUILLET 1972, GRAY & VENTILLA 1973). This approach may be useful if the organism is not dependent solely on soluble foods as a nutritional source. Further tests with protozoans would best be carried out in a dilute assay medium capable of supporting log-phase growth for several days yet simulate the organic content of most natural surface waters. In addition, since autoclaving of dosed medium may affect the availability of metals by causing precipitation or enhancing binding with components of the medium, dosing could be carried out after the medium is sterilized. Also, investigators should note that high cell concentrations alone can mediate toxic effects (STEEMAN-NIELSEN et al. 1969). One must also be aware that protozoans as a group vary greatly in their sensitivity to toxic substances, and that considerable differences may exist within a species in its response to toxicants (CAIRNS 1974). For example, our preliminary observations of the ciliate Tetrahymena pyriformis (also in a concentrated organic medium) at 20-25°C indicated no growth rate reduction at concentrations of 500 mg/L Cr^{+6}, 500 mg/L Cu^{++}, and 500 mg/L Zn^{++}. This can be compared to marked growth rate reduction of Chilomonas paramecium at less than 50 mg/L for each of these metals.

ACKNOWLEDGMENTS

We would like to thank Dr. William H. Yongue, Jr. of the Biology Department of Virginia Polytechnic Institute and State University for his assistance in the identification and maintenance of the protozoan cultures, Nancy Cromer for conducting the atomic absorption analyses, and Michael W. Newcomb for his assistance in all phases of the study. Portions of this study were supported by OWRT Grant # B-084-Va.

REFERENCES

AMERICAN PUBLIC HEALTH ASSOCIATION: Standard Methods for the Examination of Water and Wastewater. 14 ed. Washington, D. C. 1976.

CAIRNS, J., Jr.: Protozoans (Protozoa). Pages 1-28 in C. W. Hart, Jr. and S. L. H. Fuller, eds., Pollution Ecology of Freshwater Invertebrates. Academic Press, Inc. 1974.

CAIRNS, J., Jr., and K. L. DICKSON: Trans. Kans. Acad. Sci. 73, 1 (1970).
CAIRNS, J., Jr., and J. L. PLAFKIN: Arch. Protistenk. 117, 47 (1975).
CAIRNS, J., Jr., G. R. LANZA, and B. C. PARKER: Proc. Acad. Nat. Sci. Phila. 124, 79 (1972).
CARTER, J. W., and I. L. CAMERON: Water Res. 7, 951 (1973).
FRANKEL, J.: J. Exp. Zool. 159, 113 (1965).
GRAY, J. S., and R. J. VENTILLA: Ambio 2, 118 (1973)
GUILLARD, R. R. L.: Division rates. Pages 289-311 in J. R. Stein, ed. Handbook of Phycological Methods -- Culture Methods and Growth Measurements, Cambridge University Press 1973.
HANNAN, P. J., and C. PATOUILLET: Biotechnol. Bioeng. 14, 93 (1972).
KONOPIKOVA, L.: Experiment to test the select waste waters on protozoa. Acta. Univ. Carol., Biol., 3-4, 125-133 (1973). Microbiology Abstracts, Section C 5(1):5C791 (Jan. 1976).
MURPHY, K. L., R. ZALOUM, and D. FULFORD: Water Res. 9, 389 (1975).
PRESCOTT, L. M., M. K. KUBOVEC, and D. TRYGGESTAD: Bull. Environ. Contam. Toxicol. 18, 29 (1977).
PROVASOLI, L.: J. Protozool. 5, 1 (1958).
RUTHVEN, J. A., and J. CAIRNS, Jr.: J. Protozool. 70, 127 (1973).
STEEMAN-NIELSEN, E., L. KAMP-NIELSEN, and S. WIUM-ANDERSEN: Physiol. Plant. 22, 1121 (1969).
STEEMAN-NIELSEN, E., L. KAMP-NIELSEN: Physiol. Plant, 23, 828 (1970).
SUDO, R., and S. AIBA: Wat. Res. 7, 1301 (1973).
SUNDA, W. G., and J. A. M. LEWIS: Limnol. Oceanogr. 23, 870 (1978).