Evaluation of a Cell Culture Assay for Determination of Water Quality of Oil-Refinery Effluents

D. Richardson*[1], T. C. Dorris*[2], S. Burks*,
R. H. Browne**, M. L. Higgins***[3], and F. R. Leach***[4]
*Departments of Biochemistry***, Statistics**, and Zoology**
Oklahoma State University
Stillwater, Oklahoma 74074*

Assessment of the quality of water returned to the environment after industrial use is required to establish that no pollutants remain which would be injurious to various biological forms. One obvious means of establishing this point is to use bioassays with the organism involved and develop model systems for those which cannot be directly tested (e.g., humans). Bacteria, aquatic invertebrates, algae, and fish have all been used as the biological test organism.

The use of bacteria as indicators of water quality has been reviewed by BOTT (1973). CARTER and CAMERON (1973) described the determination of the toxicities of five heavy metal compounds on the survival of the ciliated protozoan Tetrahymena pyriformis. These organisms were in the maximum stationary growth phase with no reproduction occurring. ANDERSON (1948) used Daphnia magna immobilization as an indicator for toxicity thresholds of chlorides. The sensitivity of Daphnia was increased during the molting state of their life cycle, possibly due to increased permeability of the nerve covering or to greater body volume. The Daphnia were more sensitive than fish to the metal salts.

Fathead minnows (Pimephales primelus Raf.) were used by GRAHAM and DORRIS (1968) for long-term toxicity determination on wastes from four oil refineries. They found chronic or cumulative toxicity during 16-32 days of exposure. SPRAGUE (1973) described a four-day lethal concentration of lethal threshold concentration determination using rainbow trout.

In the bioassays described above, individual variations inherent in intact animals such as age, sex, reproductive condition, previous environment, and life cycle substantially increased experimental variability (LENNON, 1967; MACEK and SANDERS, 1970). The number of individual animals used also limits their usefulness.

---

[1]Taken in part from D. R.'s Ph.D. thesis submitted to the faculty of the Graduate College. D. R. was an E. P. A. Predoctoral Fellow.

[2]Send reprint request to: Dr. T. C. Dorris
School of Biological Science
Oklahoma State University
Stillwater, Oklahoma 74074

[3]Current address: Dept. Biol., Texas Women's Univ., Denton, Tx. 76204.

[4]F. R. L. was a research career awardee CA-K3-6487 from NCI.

Bulletin of Environmental Contamination & Toxicology,
Vol. 18, No. 6 © 1977 by Springer-Verlag New York, Inc.

PUCK (1972) has reviewed the use of mammalian cells in culture for genetic and biochemical studies in vitro. The cells derived from various organisms can be grown using cell culture procedures (KRUSE and PATTERSON, 1973) in sufficient numbers to add statistical reliability, they have about a 20-hour cell cycle during which the DNA is replicated, and they can be handled with almost the same ease as microorganisms.

Several experiments have theoretically studied water pollution using cells in culture, which involved adding of reference compounds to water rather than using polluted water or a major fraction derived therefrom in compounding the medium. While adding a reference compound establishes theoretical applicability, actual experimental evidence using polluted water is required to establish the applicability of the method. RACHLIN and PERLMATTER (1968) used fish cells in culture to study the toxic effects of Zn^{2+}. When they compared the toxicity of Zn^{2+} for the fathead minnow and cells in culture derived from the fathead minnow, the cells in culture were 10 times more sensitive. Single-cell plating of bovine ovary cells were used by MALCOLM, PRINGLE, and FISHER (1973) to establish the usefulness of cell cultures for detecting toxicity of Cu, Zn, Se, Cd, Sr, rotenone, and nitrilotriacetate. L cells cultivated in suspension were used by LI, et al. (1970) to demonstrate the toxicity of elemental phosphorus.

The present paper compares Daphnia and cell culture bioassays of the toxicity of a reference compound, m-cresol, and then applies those procedures to complex fractions derived from oil-refinery effluents. The cell culture assay is established as a sensitive, reliable, and reproducible method for determination of the toxicity of oil-refinery effluents.

MATERIALS AND METHODS

Cell Culture

L-M strain cells (CCL 1.2), derived by KUCHLER and MERCHANT (1956) from the NCTC clone 939 mouse fibroblast line, were purchased from the American Type Culture Collection Cell Repository. Monolayer cultures were grown in milk dilution bottles (MDB) using medium McCoy's 5a (modified) supplemented with 10% calf serum (Microbiological Associates), 100 units/ml potassium penicillin G (Squibb), and 50 µg/ml dihydrostreptomycin sulfate (Pfizer). Suspension cultures were grown in 20 ml of the same medium with 5% calf serum in 125 ml Erlenmeyer flasks on a New Brunswick G-10 gyrotory shaker at 50 oscillations/min in a 37°C room.

The McCoy's 5a medium (modified) was purchased as a dry powder (Grand Island Biological Company). In the suspension culture experiments an equal volume of water of effluent or metacresol solutions was added to the double strength medium. After the addition of the effluent to the medium the pH was adjusted with either 1N HCl or 1N NaOH, and before the addition of the cells the pH was further adjusted to between 7.2 and 7.4 with CO_2.

Stock cells growing as monolayers in MDB were harvested using 0.1% trypsin and 0.02% EDTA in a balanced salt solution (8 g NaCl, 0.2 g KCl, 0.05 g $NaH_2PO_4 \cdot H_2O$, 1 g $NaHCO_3$, 1 g glucose, and 0.02 g

phenol red/liter of water). The action of the trypsin was stopped by addition of serum-containing medium. The cells were washed in phosphate buffered saline (SZYBALSKA and SZYBALSKI, 1962), suspended in fresh medium, and inoculated at 2 x 10^5 cells/ml into 20 ml of medium in 125 ml Erlenmeyer flasks. Growth was determined by removing 0.2 ml samples at varying times and each sample was counted twice. Cell counts were made using a Coulter Model B Cell Counter.

Chemicals - Meta-cresol was obtained from J. T. Baker and diluted with glass-distilled water.

Oil-Refinery Effluents - Process effluents were obtained from three Oklahoma petroleum refineries. The first 75 ml fraction was collected from flash evaporation (110-120°C oil bath) of the total effluent and sterilized by passage through 0.45 μm Millipore filter.

Daphnia Test - Young Daphnia, <24 hr. old, were isolated from a laboratory breeding population using an eyedropper. For each test four Daphnia were placed in a 50 ml beaker containing dechlorinated tap water and the indicated amount of m-cresol or effluent fraction. The number of surviving Daphnia was determined during a 48-hour period.

Statistical Analysis - Standard deviations for repeated experiments were determined as a measure of sensitivity and reproducibility using the 95% confidence interval to determine significant differences. The binomial test with normal approximation was used to compare two proportions. Curves were compared using the nonparametric Wilcoxon test for goodness of fit. Variance within and between experiments was analyzed by the F-test (REMINGTON and SCHORK, 1970).

Expression of Results - The growth in the experimental situations was compared to growth in the absence of inhibitor and expressed as a percentage of the control value.

RESULTS

Characterization of the Cell Culture Assay Using m-Cresol

The first experiments were designed to establish the sensitivity and reliability of the cell culture assay using m-cresol as the inhibitor. Figure 1A shows the effect of varying concentrations of m-cresol expressed as parts per million on the growth of L-M cells in suspension culture. No effect was observed with 2 ppm, while a definite reduction in growth rate and final population level reached was observed with 5 ppm m-cresol. Further increases in the m-cresol concentration reduced both the growth rate and cell yield. Figure 1B shows the dose response for m-cresol of both relative growth rate and cell yield. Good linearity is obtained using either measure of inhibition.

Table I compares the relative growth rates observed with various concentrations of m-cresol with the relative cell yield measured at 66 hours. There was no significant difference between the two methods for determining the inhibition produced by m-cresol. The end point determination at 66 hours would be easier but less rapid; furthermore, the cells growing in suspension would

require scraping from the glass at the liquid-atmosphere interface and mixing (at least daily) to obtain reliable data.

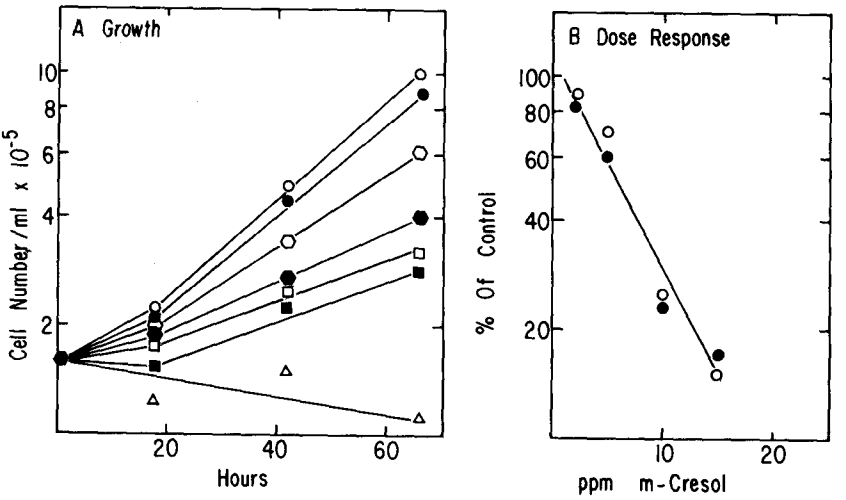

Figure 1. Effect of varying concentrations of m-cresol on growth of L-M cells in suspension. A. Growth curves. Replicate cultures in duplicate of L-M cells were established in 125 ml Erlenmeyer flask with the following concentrations of m-cresol in ppm: 0, none; ●, 2; ⬡, 5; ⬢, 7.5; □, 10; ■, 15; and Δ, 20. Growth was determined using the Coulter Counter as described in the Materials and Methods section. B. Dose Response. A logarithmic plot of relative growth rate, 0, and relative cell yield at 66 hours, ●, against m-cresol concentration.

TABLE I
Comparison of Growth Rate and Cell Yield Determination for L-M Cells in Suspension Culture

Average of four independent experiments with duplicate flasks. At 66 hours, 100% growth was equivalent to 8.6-9.8 x 10^5 cells/ml. Negative numbers represent a decrease from the inoculum indicating cell death.

Concentration, m-cresol ppm	Relative Growth Rate	Relative Cell Yield
0	100	100
2	87	82
5	72	58
10	25	23
20	-12	-6
50	-22	-12

Characterization of the Daphnia Assay Using m-Cresol

Figure 2 (parts A and B) show two experiments measuring Daphnia survival with varying concentration of m-cresol. Little effect was observed with concentrations below 20 ppm of m-cresol. In one experiment (Figure 2B) several control Daphnia died and the number of survivors with 20 ppm m-cresol was greater than the control culture. Control Daphnia growing for 48 hours under the conditions of these experiments had 15% of the population dead. In part C of Figure 2 the dose response obtained at 12 hours is shown. The wide variation makes the Daphnia bioassay unreliable except for approximation of total killing points.

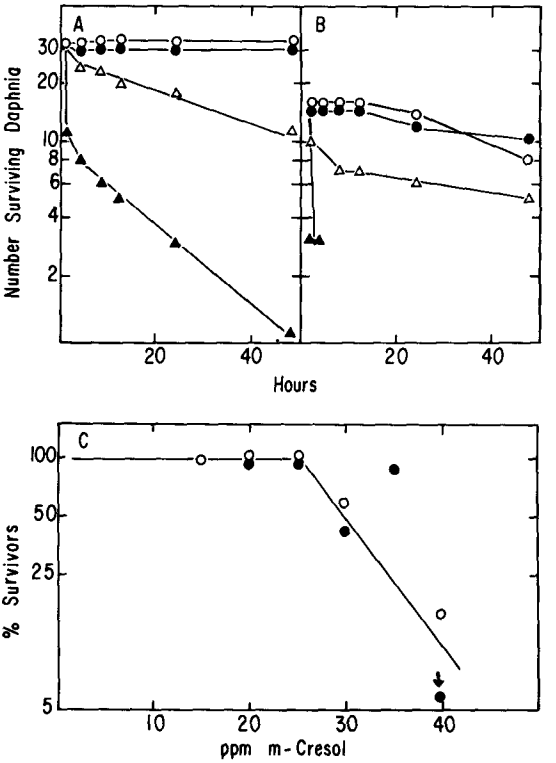

Figure 2. Dose response of Daphnia to m-cresol. Experiment A. Daphnia, 32 per condition, were incubated 4 Daphnia per 10 ml sample with no m-cresol, 0; 20 ppm, ●; 30 ppm, Δ; and 40 ppm, ▲ and the number of survivors determined at the indicated time intervals. Experiment B. Daphnia, 16 per condition, were incubated 4 Daphnia per 10 ml sample as described in part A with the same symbols indicating the m-cresol concentrations. C. Dose response curve from experiment A, 0, and experiment B, ●. Data not plotted in parts A and B is included which involves intermediate concentrations not done in both experiments. This ↓ is to indicate that there were no survivors.

Application of the Cell Culture Assay to Effluents

Figure 3A shows typical growth curves for L-M cells obtained using varying concentrations of flash evaporates from an oil-refinery effluent. The experiment represents the average of duplicate flasks for each point. There was no significant inhibition when the flash evaporate was used at a concentration of 11%, while with both 22% and 45% there was significant inhibition. Part B of the figure shows the dose response curve for this

effluent.

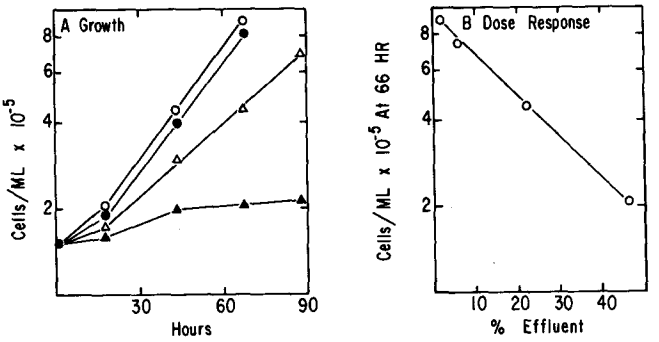

Figure 3. Cell growth and dose response curves with a flash evaporate from an oil-refining effluent. A. Effect of varying concentrations 0, 0; ●, 11%; Δ, 22%; and ▲, 45% of a flash evaporate on the growth of L-M cells grown in suspension culture. Duplicate flasks were counted for each point. B. Dose Response. The cell titer at 66 hours is plotted on a logarithmic scale against the effluent concentration as the percent of water replaced in the medium.

The reproducibility of the flash evaporation procedure and the cell assay was evaluated by flash evaporating samples of different refinery effluents on alternate days and determining the inhibition produced on duplicate assays. Table II shows typical results on a number of samples. Terminal count variation between experiments was 13% and within experiments was 8%. There was an average difference of 4.5% between replicate assays.

Comparison with the Daphnia Test

The variability of the Daphnia test was evaluated with the same concentrations of the various effluents as is also seen in Table II. Variation between experiments was 100%; variation within experiments was 50%.

DISCUSSION

Water, being the solvent upon which life on the earth is based and a participant in many of the enzymatic reactions required for life, can also serve as a vehicle for bringing inhibitory substances to their sites of action. These inhibitory substances may be residues from prior uses of the water; hence their detection would be important in protecting biological species from a deleterious exposure. With the almost endless variety of biological species available many different bioassays could be developed and each could be superior in a sharply defined set of circumstances. For a bioassay of water quality to be widely useful it must be 1) sensitive, 2) rapid, 3) based on reproduction rather than survival, and 4) statistically significant. The results presented in this paper suggest that a cell culture assay meets these goals.

TABLE II
Response of L-M Cells in Suspension and *Daphnia* to Oil-Refinery Effluent Flash Evaporates

Replicate assay values on different days are separated by a comma. The % growth is the average of duplicate flasks calculated as described in the Materials and Methods section (control 8.6-9.8 x 10^5 cells/ml). The flask evaporate concentration is 45%. The *Daphnia* experiments were performed as described in the Materials and Methods section.

Sample #	L-M Cells % Growth @66 hours	*Daphnia* Number Surviving				
		Hours				
		1	4	8	12	24
Control	100,000	4,4	4,4	4,4	4,4	4,4
2	86,86	4,3	2,1	2,1	0,0	0,0
3	14,18	1,0	0,0	0,0	0,0	0,0
4	37,30	0,1	0,0	0,0	0,0	0,0
6	31,28	4,4	4,4	3,1	0,0	0,0
8	45,44	4,4	2,4	2,0	0,0	0,0
10	74,69	4,4	4,4	4,4	4,4	0,4

The cell culture assay is more sensitive than the commonly used *Daphnia* bioassay. Figure 1B shows that the threshold toxicity of m-cresol in the cell culture assay is 2 ppm, which is 15-18 times more sensitive than *Daphnia* with a threshold level of 35 ppm (Figure 2C). Thus a concentration of m-cresol of an order of magnitude lower can be detected using L-M cells. Figure 1A shows that sufficient inhibition occurs within a 40-hour incubation period to allow determination time which constitutes 2 generations for the L-M cells. TOLMACH and ARNZEN (1975) described an instrument for the automated performance of kinetic and toxicity experiments with cultured cells which would be adaptable to the procedures described in this paper. For widest applicability the potential for automation of any bioassay should exist.

The faithful copying of genetic information in the process of replication and its subsequent passage to the progeny requires a higher fidelity of operation than acts of metabolism required for life which are detectable in acute exposures to inhibitory compounds. Therefore, the bioassay should depend upon the accomplishment of the more critical process of genetic material replication and functioning in addition to measuring the fitness of physiological functions. The cell culture assay spans two to three generations for the cells and thus certainly measures genetic replication and expression. Since whole animals have a greater generation time, a marked advantage is given to cell culture approaches. Between 3 and 15 x 10^6 individual cells are involved in each experimental determination using cell culture which greatly reduced the variability inherent in fish and *Daphnia* bioassays where only 20 or so individuals are involved. While any model system has the disadvantages of not being the real thing, determination of water quality using humans would be excluded

because of moral and temporal considerations.

The practical application of an *in vitro* cell bioassay for the determination of water quality is presented using actual oil-refinery effluents as the source of polluted water. The procedure developed should be widely applicable to water quality evaluations.

ACKNOWLEDGMENTS

This research was supported in part by Oklahoma Agricultural Experiment Station Project 1534, Oil Refiners Waste Control Council, and USDI Office of Water Resources Research. This publication is article J-3207 of the Oklahoma Agricultural Experiment Station.

REFERENCES

ANDERSON, B. G.: Am. Fisheries Soc. 78, 96 (1948).

CARTER, J. W. and I. L. CAMERON: Water Research 7, 951 (1973).

GRAHAM, R. J. and T. C. DORRIS: Water Research 2, 643 (1968).

KRUSE, P. F., JR. and M. K. PATTERSON, JR., eds.: Tissue culture methods and applications. New York: Academic Press 1973.

KUCHLER, R. J. and D. J. MERCHANT: Proc. Soc. Exptl. Biol. Med. 92, 803 (1956).

LENNON, R. E.: Prog. Fish Cult. 23, 129 (1967).

LI, M. F., G. S. TRAXLER, and L. M. LANGILLE: Can. J. Zool. 48, 133 (1970).

MACEK, K. and H. O. SANDERS: Trans. Am. Fish. Soc. 99, 89 (1970).

MALCOLM, A. R., B. H. PRINGLE, and H. W. FISHER: Chemical toxicity studies with cultured mammalian cells. In Bioassay Techniques and Environmental Chemistry. Ann Arbor: Ann Arbor Science 1973.

PUCK, T. T.: The Mammalian Cell as a Microorganism. San Francisco: Holden-Day 1972.

RACHLIN, J. W. and A. PERLMATTER: Water Research 2, 409 (1968).

REMINGTON, R. and M. SCHORK: Statistics with Application to the Biological and Health Sciences. Englewood Cliffs, New Jersey: Prentice Hall 1970.

SPRAGUE, J. B.: The ABC's of pollutant bioassay using fish. In Biological Methods for the Assessment of Water Quality. (Edited by CAIRNS, J. and K. L. DICKSON) ASTM #528, 6 (1973).

SZYBALSKA, E. H. and W. SYZBALSKI: Proc. Nat. Acad. Sci. U.S.A. 48, 2026 (1962).