EVALUATION AND APPLICATION OF A CELL CULTURE SYSTEM TO DETECT TOXICITY IN OIL-REFINERY EFFLUENTS

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CHAPTER I

INTRODUCTION

The objectives of this research are to evaluate cell culture as a technique that can be routinely used to screen effluent fractions for toxicity at the cellular level, and to investigate the efficacy of this technique in determination of those fractions of treated oil refinery effluents that contain toxicity so that the constituents may be isolated and identified.

Very little is known about the organic components of the effluents and the biological effects of refinery effluents have not been sufficiently investigated. Tissues of fish have higher concentrations of toxic material than the surrounding environment (Graham and Dorris, 1968), an indication of toxin accumulation within the fish. Since aquatic organisms are continuously exposed to toxic material in effluents, they may accumulate these compounds until lethal levels are reached. Therefore, the question of toxicity should be approached by biological as well as strictly chemical techniques.

The identity of trace toxic compounds in oil refinery effluents is being sought by sequential solvent extractions, analytical series of columns, thin layer, and gas chromatography and mass or infrared spectrometry (Burks, 1969).

Daphnia (Burks, 1970 unpublished data) and fish (Gould, 1962; Ward, 1962; Douglas, 1963; Bunting, 1963) have been used to bioassay

oil-refinery effluents. Often it is impossible to determine toxicity using the intact organism since only a small volume of test material remains after an effluent has been extracted and separated into fractions. A more sensitive test requiring smaller amounts of test material is needed.

Toxicity is not a universal constant, but must be defined in terms of a particular system. Cell culture is a system which allows many variables to be controlled (e.g., pH, temperature, salts, etc.). Toxicants can be detected more rapidly and at a lower concentration with cell culture than is possible with the intact organism (Rounds and Pomerat, 1962; Rachlin and Perlmutter, 1968; Barille and Hardegree, 1970). Toxicity is expressed in the progressive sequence of slight injury, inhibition, and death. Cell culture should be more sensitive to toxicity than the intact organism since inhibition rather than death is quantitatively measured as the index of effect.

Cell culture also avoids the individual variation inherent in intact animals because of age, sex, reproductive condition, previous environment, and other spontaneous effects that occur throughout the life cycle of the intact organism (Lennon, 1967; Maeck and Sanders, 1970). Fish test results often cannot be compared (Lennon, 1967), because of unexplained differences in species susceptibility (Henderson, 1960; Lennon, 1967) and the inability to evaluate all constituents of the aquatic system except the pollutant tested (McKee and Wolf, 1963). Water may vary in hardness, alkalinity or acidity, dissolved oxygen, CO_2, chlorine, ammonia, and other confounding constituents such as heavy metals that not only severely affect the toxicity of the test material (Standard Methods, 1971), but also prevent routine duplication

of experimental results by independent laboratories. The cell culture test system is based on a reproducible medium which may be employed by other laboratories in independent bioassays.

The toxic effects on specific cell types or organs can be evaluated without interference from other organ systems. Rachlin and Perlmutter (1968) suggested that cell culture be routinely used to set criteria for enforcement of water quality standards using the concept of "biologically effective concentrations" rather than "biologically safe concentrations".

Effects in fish and Daphnia studies are usually measured only in a dichotomous way (i.e., dead or not dead) (Anderson, 1944; Henderson, 1960; Graham and Dorris, 1968). One must study intermediate effects, investigate mechanisms, and quantify results to gain a knowledge of what is happening. Pace (1961) states,

> ... it is only through organized study at the cellular level that we may hope to gain fundamental knowledge concerning the effects of pollutants, singly or in combination. ... we now have available numerous cloned cell types which are suitable for such studies.

Cell cultures can evaluate toxicity (Haddow and Robinson, 1939; Siegel and Cailleau, 1956; Borne, 1964; Diamond, 1965; Ambrose et al., 1967; Higgins et al., 1969; Dixon and Dalmadge, 1969; Barile and Hardegree, 1970). It was not until the 1960's that the technique was used to study environmental pollutants. At first it was used to study the effects of insecticides (Gablinks and Friedman, 1965; Gablinks, 1965; Wilson and Walker, 1966; Gablinks et al., 1967; Grace and Mitsuhashi, 1971) and air pollutants. Studies of the effects of air pollutants have utilized both the component (Pace et al., 1961; Rounds, 1966; Pace et al., 1969) and the holistic (Rounds and Pomerat, 1962;

Leuchtenberger and Leuchtenberger, 1969) approaches. Basic cell culture techniques are still being developed to evaluate gas-phase pollutants (Baker and Tumasonia, 1971). Until 1968, cell cultures had not been used to study water pollutants. The major groups publishing in this field after 1968 are those of Rachlin (Rachlin and Perlmutter, 1968; Rachlin, Wolfe, and Perlmutter, 1969), Fisher (Fisher, 1969; Malcolm, Pringle, and Fisher, 1973), Li (Li and Jordan, 1969; Li, Traxler, and Langille, 1970; Li and Traxler, 1972) and Metcalfe (Metcalfe, 1971).

To date, all cell culture evaluations of water pollutants have been from the component approach. Oil refinery effluents and many other industrial wastes are so complex that it would be impossible to approach existing effluents with an additive approach. Since it is the interacting whole that affects the organisms in the aquatic environment, it seems practicable to begin with the whole effluent or large fractions. This approach would allow consideration of synergistic effects, should they occur.

Cell culture may be useful in the isolation and identification of toxic components. Cell culture provides a screening device to determine whether a fraction of the effluent containing a relatively small number of compounds is toxic at the cellular level. If toxic, the fraction can be subfractionated further to isolate and identify the toxic components. Cells, to some extent, may concentrate the toxic components and increase the sensitivity of the test and the amount of material available for chemical analysis.

Once the toxic components are identified, cell culture may be used in determining the site of the effects. Toxic components or combinations of components may differentially affect organs. Primary cultures

of different tissues might be used to determine the target organs. The mechanisms by which the deleterious effects are brought about must eventually be approached both cytochemically and physiologically.

CHAPTER II

MATERIALS AND METHODS

Materials

Cells

L cells were derived from normal subcutaneous areolar and adipose tissue taken from a male mouse in 1940 by Earle (1943). They were initially used to study carcinogenesis and are still widely used. L cells are often used in toxicology studies (Siegel and Caillean, 1956; Pace et al., 1961; Nat. Cancer Inst. Monograph, 1962; Leach et al., 1964; Gablinks, 1967; Higgins et al., 1969). The NCTC clone 929 was derived from a clone of Strain L cells in 1948. L-M cells, which were used in this study, are a fibroblastic cell line derived from NCTC clone 929 in 1952 (American Type Culture Collection, 1972).

Medium

McCoy's 5A medium without antibiotics (Table I) from Microbiological Associates, Maryland, supplemented with calf serum from Grand Island Biological Company was used exclusively. The powdered medium was made double strength (2X) and frozen at -20 C. The pH of the thawed medium was adjusted with CO_2 to 7.3, 1.1 g/liter sodium bicarbonate, antibiotics, and the proper amount of calf serum added. The antibiotics were potassium penicillin (50 mg/liter) and streptomycin sulfate (62.5 mg/liter).

TABLE I

McCOY 5A MEDIUM WITHOUT SERUM OR ANTIBIOTICS

Components	mg./liter	Components	mg./liter
Amino Acids		Vitamins	
L-Alanine	13.36	P-Aminobenzoic Acid	1.0
L-Arginine HCl	42.14	Ascorbic Acid	0.5
L-Asparagine•H_2O	45.03	D-Biotin	0.2
L-Aspartic Acid	19.97	D-Ca-Panthothenate	0.2
L-Cysteine	24.24	Choline Chloride	5.0
L-Glutamic Acid	22.07	Folic Acid	10.0
L-Glutamine	219.15	i-Inositol	36.0
L-Glycine	7.51	Nicotinamide	0.5
L-Histidine HCl•H_2O	20.96	Nicotinic Acid	0.5
L-Tryptophan	30.6	Pyridoxal HCl	0.5
L-Phenylalanine	16.52	Pyridoxine HCl	0.5
L-Tyrosine	18.12	Riboflavin	0.2
L-Lysine HCl	36.54	Thiamine•HCl	0.2
L-Methionine	14.92	Vitamin B_{12}	2.0
L-Isoleucine	39.36	Other Components	
L-Leucine	39.36		
L-Valine	17.57	Glutathione	0.5
L-Threonine	17.87	Bacto-Peptone	600
L-Serine	26.28	Phenol Red	10
L-Hydroxyproline	19.67	Dextrose	3,000
L-Proline	17.27	Inorganic Salts	
		$CaCl_2$	100
		KCl	400
		$MgSO_4 \cdot 7H_2O$	200
		NaCl	6,460
		$NaHCO_3$	2,200
		$NaH_2PO_4 \cdot H_2O$	580

Effluents

Process effluents were received from Oklahoma petroleum refineries at various stages in the treatment process. The term "treated" indicates the final effluent at the outfall. "Untreated" effluent designates the process effluent. "Partially treated" denotes holding pond samples from an intermediate step in the process. All samples were assigned a location and date code on arrival. Effluent fractions obtained by flash evaporation were given an additional code to indicate the number of weeks the effluent or flash evaporate was stored.

Under Condition I, effluents were immediately flash evaporated, filtered with a 0.45 μm microbiological filter (Millipore Filter Corp.), and stored at 4 C in glass prescription bottles inside cardboard boxes in the cold room (Glass). Time indicates the time in weeks the flash evaporate was stored in glass containers after flash evaporation and before cell assay.

Under Condition II, the raw effluent containing bacteria and sediment was stored in the original 5-gallon Nalgene container inside cabinets at room temperature (Nalgene). Flash evaporation and filtration were not performed until one or two days before the cell assay. Time indicates the time in weeks the raw effluent was stored in the Nalgene containers before flash evaporation. Under Conditions I and II, 70 to 75 ml of distillate was collected from 700 ml of petroleum refinery effluent.

When the total effluent was assayed, the effluent was filtered with a 0.45 μm microbiological filter to obtain sterility. Solvent extracts were not water-soluble and were so concentrated that there was no need for sterilization. Either hexane or methylene chloride extracts were dissolved in 2-3 ml acetone. Acetone acted as a dispersing agent rather

than a solubilizing agent. Ten ml of glass-distilled water were added and the vials heated in a 37 C water bath, and shaken for an hour. Nitrogen was bubbled through the mixture for an hour to evaporate the acetone. Nitrogen was used to prevent oxidation of compounds. Some extracts were solubilized with relative ease (ETU 9.70), while others required longer heating and shaking (ETE 9.70).

Routine assays by the petroleum companies identify only a small number of the possible water pollutants. Each plant has an analytical section that assays water samples from that refinery for specific compounds known to be toxic (phenol, ammonia, sulfides, etc.). Certain refineries assay for additional compounds that may be present in the samples (sulfates, chlorides, phosphates, etc.). Table II gives the chemical analysis of the effluents used in the cell assay and illustrates the variability in the concentration of each compound at different refineries and at the same refineries on different dates and/or different sample sites.

Methods

Stock Cell Cultures

L-M cells grow in monolayer or in suspension. Cells were obtained in the monolayer form from a stock maintained in the cell culture section of the Biochemistry Department of the Oklahoma State University. Suspension cultures were made by treating monolayer cells with a trypsin-versene (TV) salt mixture for five minutes. The TV solution contained 4.0 g trypsin, 0.08 g versene, 8.0 g NaCl, 0.2 g KCL, 0.005 g $NaH_2PO_4 \cdot H_2O$, 1.0 g $NaHCO_3$, 2.0 g glucose and 0.02 g phenol red in a liter of distilled water. Trypsin was 1-250, 10X Nat. Form, dry stable

TABLE II

CHEMICAL ANALYSES OF EFFLUENTS IN CELL ASSAYS*

Chemical Analysis	ETU 10.60 Treated 10.72	ETU 10.60 Treated 11.72	ETE-SI Treated 11.72	BQB Partially Treated 10.72	BQB Partially Treated 11.72	BQB Treated 10.72	BQB Treated 11.72
	(Date of Sample)						
pH#	9.8	8.2	7.9	9.1	9.3	7.7	7.7
phenol	4.3	1.5	0.1	5.0	2.2	.005	.004
ammonia, N	41	14	23	45	55	18	33
sulfide, S	5.0	0.5		35	14	0	0
COD#	565	630	616	290	288	106	136
alkalinity, P	152	0	0	132	122	0	0
$CaCO_3$, MO	248	86	188	318	284	100	130
BOD#	150	140	95			3	4
sulfate, SO_4				560	640	688	56
chlorides, Cl				670	690	910	790
total phosphates, PO_4				0.6	0.6	0.12	0.1

	DPQ-E Untreated 1.73	DPQ-E Untreated 2.73	DPQ-E Untreated 4.73	DPQ-E Treated 1.73	DPQ-E Treated 2.73	DPQ-E Treated 4.73
			(Date of Sample)			
pH#	7.2	7.2	6.6	8.3	8.4	7.0
phenol	.09	.25	.33	.01	.02	.03
ammonia, N	3.0	6.6	5.2	78	0.1	7.8
sulfide, S	<1	<1	<1	<1	<1	<1
COD#	102	268	360	45	57	134
alkalinity, P	0			24		
$CaCO_3$, MO	132			200		
BOD#						
sulfate, SO_4						
chlorides, Cl						
total phosphates, PO_4						

TABLE II (Continued)

Chemical Analysis	DPQ-W					
	Untreated			Treated		
	1.73	2.73	4.73	1.73	2.73	4.73
	(Date of Sample)					
pH#	8.2	8.1	8.2	7.5	7.3	7.0
phenol	0.03	0.08	1.2	0.06	0.06	0.09
ammonia, N	5.2	18	13	10.8	10.2	8.3
sulfide, S	<1	<1	<1	<1	<1	<1
COD#	82	105	182	66	72	66
alkalinity, P	0			0		
$CaCO_3$, MO	154			12		
BOD#						
sulfate, SO_4						
chlorides, Cl						
total phosphates, PO_4						

*Data from Water Laboratory Report of the individual refinery that provided the effluent. All results in ppm except where marked with a # sign.

powder standardized for cell culture work obtained from Nutritional Biochemicals Corporation in Cleveland, Ohio. The proteinase and chelating action was stopped by diluting with double volumes of medium. The cells were sedimented in an International Clinical centrifuge with head No. 804 for 5 minutes at 500-600XG and suspended in phosphate buffered saline (PBS) (Szyhabska and Szybalski, 1962). The PBS contained 7 g NaCl, 0.4 g KCl, 2.75 g Na_2HPO_4, 0.25 g $NaH_2PO_4 \cdot H_2O$ and 1 g glucose dissolved in a liter of distilled water. The pH was between 7.3 and 7.5.

Cell counts were made with the Coulter Model B cell counter. The multipliers were set at 4 for 1/amplification and ½ for 1/aperture current. The lower threshold was set at 10 to eliminate debris and the upper threshold was set at Out. These settings gave a background between 20 and 80 counts. Two 0.5 ml samples were counted from a 1:100 dilution made in 0.9% NaCl solution. Two counts per vial were taken for duplicate flasks at each concentration, averaged, background subtracted, and the result multiplied by the dilution factor of 200 to obtain the number of cells per milliliter.

The suspension cultures were grown in 125 ml Kimax Erlenmeyer flasks containing 20 ml of fluid rotated on a New Brunswick G-10 gyratory shaker at 50 oscillations per minute in a 37 C room. Suspension cultures were initiated at a concentration of 5×10^4 cells/ml. Daily counts verified that growth of stock cells was adequate. Cell concentrations were maintained between 5×10^4 and 8×10^5 cells/ml. The maintenance of stock cells as suspension cultures rather than as monolayers enabled the monitoring of cell growth, the avoidance of trypsinization, and the harvesting of cells during the active growth phase.

Monolayer cells were harvested at 50 hours by trypsinization. The cells were contact-inhibited at this stage, not undergoing logarithmic growth. The evaluation of monolayer and suspension stock cells compares the two methods of obtaining cells for experimental use, not simply the type of cell growth.

Effluents

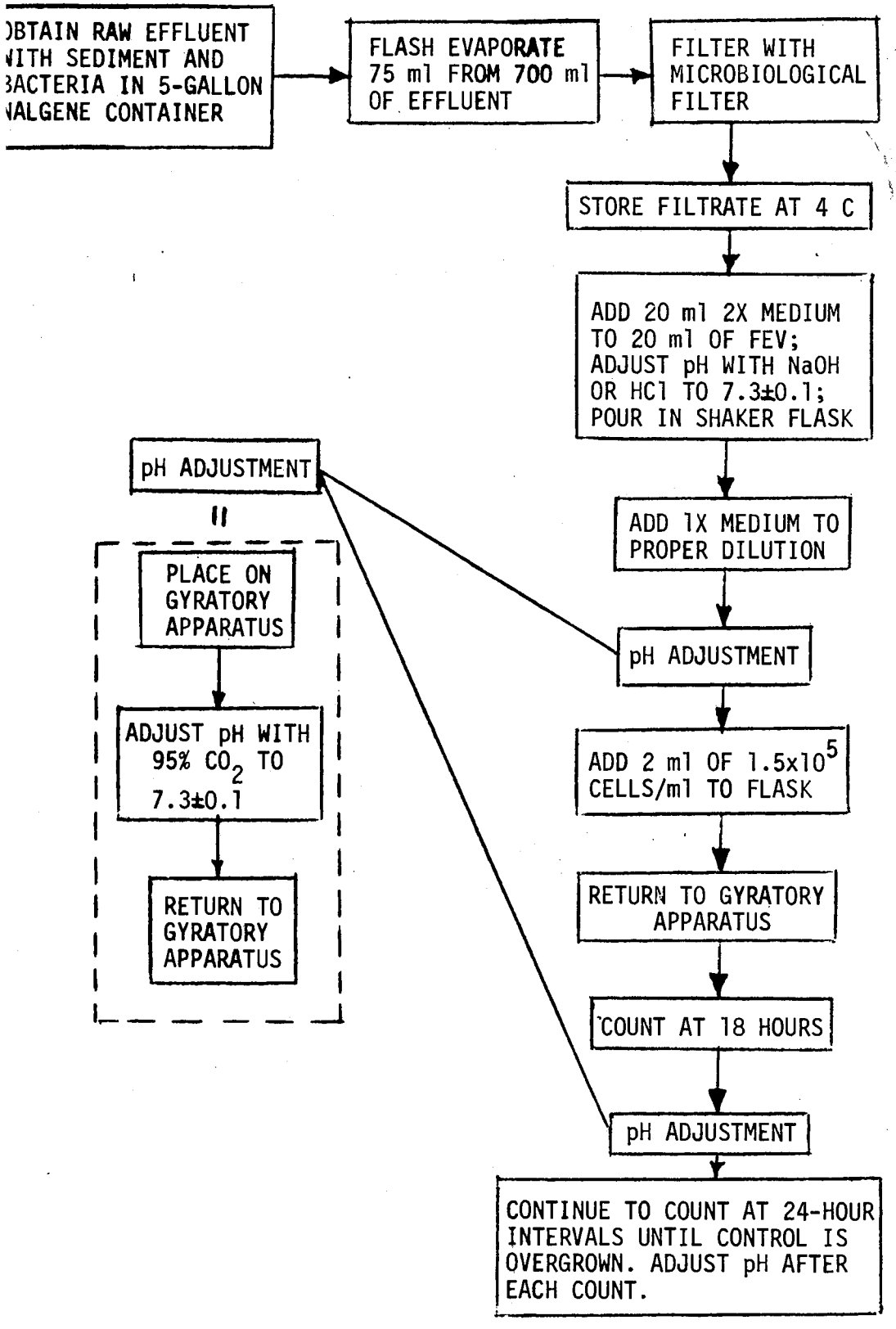
Flash evaporation was used to concentrate toxic components since the volatile portion of oil refinery effluents are more toxic to Daphnia than either the whole effluent or the non-volatile portions (Burks, 1971, personal communication). The flash evaporation apparatus consisted of two connected rotating flasks, one rotated in a hot Wesson oil bath (110-120 C) and the other in an ice bath. The first 75 ml was collected, filtered with a 0.45 μm microbiological filter, and then stored in the refrigerator. The combination of 2X medium with the effluent and adjustment of the pH to approximately 7.3 with NaOH or HCl before adding effluent to the cells avoided pH shock (Figure 1).

Experimental Procedure

The stock cells, concentrated by centrifugation and suspended in PBS, were diluted with McCoy's 5A to obtain 1.5×10^5 cells/ml. Two ml of the cell suspension were added to each of the experimental flasks. Cell counts of each individual flask were not taken at time zero since previous tests had shown cell counts at initiation to be within 0.5×10^5 cells/ml of the calculated value.

Experimental cell counts were taken 18 hours after adding the cells to the test flasks and at 24-hour intervals thereafter. Final counts

Figure 1. Cell Assay System


)BTAIN RAW EFFLUENT
VITH SEDIMENT AND
BACTERIA IN 5-GALLON
NALGENE CONTAINER
FLASH EVAPORATE
75 ml FROM 700 ml
OF EFFLUENT
FILTER WITH
MICROBIOLOGICAL
FILTER
STORE FILTRATE AT 4 C
ADD 20 ml 2X MEDIUM
TO 20 ml OF FEV;
ADJUST pH WITH NaOH
OR HCl TO 7.3±0.1;
POUR IN SHAKER FLASK
pH ADJUSTMENT
ADD 1X MEDIUM TO
PROPER DILUTION
pH ADJUSTMENT
ADD 2 ml OF 1.5x10^5
CELLS/ml TO FLASK
RETURN TO GYRATORY
APPARATUS
COUNT AT 18 HOURS
pH ADJUSTMENT
CONTINUE TO COUNT AT 24-HOUR
INTERVALS UNTIL CONTROL IS
OVERGROWN. ADJUST pH AFTER
EACH COUNT.
PLACE ON
GYRATORY
APPARATUS
ADJUST pH WITH
95% CO2 TO
7.3±0.1
RETURN TO
GYRATORY
APPARATUS

(Day 3) for the control were in the range of 10 to 13×10^5 cells/ml if the counts were taken at 24-hour intervals throughout. L cells are no longer in the logarithmic growth phase when the count exceeds 10×10^5 cells/ml. Counts of the control made before and after experimental samples were counted showed that there was no change in cell numbers during the time required to make the cell counts.

Criterion for Positive Effect

Cell counts were used as an indicator of an effect because cell populations should continue to grow at the optimal rate only if none of the stages of the growth cycle are inhibited. The index can measure inhibitory effect of the test material and should be a good indicator of any major effect that occurs.

The growth of the test cells is compared to the growth of the controls by means of the growth ratio

$$\%G = 100(T - C_o)/(C - C_o) = (100)\ \Delta \text{ treated}/\Delta \text{ untreated}$$

where

T = cell concentration of test flask at final count of control,

C = cell concentration of control flask at final count,

C_o = calculated cell concentration when cell suspension was added to each flask (Cancer Chemotherapy Reports, 1962).

A test-to-control ratio has been used in inhibition studies (National Service Center, 1962; Dixon, 1972; Cook, 1972) and to evaluate environmental influences (Rounds, 1966).

The calculated effective dose (ED_{50}) inhibits growth to 50% of

control growth (National Service Center, 1962). The ED_{50} was calculated at 66 hours in the cell assay.

If the growth ratio was 50% or less, the extract was considered to have a definite inhibitory effect. If reduction in growth ratio was statistically significant, but less than 50%, the extract was considered slightly inhibitory.

Daphnia Tests

The Daphnia test (Burks, 1972, personal communication) was used to compare the cell culture method to a routinely performed pollution bioassay. M-cresol dilutions were made as in the cell assay system, except that pond water was used instead of distilled water as the diluent. Reference compound and effluent tests were performed in the same manner as the routine bioassay except that the effluent had been filtered and that 10 ml rather than 50 ml beakers were used.

Only young adult Daphnia were used in these tests since they have a lower spontaneous death rate. They were selected on the basis of size since the first molt occurring at 16-20 hours after birth results in a doubling of body volume (Anderson, 1944). Young adults were isolated from the breeding population by means of an eyedropper and 4 Daphnia added to each beaker of premixed solutions. The pH of the solutions had been adjusted to between 6.8 and 7.8 by KOH or HCl. The number of dead Daphnia in each time interval was recorded and these numbers used to calculate the per cent survival.

Techniques of Statistical Analysis

The standard deviation obtained by repeating the test with m-cresol

served as a measure of sensitivity and reproducibility. If the 95% confidence intervals for two concentrations did not overlap, the mean cell growths for these concentrations were significantly different. 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, both within and between experiments, was analyzed by the F-test, because it is relatively insensitive to non-normality of the data. All of the above statistical tests are discussed in Statistics with Application to the Biological and Health Sciences (Remington and Schork, 1970).

CHAPTER III

RESULTS

Cell Assay Parameters

Figure 2 shows a typical growth curve obtained under the conditions described in the Materials and Methods section. There is a slight lag of less than 18 hours before exponential growth was observed. The lag is probably due to the hour the cells are not in growth medium and not at 37 C during the initiation of the experiment. The generation time was 23 hours. When the cell population exceeded 9×10^5 cells/ml, there was a much slower rate of growth.

M-Cresol Reference Compound

Cell Assay

Both monolayer and suspension cultures were used as a source of experimental cells. A comparison of these sources of cells was made using m-cresol as a reference compound. In a range of concentrations from 0.5% (5,000 ppm) to 0.0002% (2 ppm), suspension culture cells were more sensitive to m-cresol. Cells from monolayer stock cultures were totally inhibited at 100 ppm in a rangefinding assay not shown. Suspension culture stock cells were toally inhibited at 20 ppm.

Typical growth curves for monolayer and suspension-derived cells (Figures 3 and 4) illustrates the differences in sensitivity of the two

Figure 2. Control Growth Curve

Representative growth curve of L-M cells grown in suspension culture with McCoy's 5A + 5% calf serum.

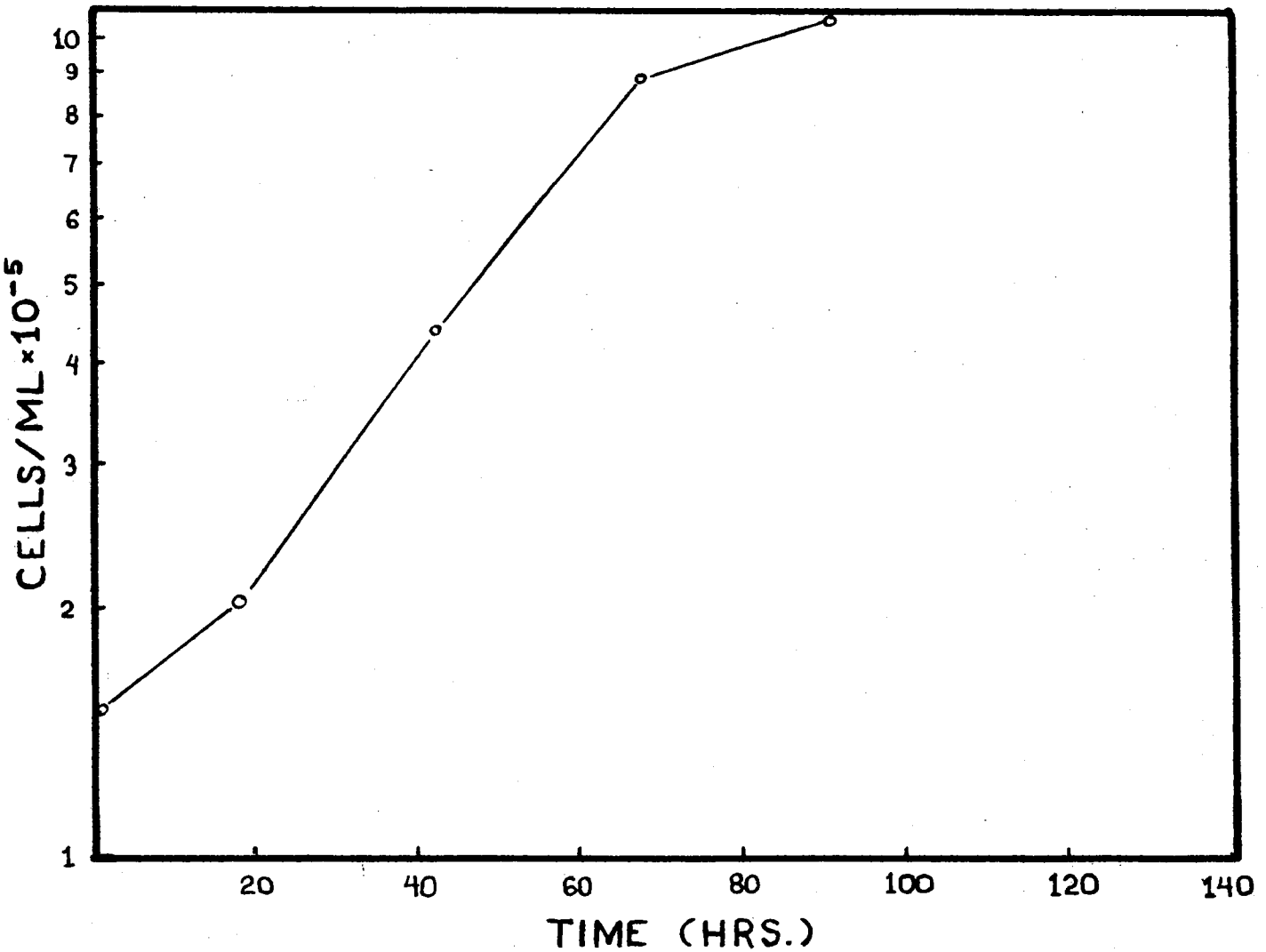

CELLS/ML ×10⁻⁵
10
9
8
7
6
5
4
3
2
1
20
40
60
80
100
120
140
TIME (HRS.)

Figure 3. Growth Curves for m-cresol Reference Concentrations; Monolayer Stock Cultures

Effect of varying concentrations of m-cresol on the growth curve of L-M cells grown in suspension culture when confluent monolayer cells were trypsinized to obtain the experimental cells. Six concentrations of m-cresol were tested: 0 ppm or control, x, 2 ppm, ■, 5 ppm, □, 10 ppm, ●, 20 ppm, ⊖, and 50 ppm, O. Counts were taken from duplicate flasks at each concentration.

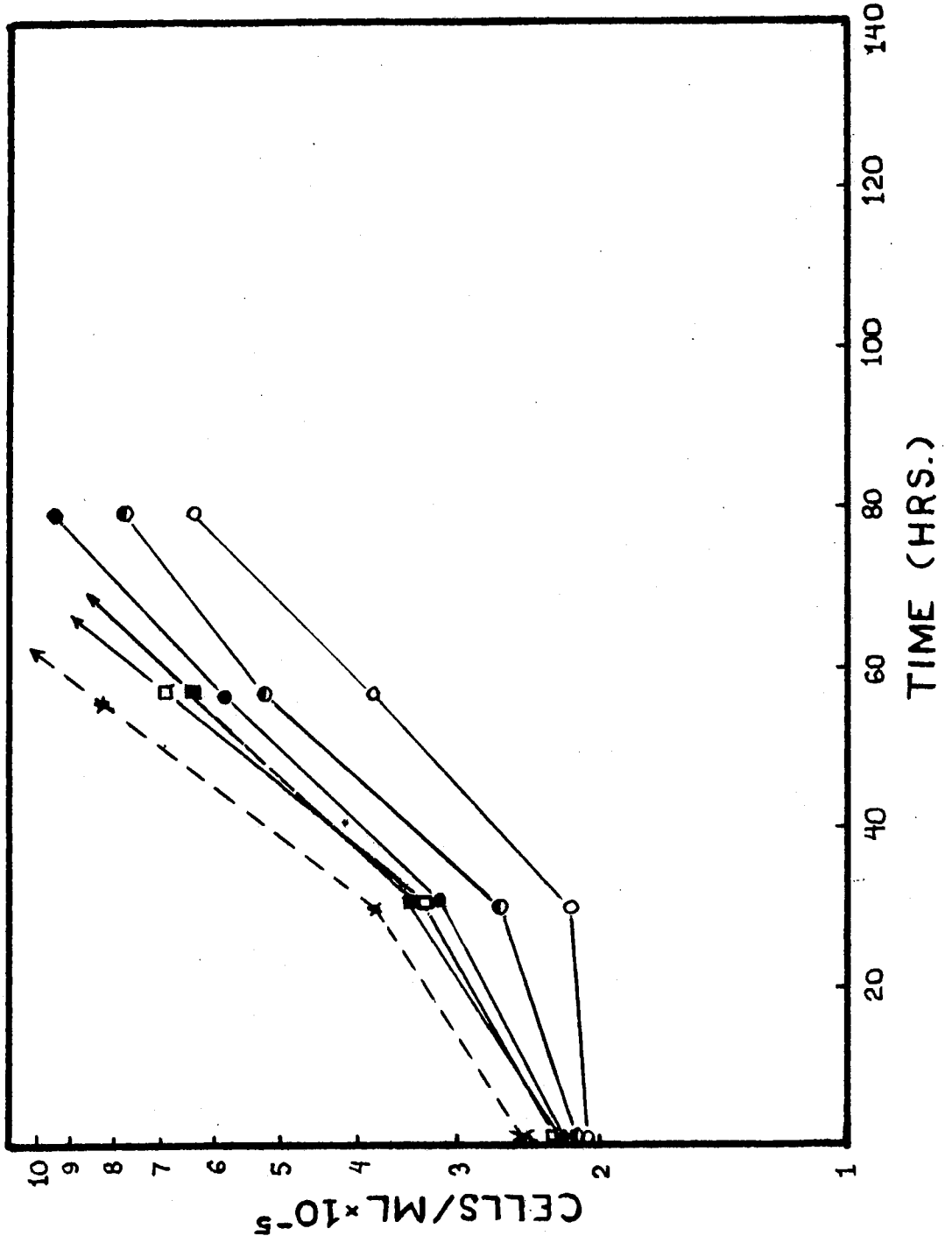
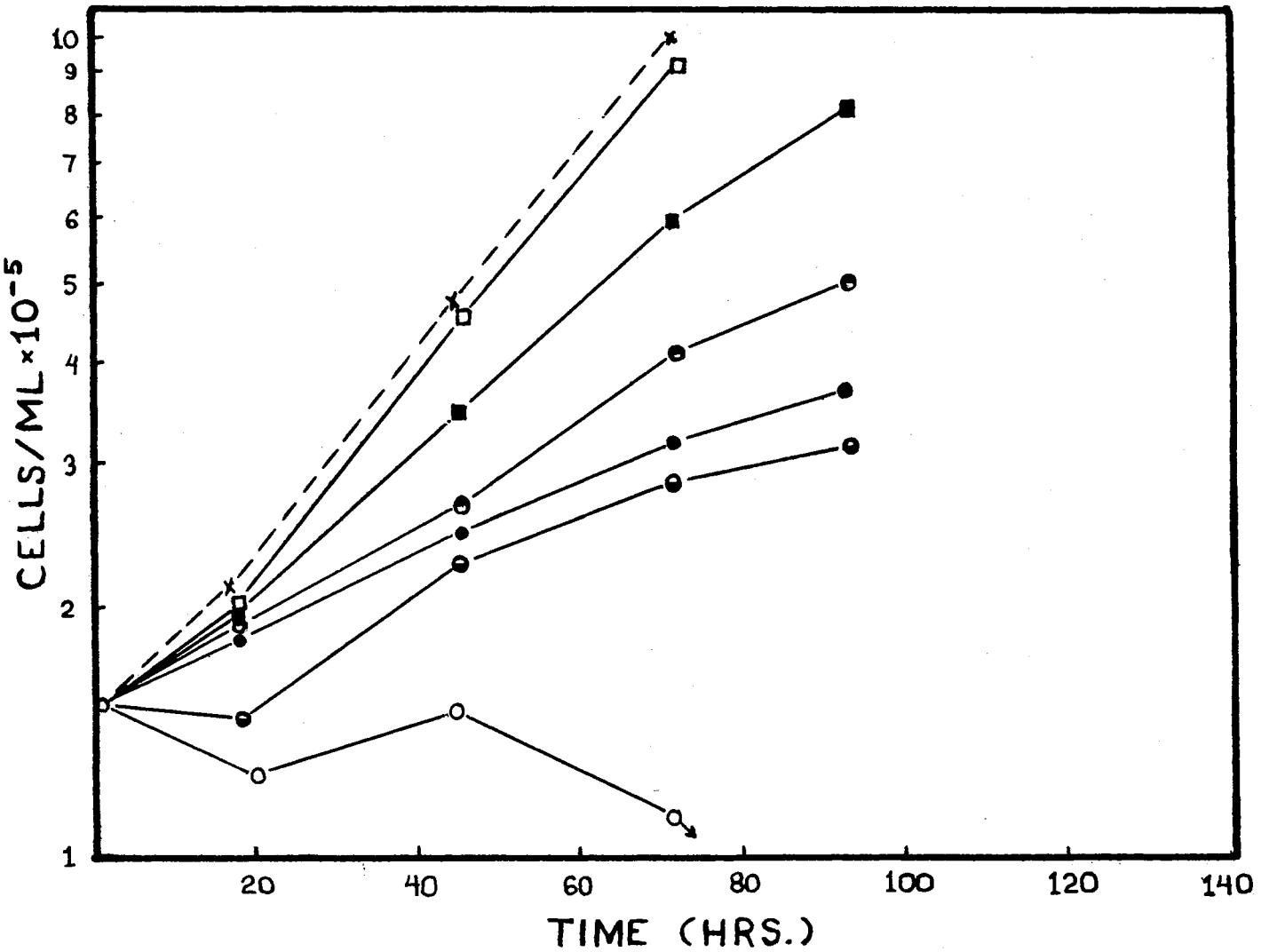

CELLS/ML×10⁻⁵
10
9
8
7
6
5
4
3
2
1
20
40
60
80
100
120
140
TIME (HRS.)

Figure 4. Growth Curves for m-cresol Reference Concentrations; Suspension Stock Cultures

Effect of varying concentrations of m-cresol on the growth curve of L-M cells grown in suspension culture when suspension cultures were the source of experimental cells. Seven concentrations of m-cresol were tested: 0 ppm or control, x, 2 ppm, □, 5 ppm, ■, 7.5 ppm, ◐, 10 ppm, ●, 15 ppm, ◒, and 20 ppm, ○. Counts were taken from duplicate flasks at each concentration.


CELLS/ML ×10⁻⁵
10
9
8
7
6
5
4
3
2
1
20
40
60
80
100
120
140
TIME (HRS.)

methods. For monolayer-derived cells, the ED_{50} was 20 ppm, and there was a lack of separation of the terminal points at 2 and 5 ppm. The overlapping of endpoints for concentrations within 5 ppm was frequently observed. For suspension-derived cells, the ED_{50} was calculated at 6 ppm and terminal points were well spread. Overlapping of terminal points was never observed with this system.

The per cent growth rate (%GR) was calculated for the reference system and compared to the per cent growth (%G) at 66 hours (Table III, Figures 5 and 6). The two values were not significantly different. Per cent growth, obtained from a ratio of cell yields, is more convenient; but either method can be used for expressing results. If the terminal point assay, i.e., %G, is used, the cells should be pipetted daily to disaggragate clumps and to remove cells from the sides of the flask to obtain consistent growth.

Statistical comparison of these two methods also demonstrated that the use of suspension cultures for initiating cell assays yielded a more reliable assay (Table III). The amount of variation between and within experiments determines the reliability of the system. There was no significant difference between the variation within experiments for the two methods, but the variation between monolayer cell experiments was almost twice that between suspension cell experiments. Therefore, the suspension stock cell system is twice as reliable as the monolayer stock cell system.

Suspension culture cells are also about twice as sensitive as trypsin-treated monolayer cells. The concentration of m-cresol that produced only 50% inhibition of monolayer-derived cells produced total inhibition of suspension-derived cells. Survival curves for both

TABLE III

COMPARISON OF GROWTH RATE (%GR) AND GROWTH (%G)
RESPONSE TO M-CRESOL

M-Cresol (ppm)	%GR	%G	Standard Deviation of %G
Experiment A			
0	100	100	0
2	97	93	10
5	87	81	16
10	60	62	24
20	47	48	18
50	20	16	11
Experiment B			
0	100	100	0
2	87	82	15
5	72	58	13
10	25	23	5
20	-12*	-6*	4
50	-22*	-12*	2

95% confidence intervals on mean %G for a particular concentration

	Experiment A	Experiment B
Within Experiments	$\bar{x} \pm 6\%$	$\bar{x} \pm 8\%$
Between Experiments	$\bar{x} \pm 29\%$	$\bar{x} \pm 15\%$

Experiment A: Average of counts at 66 hours from three independent experiments in which confluent monolayer cultures were trypsinized to obtain experimental cells.

Experiment B: Average of counts at 66 hours from four independent experiments in which suspension cultures were the source of the experimental cells.

At 66 hours, 100% growth was equivalent to $8.6\text{-}9.8 \times 10^5$ cells/ml. Dilutions of m-cresol were made twice in the series of experiments for each method of stock culture.

*Values of greater than 100% inhibition or negative numbers for the growth ratio indicates cell death.

Figure 5. Survival Curves With m-cresol Reference Concentrations; Monolayer Stock Cultures

Effect of varying concentrations of m-cresol on survival of L-M cells grown in suspension culture when confluent monolayer cells were trypsinized to obtain the experimental cells. Both per cent growth calculated at 66 hours, ●, and growth rate over a 20-hour period, O, were plotted from the average of three independent experiments with duplicate flasks at each concentration.

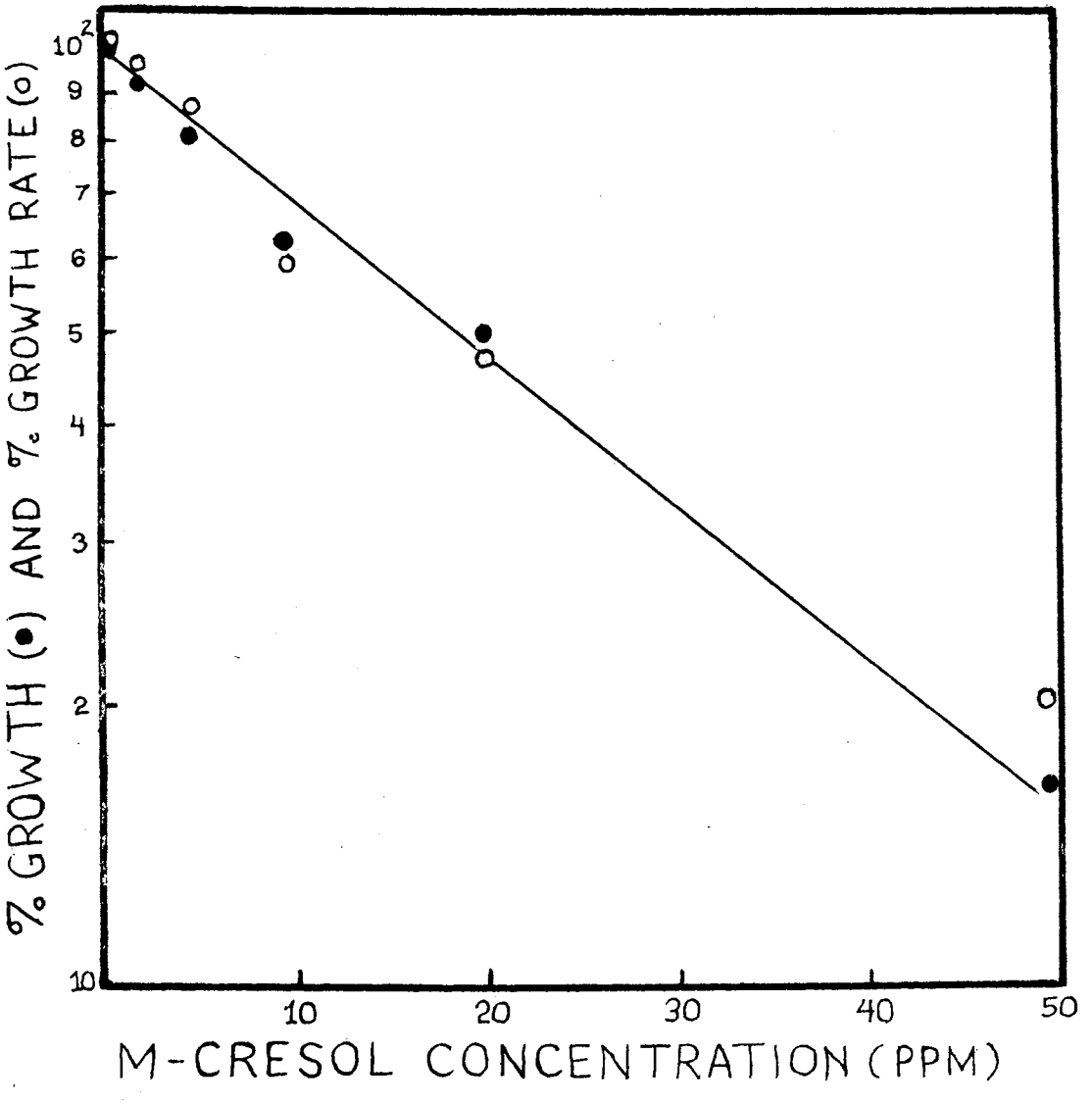
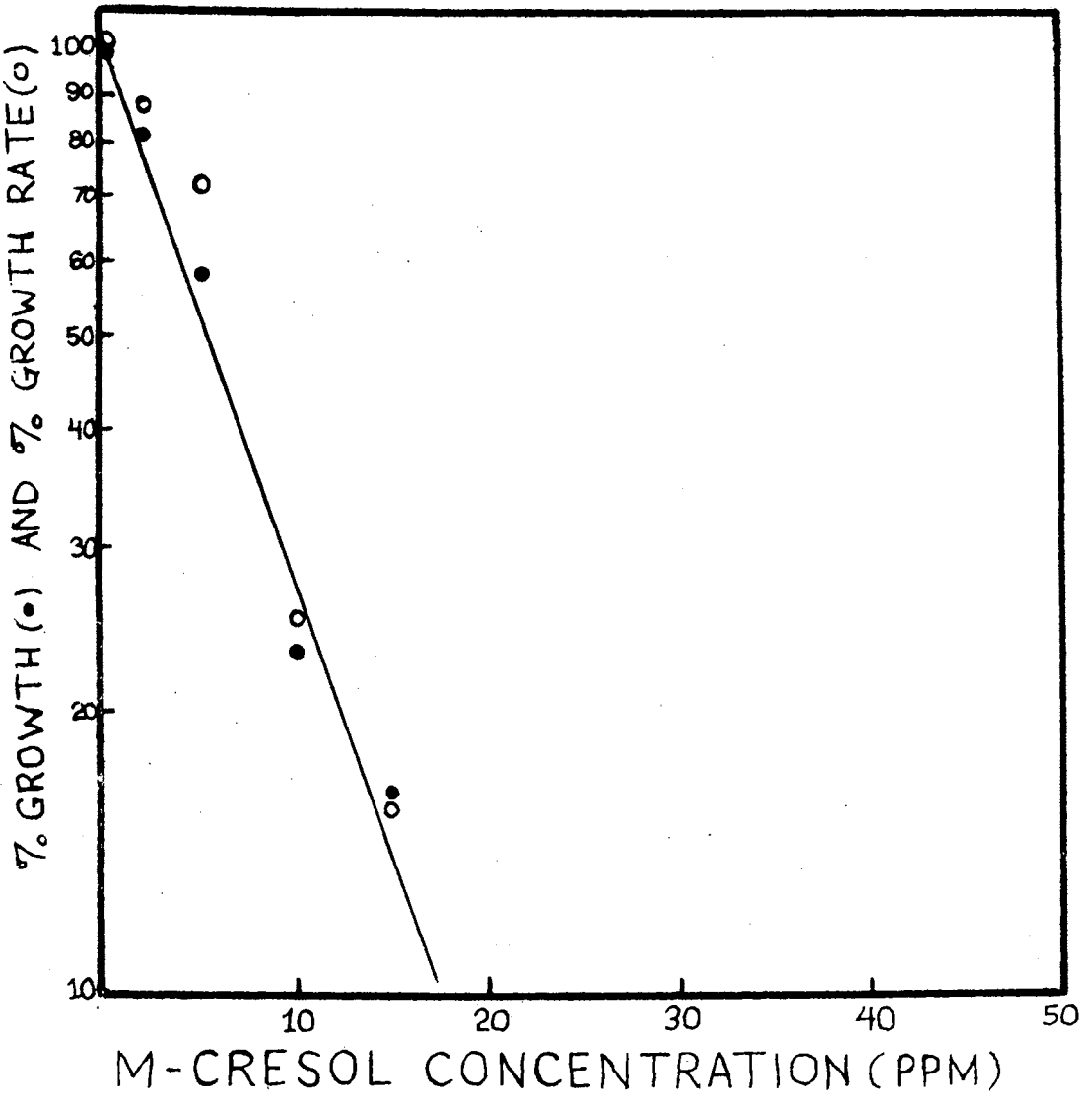

% GROWTH (•) AND % GROWTH RATE (o)
10²
9
8
7
6
5
4
3
2
10
10
20
30
40
50
M-CRESOL CONCENTRATION (PPM)

Figure 6. Survival Curves With m-cresol Reference Concentrations; Suspension Stock Cultures

Effect of varying concentrations of m-cresol on survival of L-M cells grown in suspension culture when suspension cultures were the source of experimental cells. Both per cent growth calculated at 66 hours, ●, and growth rate over a 20-hour period, O, were plotted from the average of four independent experiments with duplicate flask at each concentration.


% GROWTH (•) AND % GROWTH RATE (o)
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
M-CRESOL CONCENTRATION (PPM)

methods of obtaining experimental cells showed that the cell population was homogeneous in its sensitivity to the reference compound (Figures 5 and 6).

Daphnia Assay

Fish and Daphnia are commonly used for intact organism assays of aquatic toxicity. Daphnia are more sensitive than fish to chemical pollutants (Anderson, 1948). The Daphnia bioassay was compared to the cell culture assay to evaluate the relative sensitivities. Cell culture is a more sensitive assay than the Daphnia bioassay in detecting m-cresol toxicity. The literature value for the threshold concentration of m-cresol for Daphnia is 28 ppm (McKee and Wolf, 1963). In the cell assay system the threshold concentration is about 2 ppm and the 50% inhibitory concentration of 6 ppm is well below the Daphnia threshold concentration.

The Daphnia test was performed with various concentrations of m-cresol to determine if the literature value was valid for the population of Daphnia to be used in evaluating effluents. The Daphnia test consisted of the average of four independent experiments with duplicates at each concentration so that results would be comparable to the cell assay. Of 104 Daphnia tested for length of survival under control conditions, 5% died within 24 hours and an additional 15% died within 48 hours. For this reason tests were discontinued after 48 hours. A preliminary experiment indicated that four young adult Daphnia were the maximum number that retained the longest survival time in a control situation. Fully mature adults gave unreliable results (Table IV) since they gave birth to young, died spontaneously, and molted. Four Daphnia

are routinely used in bioassays (Anderson, 1944, 1945, 1946, 1948).

TABLE IV

DAPHNIA SURVIVAL UNDER VARIOUS CONTROL CONDITIONS

Age and Number of Daphnia	Cumulative Number of Daphnia* Dead Before the Time Listed (Time in Hours)			
	24	48	72	96
2Y	0	0	2	4
2A	0	1[B]	4	8
4Y	0	0	6	8
4A	0	2[B]	8[B]	8
6Y	0	4	10	10
6A	0	6[B]	10[B]	10
4Y-open	0	0	4	8
4A-open	0	4[B]	8[B]	8

*Duplicate beakers were tested.

A = mature adult, maybe with 1 to 3 eggs.

B = young born during test.

Open = beaker exposed to air.

Y = young adults.

Range-finding tests with the same concentrations of m-cresol as the cell assay (i.e., 0, 2, 5, 10, 15, 20, 50, 100 ppm) determined that

the threshold value for the Daphnia population was above 20 ppm but less than 50 ppm, since there was no survival above 50 ppm. In a Daphnia bioassay with 10 ppm increments of m-cresol, survival at 24 hours was not significantly affected at less than 30 ppm concentration. Mortality was almost 100% at 40 ppm. With 5 ppm increments of m-cresol, 100% mortality occurred at 40 ppm. At 25 and 35 ppm, there was no difference from the control, but the intermediate concentration produced over 50% inhibition. Both the Daphnia bioassays with 5 and 10 ppm increments indicated that the threshold was above 20 ppm and that 40 ppm was the lowest concentration of m-cresol that produced reproducible positive results (i.e., 100% mortality) (Table V).

Application to Effluents

Typical Curves

The cellular response to different effluents was variable as expected (Figures 7 and 8) since effluents are complex and variable. Those cells which were inhibited did not recover their growth rate (Figure 7, line 5 and 6), completely recovered (Figure 8), partially recovered (Figure 7, line 4), or partially recovered and later were inhibited at a different level (Figure 7, lines 2 and 3).

If the plot of the log of the survivors versus the dose is linear, it indicates that there is either a single inhibitor and single sensitivity of the cell population or that there is a mixture such that in combination the components have similar thresholds and the whole cell population has a constant sensitivity. If there are breaks in the curve, there are either two or more inhibitory substances present or the cell population has heterogeneous sensitivities. One can only

TABLE V

SENSITIVITY AND RELIABILITY OF *DAPHNIA* TEST WITH M-CRESOL

M-Cresol (ppm)	Number Surviving at Time Indicated (Time in Hours) 0.5	1	2	4	8	12	24	48
Experiment A								
0	32	32	32	32	32	32	32	32
15	31	31	31	31	31	31	31	25
20	32	32	32	32	32	32	31	30
30	29	28	27	26	24	20	18	11
40	11	9	8	8	6	5	3	1
50	0	0	0	0	0	0	0	0
Experiment B								
0	16	16	16	16	16	16	14	8
20	16	16	16	16	16	16	12	10
25	16	16	16	16	16	16	14	11
30	10	8	8	7	7	7	6	5
35	16	16	15	15	15	15	13	9
40	3	3	0	0	0	0	0	0

In Experiment A, 32 *Daphnia* were used in a total volume of 10 ml of test solution for each set of 4 *Daphnia*. In Experiment B, 16 *Daphnia* were used in a total volume of 10 ml of test solution for each set of 4 *Daphnia*.

Figure 7. Growth Curves With Stored Effluent: DPQ-E January 1973 Samples

Effect of varying concentrations of effluent flash evaporate on the growth curves of L-M cells grown in suspension culture. Seven conditions are shown, the control, x; 45% untreated effluent stored for two weeks in nalgene under Condition II, ■, the same concentration of the effluent after two weeks storage in glass under Condition I, ●; 45% treated effluent stored for two weeks in nalgene under Condition II, ◓, and two weeks storage in glass under Condition I, O; 22% concentration of treated effluent stored two weeks under Condition II, ⊖, and two weeks in glass under Condition I, □.

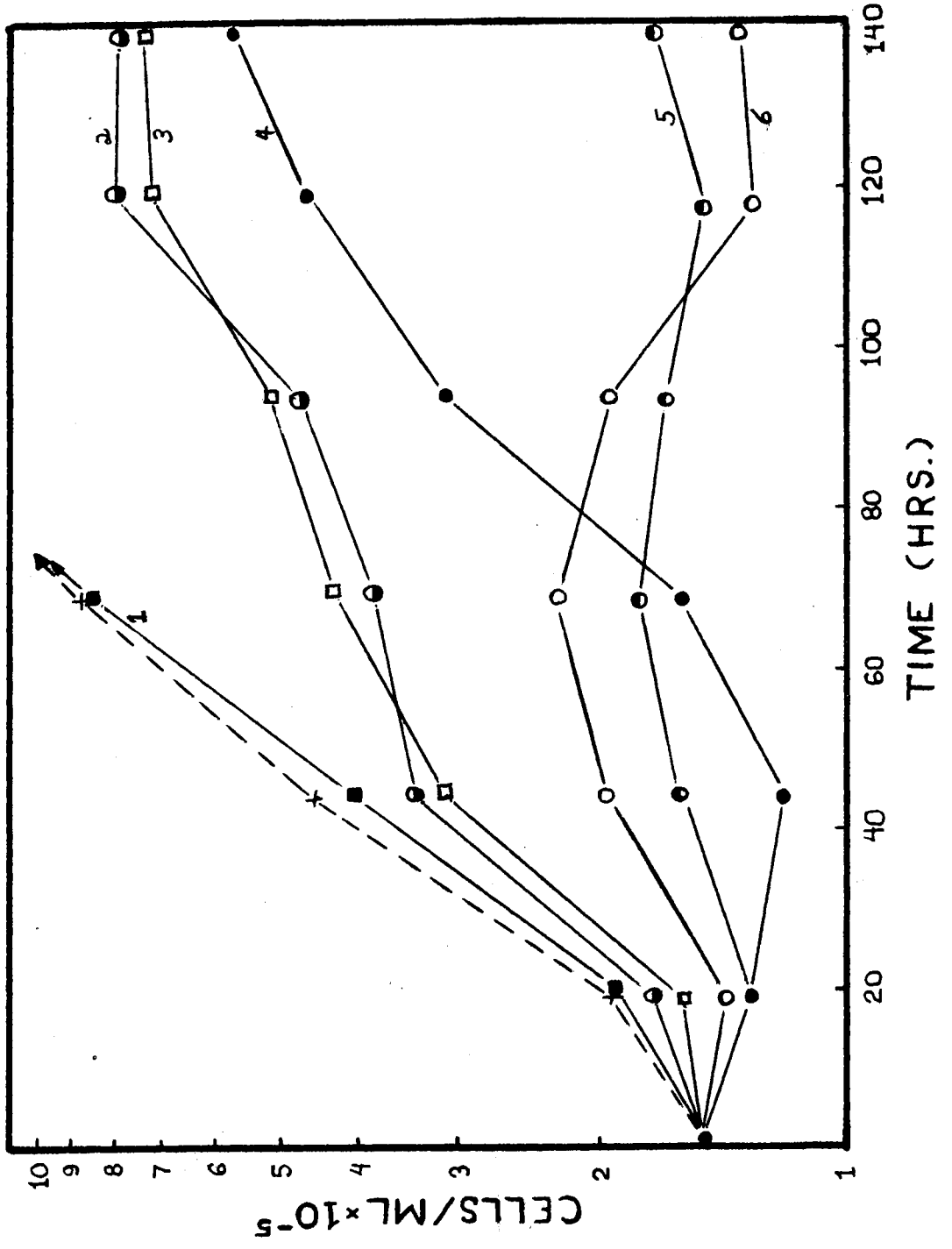
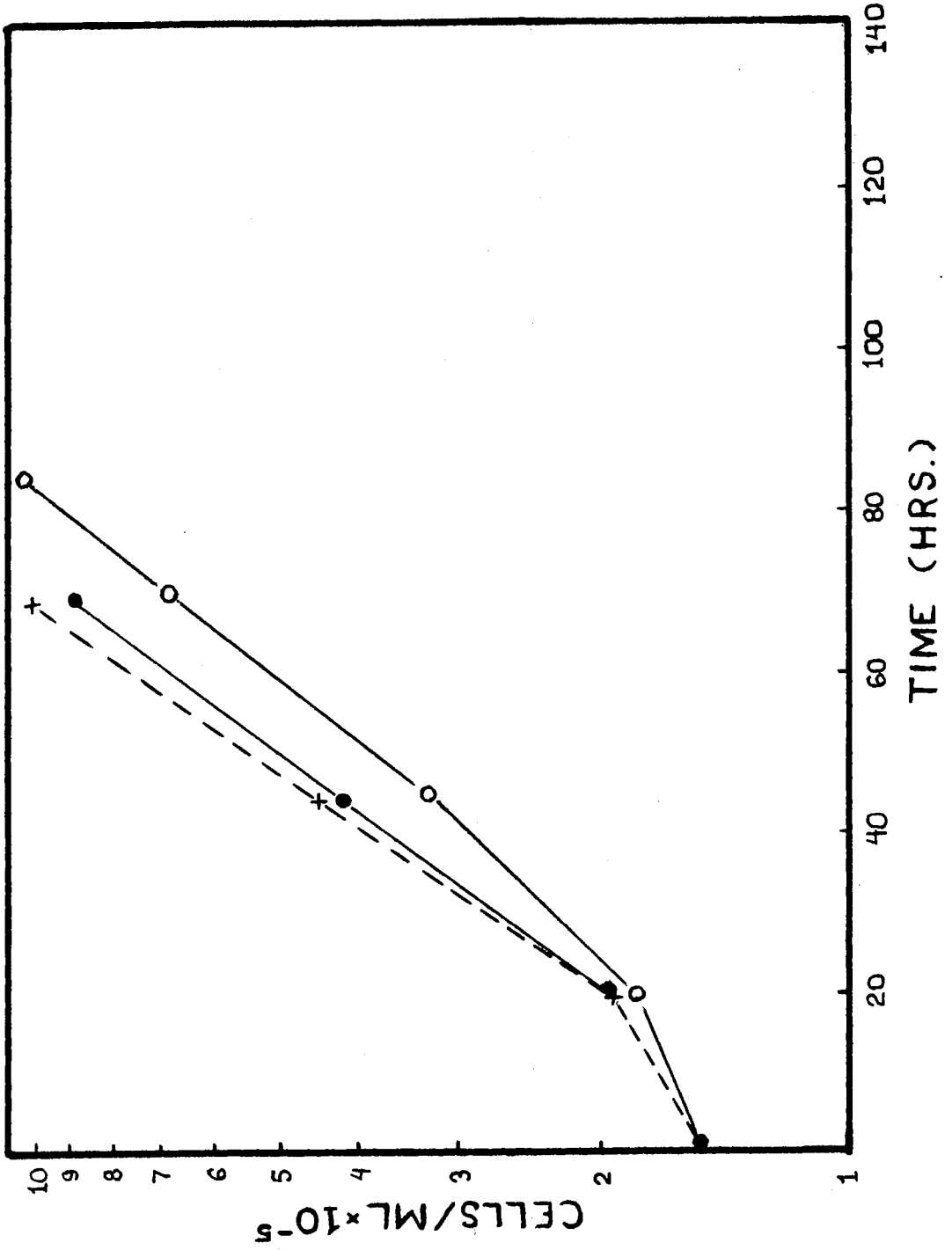

CELLS/ML×10⁻⁵
TIME (HRS.)
1
2
3
4
5
6
7
10
9
8
7
6
5
4
3
2
1
20
40
60
80
100
120
140

Figure 8. Growth Curves With Stored Effluent: DPQ-E February 1973 Samples

Effect of varying concentrations of effluent flash evaporate on the growth curves of L-M cells grown in suspension cultures. The control, x, and two examples of complete recovery of growth rate are shown; 45% treated effluent stored one week in glass under Condition I, ●, and 45% untreated effluent stored under the same condition, O.


CELLS/ML×10⁻⁵
10
9
8
7
6
5
4
3
2
1
20
40
60
80
100
120
140
TIME (HRS.)

speculate on the meaning of the survival curves obtained from three of the effluent samples since all are complex. The presence of two toxic components may be indicated by survival curves for April samples from DPQ-W (Figure 9). Essentially the same pattern was exhibited by February samples from the same location. Survival curves for April samples of BQB are different for treated and untreated samples (Figure 10). The treated effluent curve indicates a threshold concentration before toxicity was expressed. The untreated effluent curve may be explained by a cell population with different sensitivities. Only the more resistant cells may have remained at the higher concentrations of effluent. These plots indicate that inactivation of cells by effluent is a complex process (Figures 9 and 10). Therefore, intensive study of cellular response should be reserved until fractions can be obtained which contain a smaller number of compounds.

Techniques for Analysis. Rangefinding cell assays were performed on the day following the collection of the flash evaporate. Treated effluent was routinely tested at 45 and 22% and untreated effluent at 45, 22, 11, and 5% flash evaporate. If the refinery under investigation had a prior history of highly toxic effluents, additional concentrations were tested. Typical rangefinding assays are presented in Figures 11 - 14. These results show that when the concentration of effluent was decreased, the cell count was increased. A plot of the log of growth rate versus concentration of effluent shows that response was linear (Figure 15). Further experiments were performed with the concentration of effluent that gave approximately 50% inhibition in the rangefinding assay.

The reproducibility of flash evaporation and the cell assay were

Figure 9. Survival Curves With Effluent: DPQ-W April 1973 Untreated Samples

Effect of increasing concentrations of untreated effluent flash evaporate on survival of L-M cells in suspension culture. Effluent was not stored. The per cent growth at 66 hours was plotted. Duplicate flasks were counted at each concentration.

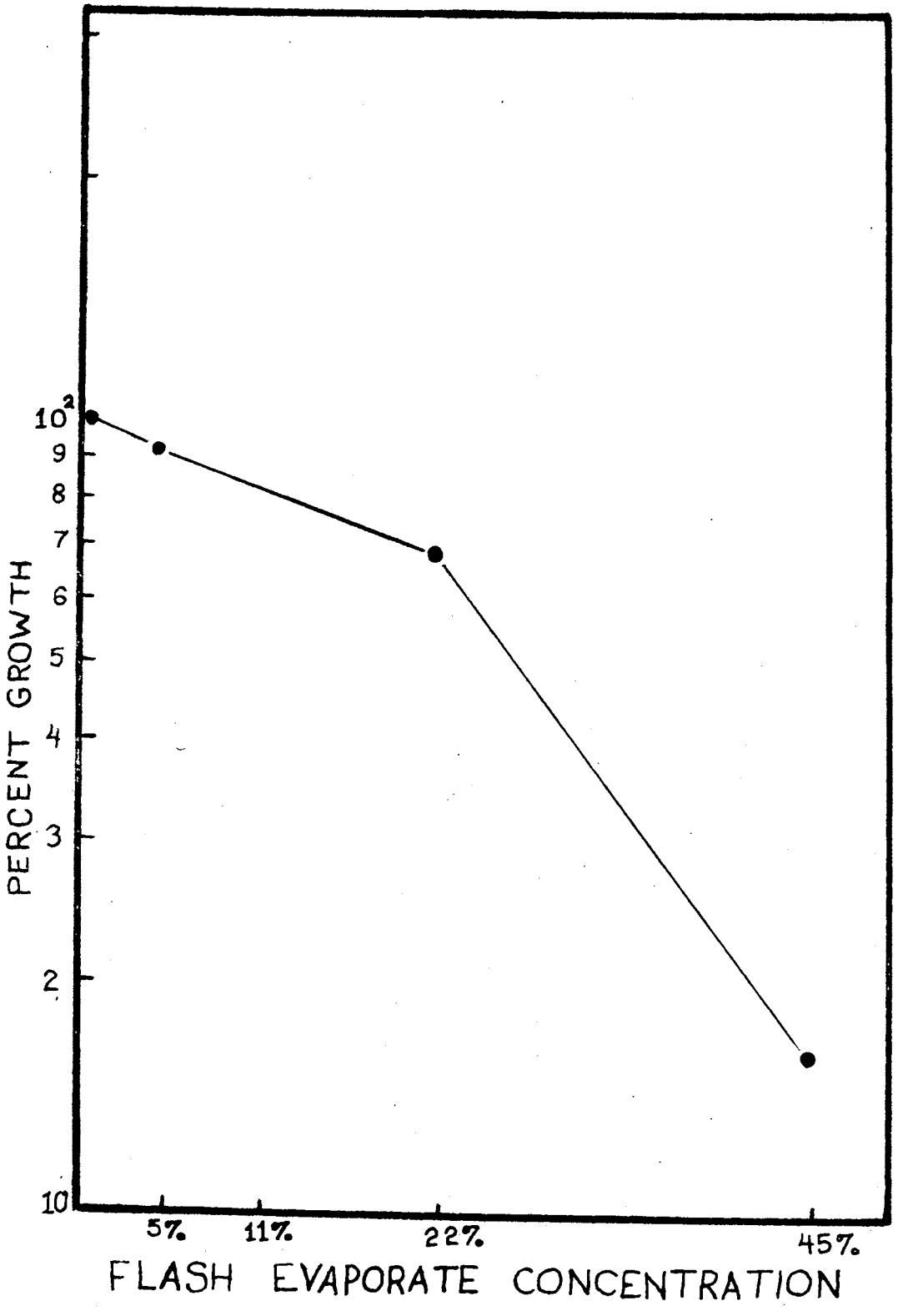

PERCENT GROWTH
10^2
9
8
7
6
5
4
3
2
10
5%
11%
22%
45%
FLASH EVAPORATE CONCENTRATION

Figure 10. Survival Curves With Effluent: BQB April 1973 Samples

Effect of increasing concentrations of effluent flash evaporates on survival of L-M cells in suspension culture. Effluent was not stored. The per cent growth at 66 hours was plotted for treated, ●, and partially treated, O, effluent. Duplicate flasks were counted at each concentration.

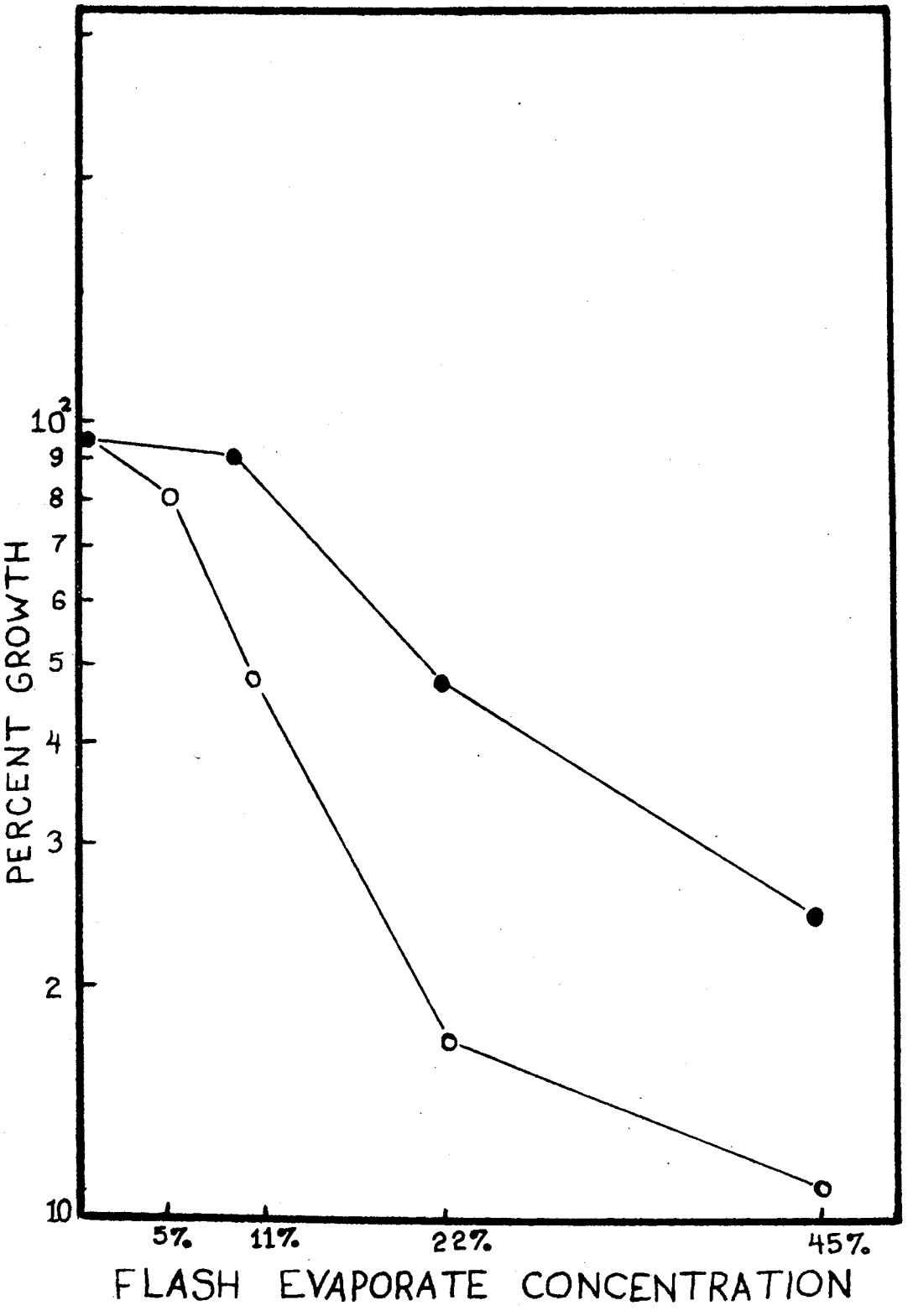

PERCENT GROWTH
10²
9
8
7
6
5
4
3
2
10
5%
11%
22%
45%
FLASH EVAPORATE CONCENTRATION

Figure 11. Growth Curves With Effluent: BQB April 1973 Treated Samples

Effect of decreasing concentrations of effluent flash evaporate on the growth curves of L-M cells grown in suspension culture. Treated effluent was tested at concentrations of 45%, O, 22%, □, and 11%, ●. Duplicate flask were counted for each concentration.

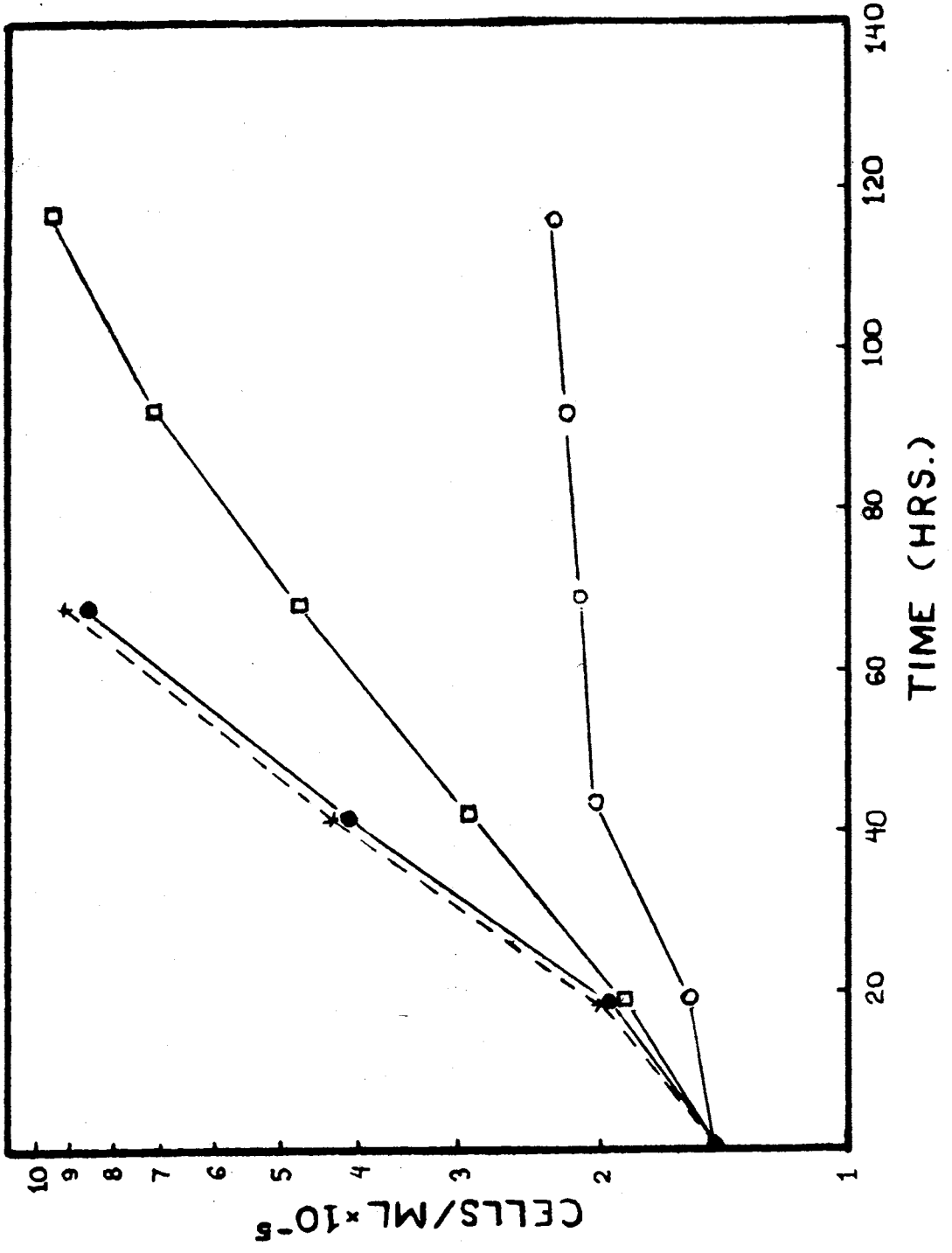

CELLS/ML×10⁻⁵
10
9
8
7
6
5
4
3
2
1
20
40
60
80
100
120
140
TIME (HRS.)

Figure 12. Growth Curves With Effluent: BQB April 1973 Partially Treated Samples

Effect of decreasing concentrations of effluent flash evaporate on the growth curves of L-M cells grown in suspension culture. Partially treated effluent was obtained from a holding pond and tested at concentrations of 45%, O, 22%, ◑, 11%, ●, and 5%, □. Duplicate flasks were counted for each concentration.

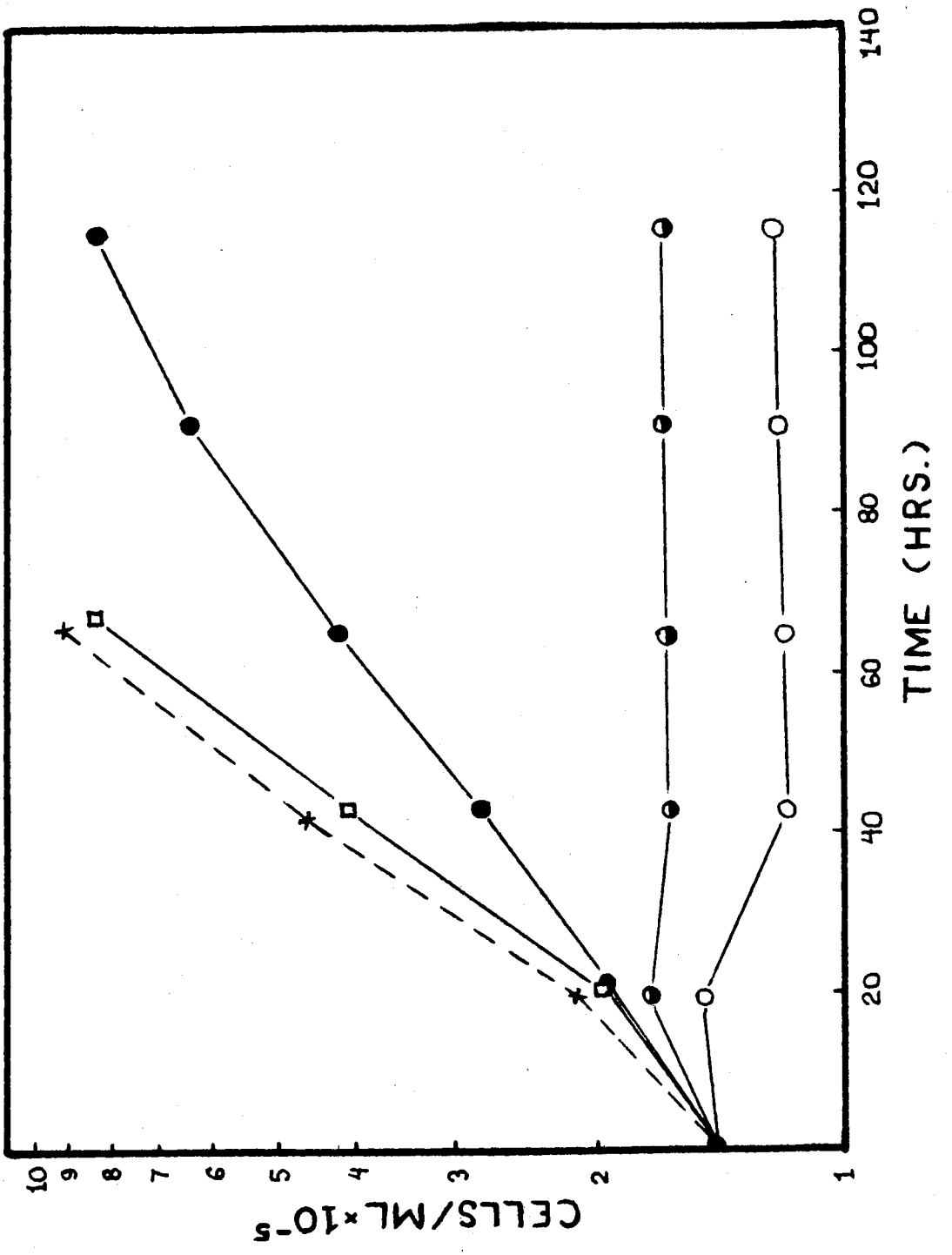

CELLS/ML×10⁻⁵
10
9
8
7
6
5
4
3
2
1
20
40
60
80
100
120
140
TIME (HRS.)

Figure 13. Growth Curves With Effluent: DPQ-E February 1973 Treated Samples

Effect of decreasing concentrations of effluent flash evaporate on the growth curves of L-M cells grown in suspension cultures. Treated effluent was tested at concentrations of 45%, ■, and 22%, O. Duplicate flasks were counted at each concentration.

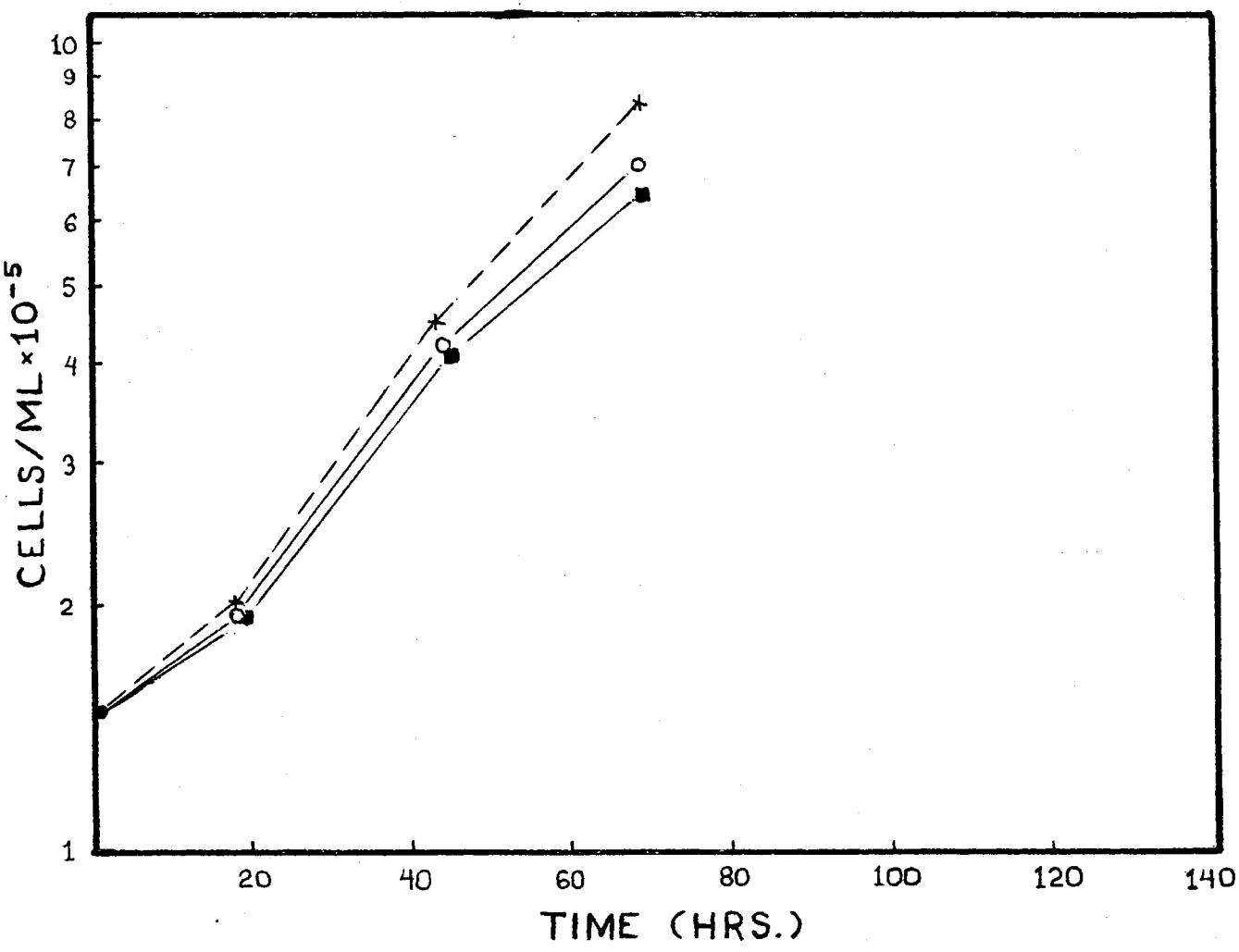

CELLS/ML x10⁻⁵
10
9
8
7
6
5
4
3
2
1
20
40
60
80
100
120
140
TIME (HRS.)

Figure 14. Growth Curves With Effluent: DPQ-E February 1973 Untreated Samples

Effect of decreasing concentrations of effluent flask evaporate on the growth curves of L-M cells grown in suspension culture. Untreated effluent was tested at concentrations of 45%, □, 22%, O, 11%, ■, and 5%, ●. Duplicate flasks were counted at each concentration.

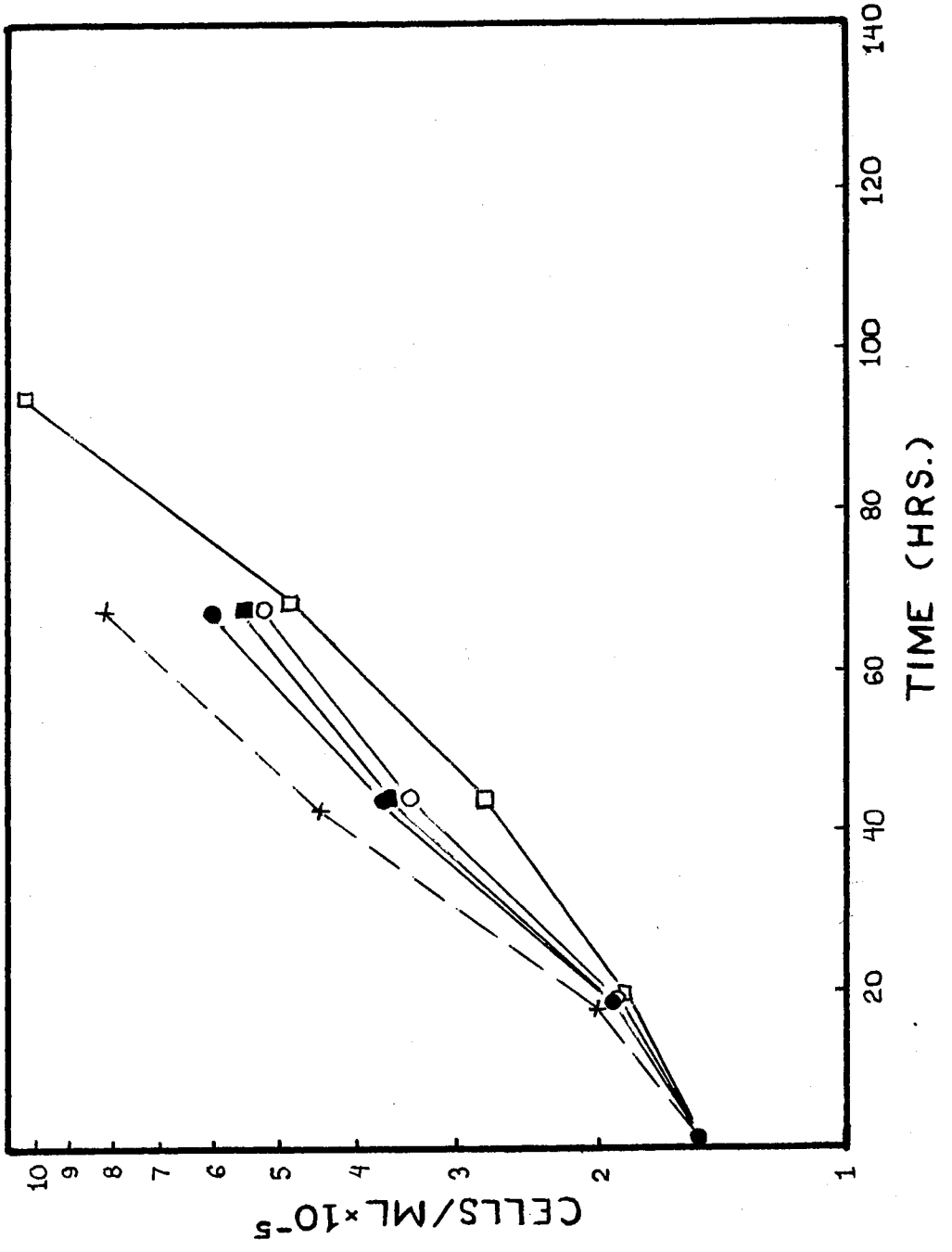
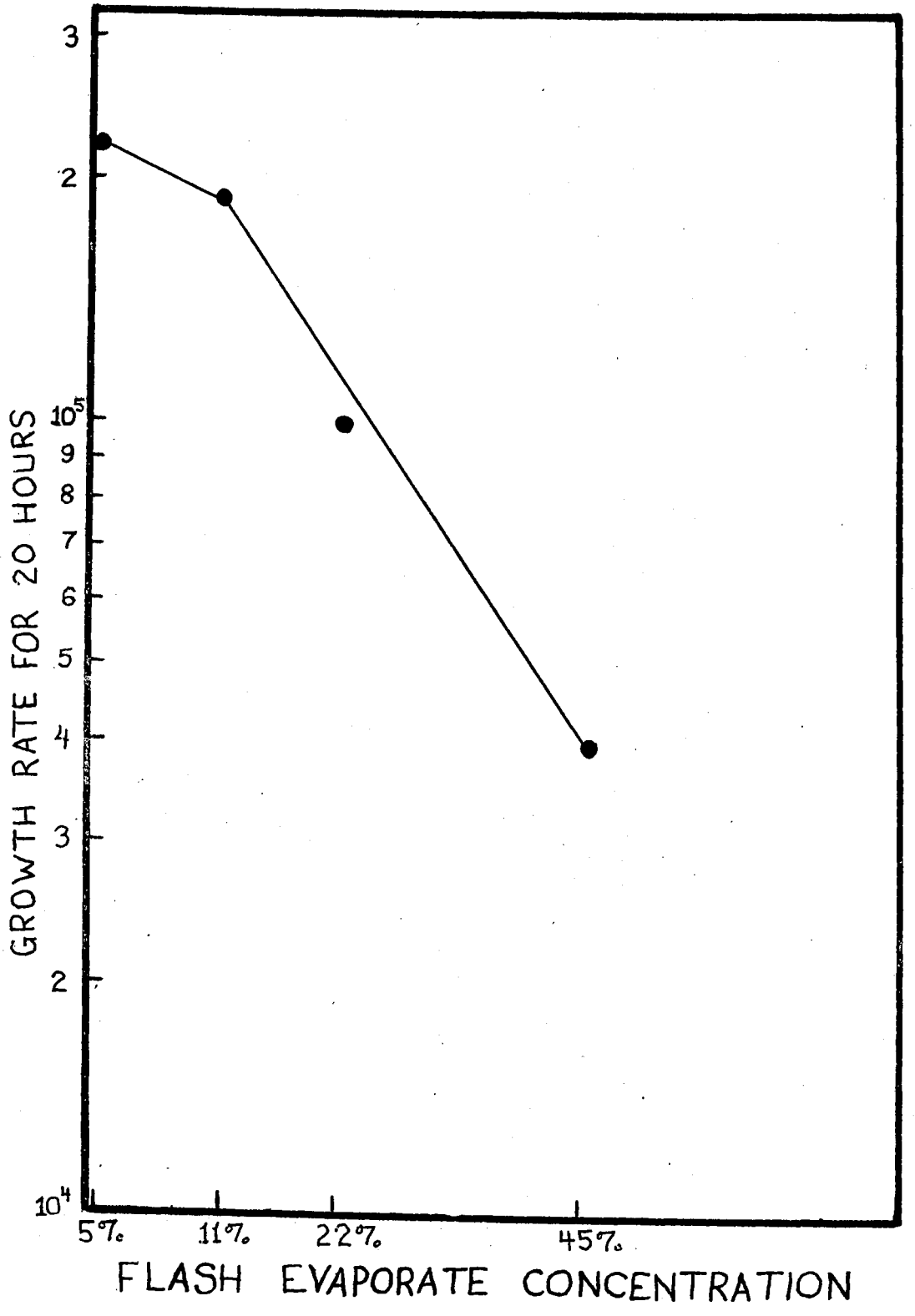

CELLS/ML×10⁻⁵
10
9
8
7
6
5
4
3
2
1
20
40
60
80
100
120
140
TIME (HRS.)

Figure 15. Change in Growth Rate With Effluent: BQB April 1973 Treated Samples

Effect of decreasing concentrations of effluent flash evaporate on the growth rate of L-M cells grown in suspension culture.


3
2
10^5
9
8
7
6
5
4
3
2
10^4
GROWTH RATE FOR 20 HOURS
5%
11%
22%
45%
FLASH EVAPORATE CONCENTRATION

evaluated to determine the feasibility of the entire system. The variation in toxicity due to the concentration process was investigated by flash evaporating two fractions from the same effluent on the same day. In all cases, the results from the two flash evaporations of one sample closely approximated each other (Table VI).

TABLE VI

DUPLICATE FLASH EVAPORATIONS FOR TWO EFFLUENTS

Source	Month	% Flash Evaporate	% Growth at 66 Hours Weeks Stored (Condition I) 0	1	2
NDD	10.70	45	62,67		33,32
		22	56,54		67,64
ETU 10.60	10.70	22		54,52	
		11		52,48	

At 66 hours, 100% growth is equivalent to $9.5 - 10.0 \times 10^5$ cells/ml. The first per cent growth number represents the cell growth using the proper dilutions from 50 ml of flash evaporate from the first 500 ml of total effluent in the Nalgene container; the number following the comma represents the cell growth using the proper dilutions from 50 ml of flash evaporate obtained from the second 500 ml of total effluent in the Nalgene container.

Replicate cell assay results using treated and untreated samples obtained over a three-month period showed a high degree of reproducibility for the cell assay. Samples were obtained from three different refineries and the storage time in glass and Nalgene was varied (Table VII).

TABLE VII

REPLICATE CELL ASSAYS WITH FLASH EVAPORATES
FROM STORED EFFLUENTS

Effluent Flash Evaporate at 45%*	Storage Time in Weeks Cond. I (glass)	Cond. II (Nalgene)	% Growth at 66 hours**
DPQ-E, January			
Treated	5	0	4,0
	4	1	86,86
	3	2	14,18
	2	3	37,30
	1	4	34,26
Untreated	5	0	31,28
	4	1	34,23
	3	2	45,44
	2	3	51,59
	1	4	74,69
DPQ, February			
E Treated	3	0	95,82
	0	3	83,86
E Untreated	3	0	70,65
	0	3	60,57
W Untreated	3	0	64,55
	0	3	16,17
BQB, April			
22% Treated	1	0	46,46
	0	1	48,48
11% Part. Treated	1	0	43,50
	0	1	55,59

*Unless otherwise indicated.

**The original and replicate assay values are separated by a comma. At 66 hours, 100% growth is equivalent to $8.6 - 9.8 \times 10^5$ cells/ml.

The reliability of the cell assay with effluents was determined by measuring the variation between and within experiments for several effluents. This information was obtained by repeating assays one day apart to avoid storage time changes in the effluent. Variation between experiments was 13% and within experiments was 8%. It is apparent from these data that the toxicity of the effluents varied with time and method of storage.

The variability of the Daphnia test was evaluated with the same concentrations of effluent as in the replicate cell assay (Table VIII). Variation between experiments was 100%; variation within experiments was 50%.

Survey of Effluents

A survey of effluents was designed in which samples obtained by immediate flash evaporation were tested to determine the relative toxicity of several refineries (Table IX) and stored for different periods in glass (Table X) or Nalgene (Table XI) and retested. Among eight samples from four locations, only three samples caused more than 50% inhibition at a 45% concentration of flash evaporate. Only one sample was toxic at an 11% dilution of flash evaporate. Although trends existed in the corresponding Daphnia test, results did not correlate with the cell assay (Table XII).

In this survey, toxicity of some effluents apparently changed during storage (Tables X and XI). To evaluate the change in toxicity during storage, a sequential study was performed with samples from two refineries in the same city, enabling storage and transportation times to be equal. Both refineries had untreated process effluents and

TABLE VIII

REPLICATE *DAPHNIA* ASSAYS WITH FLASH EVAPORATES FROM STORED EFFLUENTS

Effluent Flash Evaporate*	Storage Time (weeks) Cond. I (glass)	Cond. II (Nalgene)	Number Surviving** Time in Hours 1	2	4	8	12	24	48
DPQ-E, January	Control		4,4	4,4	4,4	4,4	4,4	4,4	4,4
Treated	5	0	1,0	0,0	0,0	0,0	0,0	0,0	0,0
	4	1	4,3	4,1	2,1	2,1	0,0	0,0	0,0
	3	2	1,0	0,0	0,0	0,0	0,0	0,0	0,0
	2	3	0,1	0,1	0,0	0,0	0,0	0,0	0,0
	1	4	4,3	0,3	0,1	0,1	0,0	0,0	0,0
Untreated	5	0	4,4	4,4	4,4	3,1	0,0	0,0	0,0
	4	1	4,4	4,4	4,4	2,2	2,1	1,1	0,0
	3	2	4,4	2,4	2,4	2,0	0,0	0,0	0,0
	2	3	4,4	4,4	4,4	4,3	0,4	0,4	0,3
	1	4	4,4	4,4	4,4	4,4	4,4	0,4	0,3
DPQ, February	Control		4,4	4,4	4,4	4,4	4,4	3,4	1,4
E Treated	3	0	4,4	4,4	4,4	4,4	3,4	1,4	0,1
	0	3	4,4	4,4	4,4	4,4	4,4	4,4	1,1
E Untreated	3	0	4,2	4,1	1,0	1,0	0,0	0,0	0,0
	0	3	4,4	4,4	2,3	2,3	0,3	0,0	0,0
W Untreated	3	0	4,2	4,0	4,0	2,0	2,0	2,0	1,0
	0	3	4,4	4,4	4,4	1,2	0,1	0,0	0,0
BQB, April	Control		4,4	4,4	4,4	4,4	4,4	4,4	3,4
22% Treated	1	0	4,4	4,4	4,1	2,1	2,0	0,0	0,0
	0	1	4,4	4,4	3,4	3,2	2,2	0,1	0,0
11% Partially Treated	1	0	4,4	4,4	4,4	4,3	2,2	2,1	0,2
	0	1	4,4	4,4	3,4	1,4	0,4	0,4	0,4

*45% dilution, unless otherwise indicated.

**The data are listed to indicate the number surviving at the time indicated in the original and replicate assay. For example, 4,1 indicates that all 4 *Daphnia* have died in the original assay, but only 1 of 4 has died in the replicate assay at the time indicated.

TABLE IX

SURVEY OF EFFLUENTS IMMEDIATELY FLASH EVAPORATED

Source	Month	% Flash Evaporate	% Growth at 66 Hours
NDD	10.72	45	62
		22	56
	11.72	45	70
		22	71
ETU 10.60	11.72	45	99
		22	99
ETE-SI	11.72	22	65
		11	79
		5	80
BQB #3 oil	11.72	22	70
		11	80
		5	99
BQB final	11.72	22	87
		11	83

TABLE X

STUDY OF EFFLUENT IMMEDIATELY FLASH EVAPORATED AND STORED IN GLASS

Source	Month	% Flash Evaporate	% Growth at 66 Hours Weeks Stored in Glass (Cond.I) 0	1	2	3	4
NDD	10.72	45	60		33		
		22	56		67		
ETU 10.60	10.72	45		37	33		
		22		54	68		
BQB#3 oil	10.72	45				0	
		22				31	0
		11					83

TABLE XI

COMPARISON OF STORAGE OF FLASH EVAPORATES AND EFFLUENTS

Storage Condition	% Flash Evaporate	% Growth at 66 Hours
I	45	37
	22	54
	11	52
II	45	71
	22	85

Sample is from ETU 10.60 10.72.

Storage Condition I = two weeks storage.

Storage Condition II = two weeks storage.

At 66 hours, 100% growth is equivalent to $8.6 - 9.5 \times 10^5$ cells/ml.

TABLE XII

DAPHNIA SURVIVAL IN EFFLUENTS IMMEDIATELY FLASH EVAPORATED

Effluent	Number of Surviving of 4 Daphnia — Time in Hours						
	1	2	4	8	12	24	48
Pond	4	4	4	4	4	3	1
NDD 10.72	0	0	0	0	0	0	0
11.72	4	4	4	2	2	0	0
ETU 10.72	0	0	0	0	0	0	0
11.72	0	0	0	0	0	0	0
ETE 11.72	4	4	2	0	0	0	0
BQB#3 10.72	4	0	0	0	0	0	0
11.72	0	0	0	0	0	0	0
BQB final							
10.72	4	4	4	4	4	3	3
11.72	4	3	3	1	1	0	0

Four Daphnia were tested in a total volume of 10 ml of 45% flash evaporate from the effluent indicated.

treated final effluents. All samples were stored six days in the original containers at room temperature before flash evaporation. The conditions for storage in glass and Nalgene were discussed in Materials and Methods. Less changes occurred after immediate flash evaporation than when the effluent was stored in Nalgene (Tables XIII - XIV). Changes in samples stored in glass in the cold were less frequent and less severe than for samples stored in Nalgene at room temperature. While none of the glass-stored flash evaporates had more than one change, they may increase, decrease, or remain the same in toxicity. None of the flash evaporates stored in glass changed during the first week. This time is sufficient to perform the necessary rangefinding assays. During the same period of time, only 60% of the Nalgene-stored samples at room temperature retain the original level of toxicity. After one month of storage, only 20% of the Nalgene-room temperature-stored samples remained stable in toxicity, while 60% of the glass-stored samples remained toxicity. A summary of the number of flash evaporates that did or did not change in toxicity is presented in Table XVI. Although stored samples could either increase or decrease in toxicity, decreases were much more common. Seventeen effluents were tested for one week of storage. Ten effluents were tested throughout one month of storage.

TABLE XIII

EFFECT OF STORAGE OF FLASH EVAPORATES OF DPQ-E ON CELL GROWTH

Date: Site	%FEV*	% Growth at 66 Hours								
		No Storage	Weeks Stored Condition I				Weeks Stored Condition II			
			1	2	3	4	1	2	3	4
January, 1973:										
Treated	45	0	12	11	0	4	53	4	45	52
	22	36	42	38	37	49	97	31	78	83
Untreated	45	0	--	0	74	81	--	0	72	82
	22	92	91	--	--	--	91	--	--	--
February, 1973:										
Treated	45	73	88	98	95	96	81	95	83	99
	22	83	--	--	--	--	--	--	--	--
Untreated	45	55	69	67	70	66	78	83	60	87
April, 1973:										
Treated	45	94	96	--	--	--	58	--	--	--
Untreated	45	94	90	--	--	--	87	--	--	--

*FEV = flash evaporate.

At 66 hours, 100% growth is equivalent to $8.6 - 9.9 \times 10^5$ cells/ml.

TABLE XIV

EFFECT OF STORAGE ON FLASH EVAPORATES OF DPQ-W ON CELL GROWTH

Date: Site	%FEV*	% Growth at 66 Hours								
		No Storage	Weeks Stored Condition I				Weeks Stored Condition II			
			1	2	3	4	1	2	3	4
January, 1973:										
Treated	45	77	91	63	62	60	98	96	78	97
	22	90	95	98	88	86	99	99	96	99
Untreated	45	71	80	72	68	66	88	84	68	8
	22	96	--	--	--	--	--	--	--	--
February, 1973:										
Treated	45	96	84	92	--	88	--	--	--	93
Untreated	45	36	23	31	55	52	23	33	16	83
	22	72	--	--	--	--	--	--	--	--
April, 1973:										
Treated	45	76	85	--	--	--	72	--	--	--
Untreated	45	16	8	--	--	--	12	--	--	--
	11	68	--	--	--	--	--	--	--	--

*FEV = flash evaporate

At 66 hours, 100% growth is equivalent to $8.7 - 9.5 \times 10^5$ cells/ml.

TABLE XV

EFFECT OF STORAGE OF FLASH EVAPORATES OF BQB ON CELL GROWTH

Date: Site	FEV* Conc.	% Growth at 66 Hours		
		No Storage	Stored One Week	
			Condition I	Condition II
4.73:				
Treated	45%	13	--	--
	22%	42	46	48
	11%	97	--	--
Partially Treated	45%	4	--	--
	22%	9	--	--
	11%	34	43	55
	5%	90	--	--

*FEV = flash evaporate.

At 66 hours, 100% growth is equivalent to $9.1-9.3 \times 10^5$ cells/ml.

TABLE XVI

NUMBER (PER CENT) OF FLASH EVAPORATES AND EFFLUENTS THAT REMAINED STABLE DURING STORAGE

Storage Time	Flash Evaporate			Effluent		
	A	B	C	A	B	C
Tested after:						
One Week	17(100)	0(0)	0(0)	10(59)	2(12)	13(76)
First Week	10(100)	0(0)	0(0)	6(60)	0(0)	4(40)
Second Week	7 (70)	1(10)	2(20)	3(30)	2(20)	0(0)
Third Week	6 (60)	0(0)	1(10)	3(30)	0(0)	3(30)
Fourth Week	6 (60)	0(0)	0(0)	2(20)	0(0)	4(40)
Summary of One Month	6 (60)	1(10)	3(30)	2(20)	2(20)	11(60)

Data for this summary table was obtained from Tables XIII - XV. Samples from 17 sources were tested for one week storage. Ten samples were tested for each week throughout a month.

A = no statistically significant change in toxicity during storage.

B = increase in toxicity during storage.

C = decrease in toxicity during storage.

Application to Effluent Fractions

Flash Evaporate Fractionation

One of the applications of the cell assay is the identification of toxic fractions in an effluent. Identification can be attempted by using different methods of extraction and comparing results, or by comparing smaller fractions obtained by one method to a larger fraction of known toxicity.

Routine analysis of an effluent involved the collection of the first 75 ml of flash evaporate. Three 25 ml portions of flash evaporate were then collected sequentlly from a second portion of effluent from the same Nalgene container. These fractions were used to locate those portions of the flash evaporate which were most toxic. Replicate cell assays were performed with a series of individual 25 ml fractions, a combination of these fractions, and the total 75 ml fraction (Table XVIIA). There was no loss in toxicity in the fractionation process since the combination and total flash evaporate produced the same degree of inhibition. The test demonstrated excellent reproducibility when the cell assay was used. The Daphnia test did not; it had as much as 75% variability (Table XVIIB).

From a series of five samples with known toxicity, the particular subfraction(s) which contained the major portion of the toxicity and the pattern of volatilization of the toxic portions varied among the effluents (Table XVIII). Certain effluents had fractions which were all toxic. Other effluents contained fractions which were totally nontoxic. The third fractions were always toxic. Toxicity could be either marked or just statistically significant. In two samples, non-inhibitory or

TABLE XVII

REPLICATE ASSAYS WITH FRACTIONS OF FLASH EVAPORATES

A. Cell Assay

Effluent Flash Evaporate at 45%*	% Growth at 66 Hours**
Treated	
Total 75 ml	79,72
Untreated	
Total 75 ml	14,12
First 25 ml	8,5
Second 25 ml	30,28
Third 25 ml	40,36
Combination of 3 fractions	15,11

B. Daphnia Assay

Effluent Flash Evaporate at 45%*	Number Surviving of 4 Daphnia** Time in Hours 1	2	4	8	12	24	48
Control	4,4	4,4	4,3	4,3	4,3	4,3	4,3
Treated							
Total 75 ml	4,4	1,1	0,0	0,0	0,0	0,0	0,0
Untreated							
Total 75 ml	0,0	0,0	0,0	0,0	0,0	0,0	0,0
First 25 ml	1,3	0,1	0,0	0,0	0,0	0,0	0,0
Second 25 ml	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Third 25 ml	2,0	1,0	0,0	0,0	0,0	0,0	0,0
Combination of 3 fractions	3,0	2,0	0,0	0,0	0,0	0,0	0,0

*Fractions obtained from DPQ-W April samples, stored for one week in Nalgene before flash evaporation.

**The data are listed with the original assay value first and the replicate assay value following the comma.

TABLE XVIII

EFFECT OF FLASH EVAPORATE FRACTIONS ON CELL GROWTH

Flash Evaporate (FEV)	Fraction	Weeks Stored (Condition I)	% Growth at 66 Hours
DPQ-E, February, 1973; Untreated; 45% FEV	Total (75 ml)	2	86
	First 12 ml		75
	Second 25 ml		58
	Third 25 ml		81
DPQ-W, February, 1973; Untreated; 45% FEV	Total (75 ml)	2	34
	First 25 ml		45
	Second 25 ml		77
	Third 25 ml		24
	Calculated Average		48
DPQ-W, April, 1973; Untreated; 45% FEV	Total (75 ml)	1	12
	First 25 ml		5
	Second 25 ml		28
	Third 25 ml		36
	Combination		11
	Calculated Average		27
BQB, April, 1973; Partially Treated; 11% FEV	Total (75 ml)	1	59
	First 25 ml		100
	Second 25 ml		24
	Third 25 ml		79
	Calculated Average		67
BQB, April, 1973; Treated; 22% FEV	Total (75 ml)	1	48
	First 25 ml		63
	Second 25 ml		97
	Third 25 ml		9
	Calculated Average		56

At 66 hours, 100% growth is equivalent to $8.7 - 9.9 \times 10^5$ cells/ml.

slightly inhibitory fractions occurred between two fractions with greater toxicity. A toxic fraction also occurred between two less toxic fractions in two samples. In four cases for which the arithmetic average of the subfractions was compared to that for the total fraction, no significant difference occurred. This result verified that no toxicity was lost in any of the fractionation processes (Table XVIII).

Other Methods of Fractionation

Solvent extraction was utilized as an additional method of concentrating effluents. Both methylene chloride and hexane extracts were tested. The cell assays were performed to determine if the preparatory procedure for solvent extracts increased toxicity. Flasks with and without nitrogen bubbled through the medium yielded the same cell counts as the control flasks. Four solvent extracts were analyzed. Methylene chloride extracts of effluents for two refineries were not inhibitory at 10 ppm. The methylene chloride extract of a later sample from one of these refineries inhibited growth 73% at 50 ppm. The same concentration of hexane extract of that effluent inhibited growth 90%. The lack of inhibition with certain extracts demonstrated that residual solvent was not creating toxicity. Toxicity could be detected, since inhibition was exhibited by two of the four extracts. Assays were not repeated to obtain a measure of reproducibility.

Cell assays with the filtered total effluent were performed on samples taken at the same time from two sources within the same refinery. Increased concentrations of effluent caused an increase in cell growth inhibition. There was no difference between the level of toxicity of the degradation ditch samples and the final effluent (Table XIX).

TABLE XIX

EFFECT OF FILTERED TOTAL EFFLUENT ON CELL GROWTH

Sample	Concentration of Flash Evaporation	% Growth at 66 Hours
Control		100
Degradation Ditch	5%	70
	15%	43
	25%	33
	50%	3
Final Effluent	5%	60
	15%	43
	25%	30
	50%	10

At 66 hours, 100% growth is equivalent to 8.6×10^5 cells/ml.

Variabilities of Effluents and *Daphnia*

Effluent

Samples from two refineries were analyzed for three months to determine the variability between and within refineries and toxicity changes during storage. In the series of DPQ samples (Table XIII), the treated effluent was the most toxic for January samples, the untreated effluent the most toxic for February samples, and neither effluent was toxic enough to cause cell inhibition in April. This monthly pattern illustrates the variability that occurs within a refinery.

DPQ-W samples also demonstrated variability when the same sample sites were assayed (Table XIV). Treated and untreated January samples caused the same degree of inhibition, but the untreated effluent was

more toxic than the treated in February and April. Treated April samples caused inhibition; treated February samples did not.

Considerable variability also occurred between refineries (Tables XIII - XV). Untreated April samples were not toxic for DPQ-E, were toxic for DPQ-W, and were extremely toxic for BQB. Treated samples were non-toxic for DPQ-E, slightly toxic for DPQ-W, and totally inhibitory for BQB.

The storage properties of the effluent also depended on the nature of the effluent. The number of toxicity changes in effluents stored in Nalgene depended on the particular sample (i.e., date of sample). DPQ-E samples decreased, were marginally variable, and stable in toxicity for 45% effluent from the same sample site for January - April samples (Table XIII). DPQ-W samples demonstrated a marked increase, decrease, and stability in toxicity for 45% effluent from untreated sample sites for January - April samples (Table XI). A summary of the proportion of samples that changed during storage is presented in Table XVI. The number and severity of changes that occurred in a sample depend on its components, which are variable. Storage properties could not be predicted.

Daphnia Test

The variable susceptibility of individual Daphnia produced unreliable results for concentrations of m-cresol below 40 ppm, even with four times the usual number of test organisms (Table V). The variability of Daphnia was also demonstrated with effluents (Table VIII). The cell assay could not be correlated with the Daphnia test because the Daphnia results themselves were inconsistent. Hence, any attempt to

compute correlation coefficients would be fruitless, and possibly misleading. Trends were observed, but variability precluded quantitative examination via linear regression techniques.

CHAPTER IV

DISCUSSION

Advantages of Cell Culture

Cell culture techniques are more sensitive and reliable than are studies with intact organisms. Reproducibility is better with the cell assay because it decreases the effect of individual variability due to such factors as age, sex, and previous environmental conditions and avoids the problem of water variability due to such factors as pH, hardness, and other complicating factors. Fish bioassays often are not reproducible because of the varying contents of the diluent water. Cell culture medium is commerically available and, therefore, should make assays done in different laboratories comparable. Cell assays are initiated at 3×10^6 cells/flask and completed at approximately 15×10^6 cells/flask. Intact organism bioassays are performed with a very much lower number of organisms in the test population. Daphnia assays are generally performed with four or fewer individuals and fish bioassays often involve 10 fish at each concentration. The larger the population tested the better the estimates of the true population value, and the larger the sample the more the error of estimation is decreased. Therefore, the cell assay should give more reliable results than the intact organism assays.

The action of a toxic material may be too weak to be expressed at

lower concentrations, cause inhibition at intermediate concentrations, or cause death at higher concentrations. Intact organism bioassays use death as the criterion for a positive effect. Since the cell assay uses inhibition as the indicator of an effect, it should detect lower concentrations of toxic components in the effluents. Toxicity is detected if cell replication is affected. Organism assays usually only involve one state in the life cycle; i.e., the reproductive stage is not tested.

The Daphnia bioassay was compared to the cell culture assays to evaluate the relative sensitivities of the two methods. Cell culture was more sensitive than the Daphnia bioassay in detecting m-cresol toxicity. The threshold concentration for the Daphnia assay was above 20 ppm, but was only about 2 ppm for the cell population. Variability was less and, therefore, the reproducibility better with the cell assay (15% variation between experiments) than for the Daphnia bioassay (100% variation between experiments). The variable susceptibility of individual Daphnia produced unreliable results even with four times the usual number of test organisms. A small number of individuals can give reliable results only if they have uniform susceptibility, which Daphnia do not. The Daphnia test is less time consuming to perform than cell culture and, therefore, would be useful for broad screening of effluents to determine what concentrations of effluent to test in the range-finding cell culture assay.

Evaluation of Cell Culture Technique

A reference compound was used to evaluate the parameters of the cell assay, to estimate the sensitivity and reliability of the test, and to compare the cell assay to a routinely used method. The reliability

of the assay was doubled when suspension rather than monolayer cultures were used as the source of experimental cells. The 66-hour reading had the widest spreading of data points and was the last count before uninhibited cells were more affected by density-dependent inhibition than by test material. Therefore, per cent growth (%G) at this point was used for the comparison of different experiments. Other investigators have also used end-point values of some type.

Rachlin and Perlmutter (1968) were the first to suggest cell assay of aquatic pollutants. A commonly available, easily maintained cell line derived from the fat head minnow (FHM) was compared to the intact fathead minnow. The TL_m (the concentration that kills 50% of the organisms by a specified time) for the fish was 8.9 ppm zinc for 24 hours, 7.8 ppm for 48 hours, and 7.6 ppm for 96 hours. The sublethal concentration of 1.8 ppm zinc reduced the mitotic index in the cell culture from the normal 1.3% to 0.5%, indicating that tissue culture responded with greater sensitivity to the toxicant than did the intact organism. It was suggested that water quality standards be based on the "biologically effective concentration" (i.e., the concentration that exerts no toxic action on cell culture) rather than the currently accepted "biologically safe concentration". One reason is that only adults are now tested, while embryos and newly hatched fish are more sensitive to zinc. Rachlin and Perlmutter (1969) later expanded their assay to an additional fish cell line (RTG-2).

Fisher (1969) was the first to use mammalian cell culture to evaluate possible water pollutants. Toxicity was indicated by a decrease in relative plating efficiency (RPE) of human or bovine cells. Since cells were stained before counting, this was an end-point

determination. Metals such as zinc, cadmium, iron, and mercury were tested. For the first time, more complex possible pollutants were evaluated. Pesticides such as DDT, Rotenone, and Dieldrin, herbicides such as 2,4D (2,4-dichlorophenooxacetate) and 2,4,5T (2,4,5-trichlorophenooxyacetate), and mutagens such as thalidomide and lysergic acid were analyzed for toxic action. Some of the commonly used pesticides and herbicides were more toxic than "emotionally charged compounds" such as thalidomide and lysergic acid. The conclusion was that cell assays should be used to evaluate alternatives before banning some pollutants. Alternative pesticides were 100-fold more toxic than dieldrin and DDT. The plating technique has been utilized by Malcolm to study the mechanism of toxicity. This assay now appears in the book Bioassay Techniques and Environmental Chemistry (Malcolm, Pringle, and Fisher, 1973).

Monolayer cells were used in the fish and mammalian cell assays discussed above. Li and Jordan (1969) developed a suspension cell system to evaluate a number of pesticides and herbicides as possible pollutants. Suspension cultures allow sampling from the same individual culture throughout the experiment. L cells were kept in suspension in spinner cultures. Test material was added on Day 0. Initial cell concentration was between 1.5×10^5 and 2.5×10^5 cells/ml. Daily counts were taken with a Coulter Counter for three days. Inhibition was indicated if cell growth was inhibited 50% or more. Malathion was inhibitory at 25 ppm, DDT and Lindane both at 10 ppm, and 2-4-D ester at 5ppm. One experiment was repeated with 50 ppm DDT at initial cell densities of 1.6×10^5 and 3.2×10^5 cells/ml. The respective replicate values were 48.6 (51.4) and 15.2 (17.4) per cent inhibited. This is the

same degree of replication obtained in the present investigation (Tables III and VII). Since Li and Jordan used pure compounds and replication obtained in the present study with both an organic reference compound and actual effluents were comparable to theirs, it may be concluded that cell cultures can be effectively used to assay toxicity of complex mixtures of materials.

Li and Traxler (1972) studied the factors governing the sensitivity of their bioassay system developed earlier (Li and Jordan, 1969; Li et al., 1970). This system, developed independently, is essentially the same as that described and evaluated in the present study. The major difference is that Li used spinner cultures rather than shaker cultures to maintain the L cells in suspension. This difference should not affect the parameters of the cell assay.

The initial concentration used by Li and Traxler (1972) was 2×10^5 cells/ml. When the cells were obtained from the actively growing phase, 10 µg/liter (10 ppm) HgCl gave the first significant inhibition while cells from the stationary phase were equally sensitive to 2.5 ppm. Sensitivity was increased by decreasing medium supplements from 10% calf serum to no calf serum and 0.5% peptone. It was hypothesized that the binding of HgCl to serum protein makes less Hg^{++} available or that bound mercury could be less toxic to the cells. It would be interesting to determine if reduced serum content would increase the sensitivity of the cell assay with an organic reference compound.

Growth curves were presented, but all comparisons between experiments were made with the per cent inhibition on Day 3. Comparison of the effects of HgCl on cell respiration and cell growth determined that the cell assay (10 ppm) was much more sensitive than cell respiration

(1000 ppm). Respiration may be a less sensitive indicator because cell replication is not necessary. Since the cell respiration tests take five hours rather than three days, it was suggested that respiratory measurement be used as a preliminary test for the screening of heavily polluted samples.

Cell assays discussed in this study were initiated with L-M cells in suspension at 1.5×10^5 cells/ml and counts taken with a Coulter Counter for three days. A statistically significant effect on cell growth occurred with 2 ppm m-cresol. This is the same sensitivity Li observed with HgCl.

Although titles such as "Fish Cells in Culture for Study of Aquatic Toxicants" (Rachlin and Perlmutter, 1968), "Bioassay of Water Pollutants with Cultured Mammalian Cells" (Fisher, 1969), "Use of Spinner Culture Cells to Detect Water Pollution" (Li and Jordan, 1969), and "Tissue Culture Bioassay Method for Water Pollution with Special Reference to Mercuric Chloride" (Li and Traxler, 1972) strongly imply the application of the particular cell assay to polluted water samples, only pure reference compounds have been tested. These published papers have discussed the inherent variabilities in fish assays and suggested application of cell culture to water pollution samples, but only commercially available potential pollutants have actually been assayed with cell culture.

Intensive investigation by the analytical section of the Reservoir Research Center over a four-year period has identified one compound. One gas chromatograph tracing may have as many as 15 poorly separated peaks in several locations. Effluents contain heavy metals, several families of organic compounds, and complexes of organics and inorganics. In a mixture as complex as effluents, synergism is likely to occur; that

is, the compounds present may interact in such a way as to be either more toxic or less toxic than the individual components. The step-by-step approach would not only involve the testing of all possible pollutants but all combinations thereof. If the water samples are assayed, they can be tested for their biological effect "in toto".

Effluents are too complex and incompletely known to be evaluated by an additive approach. The feasibility of the application of cell culture technique to effluent as it exists in nature was evaluated in this study. Cell culture assays have the same degree of reproducibility with effluents (variation between experiments of 13%) as with a reference compound (variation between experiments of 15%), Table III.

Application to Effluents

The cell assay was reproducible with petroleum refinery effluents (Table VII). Samples obtained during a four-month period demonstrated reproducibility with treated and untreated effluent from different refineries. Replicate cell assays with serial fractions were also reproducible. Therefore, the cell assay can be applied to the study of effluents.

The cell assay was applied to a series of 22 samples from eight plants to evaluate their relative toxicities. Extended analysis of three refineries revealed properties that should be considered in future experimental designs. The toxicity of the effluents changed during storage. Immediate flash evaporation, filtration, and storage at 4 C in glass prescription bottles decreased both the number and severity of toxicity changes. This procedure should simplify the complex mixture, remove bacteria which would normally continue to act on the effluent,

and reduce the rate of further chemical reactions by reducing the temperature of the liquid. Immediate flash evaporates did not change during the first week of storage. This is sufficient time to perform range finding and fractionation assays before storage changes occur. In the future, effluent samples should be scheduled to be received so that it is possible to process them immediately.

Effluents had a high degree of variability. Extreme variability occurred both between and within refineries. Effluent from a certain site at a particular refinery may have extreme toxicity one month and be non-toxic the next (Table XIII). Therefore, frequent sampling is necessary to evaluate a particular refinery adequately. The extreme differences between refineries suggests that a study designed to ascertain characteristics of effluents incorporate several refineries into the study. Generalizations about effluents made from a small number of sample sites is likely to be misleading.

More than a week often passes between the receiving of an effluent to be treated at a refinery and the release of the same effluent in treated form. Effluent samples from different sites within a refinery are separate entities and are not from a single effluent in various stages of treatment. For this reason, treatment processes cannot be compared if untreated and treated samples are taken on the same day. In the extended study of effluents, the treated samples were more toxic than the untreated samples for two independent refineries in April. This does not necessarily indicate that the treatment process created toxicity, but could suggest that a more toxic process effluent had been received at an earlier date. If treatment processes are to be compared,

samples should be collected sequentially as an individual process effluent is treated.

Sequential fractionation of flash evaporates revealed that toxicity could be expressed by any or all of the fractions. Since exposure to air decreased toxicity and the flash evaporation process concentrated toxicity, it has been assumed that toxic components were in the more volatile fraction. For this reason, only the first 25 or 50 mls had been collected for biological evaluation. The data on sequential fractionation show that if an additional volume of test material is needed, samples from the original effluent must be flash evaporated, rather than double volumes collected. In addition, the data demonstrated that toxicity is not found only in the first portion of the flash evaporate. Further studies should involve additional fractions. Sequentially collected 5 to 10 ml volumes of up to 20 fractions should be assayed by both chemical and biological means. Each fraction would probably contain many of the same compounds as the previous fraction in the series with either addition or deletion of a component(s). Cell assays would identify the fractions with biological activity. Chemical analysis of the fractions would locate the similar compounds. Identification of compounds could then be limited to the chemical entities found only in toxic fractions. This correlation of biological and chemical analysis may lead to the identification of toxic compounds. The analysis of effluents has a twofold purpose: to designate which effluents are so toxic that they should not be released until the biological effect is negated, and to identify the toxic components of the effluent so that the refinery involved can locate the process that is creating the toxic material.

The patterns of toxicity observed in sequential fractionation suggest that more than one component is responsible for toxicity (Table XVIII). The particular fraction that contained the major portion of the toxicity varied; it could be any or all of the fractions. Non-inhibitory or slightly inhibitory fractions occurred between two fractions with greater toxicity. This also indicates that at least two compounds or groups of compounds were responsible for cellular toxicity. Survival curves may also suggest that more than one toxic component is present.

CHAPTER V

SUMMARY AND CONCLUSIONS

Cell culture can be applied to the study of effluents or effluent fractions.

Reproducibility with the cell assay was shown with m-cresol as the reference compound. The precision of the assay was doubled when suspension rather than monolayer cells were used to initiate experiments.

Cell culture technique controls a greater number of variables and is a more sensitive and reliable assay than the Daphnia test. The variability of the Daphnia test suggests that it should only be used in the broad screening of effluents, and even for this use controls and multiple test organisms per sample are necessary.

The cell assay was applied to petroleum refinery effluents and was reproducible. Effluents had a high degree of complexity and variability. Variability occurred between refineries, within refineries, between samples, and under varying storage conditions. Therefore, samples should be processed immediately. Frequent sampling is necessary to evaluate a particular refinery adequately. There were several indications that toxicity was caused by more than one agent.

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