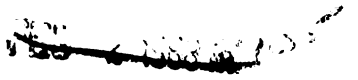
A CONTINUOUS MICROBIAL GROWTH APPARATUS FOR PESTICIDE-MICROORGANISM INTERACTION STUDIES

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
Elwin D. Evans
1966



ABSTRACT

A CONTINUOUS MICROBIAL GROWTH APPARATUS FOR PESTICIDE-MICROORGANISM INTERACTION STUDIES

by Elwin D. Evans

A continuous turbidiometric, recording, microbial growth apparatus was designed to study the effect of selected insecticides on microorganism activity. Escherichia coli B strain was cultured under continuous and static aerobic conditions with Anderson's minimum salt nutrient media. Culture cell densities were controlled under continuous culture conditions by the culture wash-out rate or by using glucose at the rate of 0.1 gm./liter as a limiting growth factor. Use of a limiting growth factor was most amenable to pesticide-microorganism interaction studies. Lindane, DDT, carbaryl and aldrin had no effect on the growth rate or culture cell densities. Malathion increased cell densities when added to a glucose limited media. This was an indication that the malathion was metabolized as a nutrient.

Only small fractions of lindane and malathion were removed from the culture media by the bacterial cells under continuous culture conditions with a glucose limited media.

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Elwin D. Evans

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Entomology

1966

ACKNOWLEDGMENTS

I wish to thank the members of my Guidance Committee for their contributions to this thesis. Dr. Roger Hoopingarner, Department of Entomology for the use of his laboratory facilities; Dr. Gordon Guyer, Chairman, Department of Entomology and Dr. James Butcher, Department of Entomology and Dr. Robert Ball, Department of Fisheries and Wildlife. I would also like to thank the Michigan State University Institute of Water Research for the generous grant and Jack Harten for analyzing the many insecticide samples by gas chromatography.

Lastly, I wish to thank my wife, Marlene, for her innumerable contributions and concessions, but mostly for caring for the children, Cheryl, Loreene, Dana, Shelly and Jay, while this was completed.

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INTRODUCTION

Research on the interactions of pesticides and microorganisms has been seriously hindered by the inadequacy of available microbial culture methods. Static or batch culture methods have the inherent problem of continuous environmental change during the culturing period.

Accurate measurement of the environmental conditions under which specific interactions occur requires a constant and continuous culture method in order to maintain constant experimental conditions. The objective of this research was to design and assemble a simple, continuous, microbial culture apparatus and to devise the operational procedures while evaluating the applicability of the equipment to pesticide microorganism research.

LITERATURE REVIEW

The use of the continuous culture technique in the study of the interactions between microorganisms and hydrocarbon pesticides has not been reported in the literature, although interactions between microorganisms and certain hydrocarbons have been known for over seventy years. Zobell (1946) reviewed the bacteriological aspects of hydrocarbon utilization by microorganisms. He reported that microorganisms are widely distributed in nature, having been isolated from soil, sewage effluent, water, marine sediments, oil fields, etc. Zobell (1950) demonstrated that the susceptibility of a hydrocarbon to attack is directly related to the molecular configuration of the hydrocarbon molecule. Long, unsaturated hydrocarbon molecules are attacked quite easily. Shorter saturated forms are even more susceptible, and branched forms are the most easily attacked. Polycyclic molecules have not been studied as extensively. Polycyclic molecules having side chains or carbon molecules attached to the ring structures are more readily attacked than are those which lack attached carbon molecules or which have a molecule or molecules other than carbon attached to the ring structure. A review by Fuhs (1961) lists over 100 species of yeasts, bacteria and fungi that utilize hydrocarbons for an energy source. Foster (1962) reviewed hydrocarbon metabolism research in relationship to industry. Trecanni (1963) described the general metabolic pathways exhibited by microorganisms in the degradation of hydrocarbons and

their derivatives. A more recent review of naturally occurring hydrocarbons is that of Kallio and McKenna (1965).

Although interactions between soil microorganisms and the organic pesticides have been studied for many years, an extensive literature on the subject has appeared only recently. Since the work of Wilson and Choudhri (1946) with DDT, in which no effect was noted in the soil microorganisms or their processes, many conflicting reports have appeared. These studies indicate that soil as an ecosystem is highly complex and variable. Under certain conditions pesticides can inhibit or destroy soil microorganisms or increase other microorganisms by acting as a stimulant, a substrate or by eliminating competitors or predators in the soil. Pesticide-microorganism research is the subject of reviews by Martin et al. (1958), Fletcher (1960) and Bollen (1961).

In foregoing studies, the enrichment culture technique universally has been used in laboratory research. This procedure yields useful information but will not always explain or corroborate field experimental results. Furthermore, the culture techniques used will not always allow the investigator to ascertain pesticide degradation sequences nor the exact environmental condition at the time these reactions occur. Constantly changing environmental conditions are inherent in this culture method.

The continuous culture of microorganisms has been used in industry since the early 1900's with limited knowledge of the kinetics of the apparatus being used. Schniebel (1902), Schalk (1906), Vas Rijn (1906) received patents for apparatus used in the brewing industry. These are being used today in many associated industries (Green, 1962). Perhaps the first instrument devised to grow cells at their maximum growth rate

in the laboratory was that devised by Moyer (1929) for Chlorella spp., Euglena spp. and several other species of blue-green algae. Myers and Clark (1944) devised a mechanically operated device to culture Chlorella spp. for chemical study.

Almost simultaneously Monod (1950) and Novick and Szilard (1950) elucidated the basic principles involved in the continuous culture of microorganisms in devices called "Bactogen" and "Chemostat" respectively. Additional contributions on the mathematical aspects have been made by Finn and Wilson (1954), Spicer (1955), Fox (1955), Herbert, Elworth and Telling (1956), Kunitz (1957), Moser (1957, 1958) and Herbert (1959, 1961). Symposia were held in Prague, Stockholm and Chicago in 1958 and in London (1960) on the continuous culture of microorganisms and application (James 1961). The first review of the literature of continuous culture was by Novick (1955) followed by Gerhardt and Bartlett (1959) and more recently by James (1961) and Holmes (1962).

The literature has many reports of devices for the continuous culture of microorganisms in the laboratory. The review of Novick (1955) covered this aspect of continuous culture. Since that time Elsworth and Meakin (1956), Perret (1957) and Dawson (1963) have produced other designs. The cyclone column unit of Dawson apparently has certain advantages over many of the other devices, in that single-celled, as well as filamentous microorganisms can be grown continuously for periods of time in excess of three months without growth appearing on the walls of the culture chamber. Anaerobic as well as aerobic conditions for culturing are easily obtained.

The use of a continuous culture apparatus as a tool for analysis or cell propagation in the laboratory is infrequently reported in the

literature. Pollock (1952) reported on penicillinase adoption of Bacillus cereus by using continuous culture techniques. Di Cuollo (1964) used this technique as a bioassay tool for antibiotics.

Perhaps the most interesting use to which this technique is applied is in the area of rumen research. Adler (1958), Stewart (1961), Quinn (1962), Hobson (1962), Rufne (1963) and Sylter (1964) have used these techniques to culture the microorganisms of the rumen gastro-intestinal tract, either in pure culture or as natural populations to ascertain the role of these microorganisms in the digestive processes of the rumen.

The use of the continuous culture technique as a laboratory tool is relatively new and will undoubtedly find widespread use in the fields of metabolism, aquatic and soil microbiology research, cell propagation and theoretical thermodynamics of growth.

MATERIALS AND METHODS

Apparatus

A schematic diagram of the apparatus used in this study is illustrated in Fig. 1. Fig. 2 shows the apparatus as it appeared during operation. The sterile nutrient reservoirs of the continuous culture apparatus consisted of two pyrex glass bottles. The larger bottle had a capacity of 12.5 gals.; the smaller 5 gals. The media outlet assemblies were composed of a neoprene rubber stopper, a drying tube which acted as a vent, and 6 mm. glass tubing which extended from the bottom of the bottle through the stopper for a short distance. The bottles and outlet assemblies were connected by 1/8" I. D. latex rubber tubing, and a 4 mm., pyrex, 3-way stop cock which was in turn connected to the nutrient input port of the growth chamber. The rubber tubing between the stop cock and the growth chamber was of sufficient length to accomodate a cam type, peristalic, fixed speed pump (Sigma motor, Co. model AL-4).

The growth chamber effluent was connected to a Bausch and Lomb transistor regulated Spectronic 20 spectrophotometer, by a modified sample tube assembly (Fig. 3), which acted as a flow-through cuvette. Continuous spectrophotometer readings of culture turbidity were recorded by an attached strip chart recorder. After passing through the spectophotometer the growth chamber effluent dropped into a disposal receptacle. Constant culture temperatures were maintained by a constant temperature water bath. Aeration was supplied from compressed air tanks.

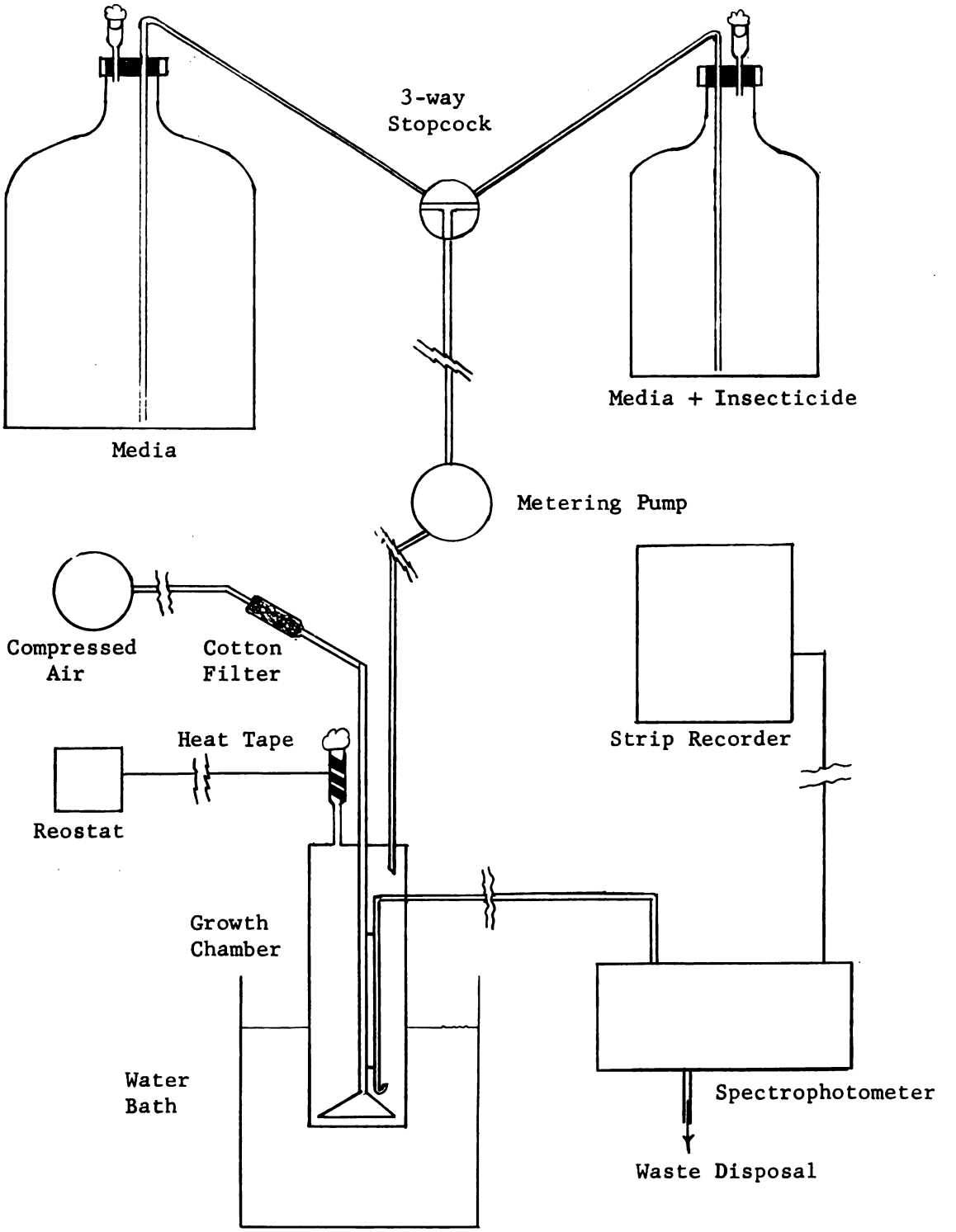


Fig. 1.--Schematic diagram of the continuous growth apparatus.

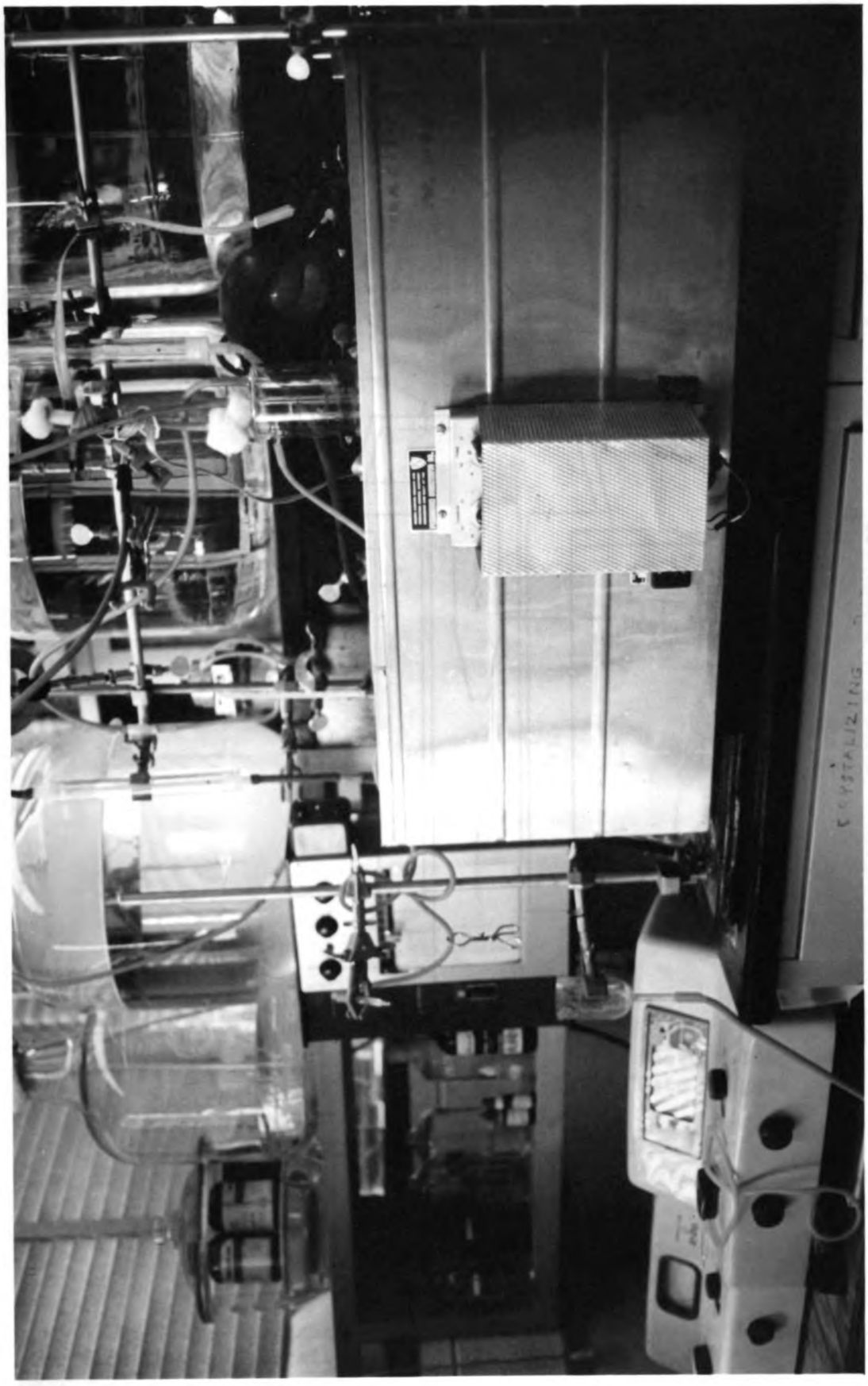


Fig. 2.--The continuous growth apparatus.

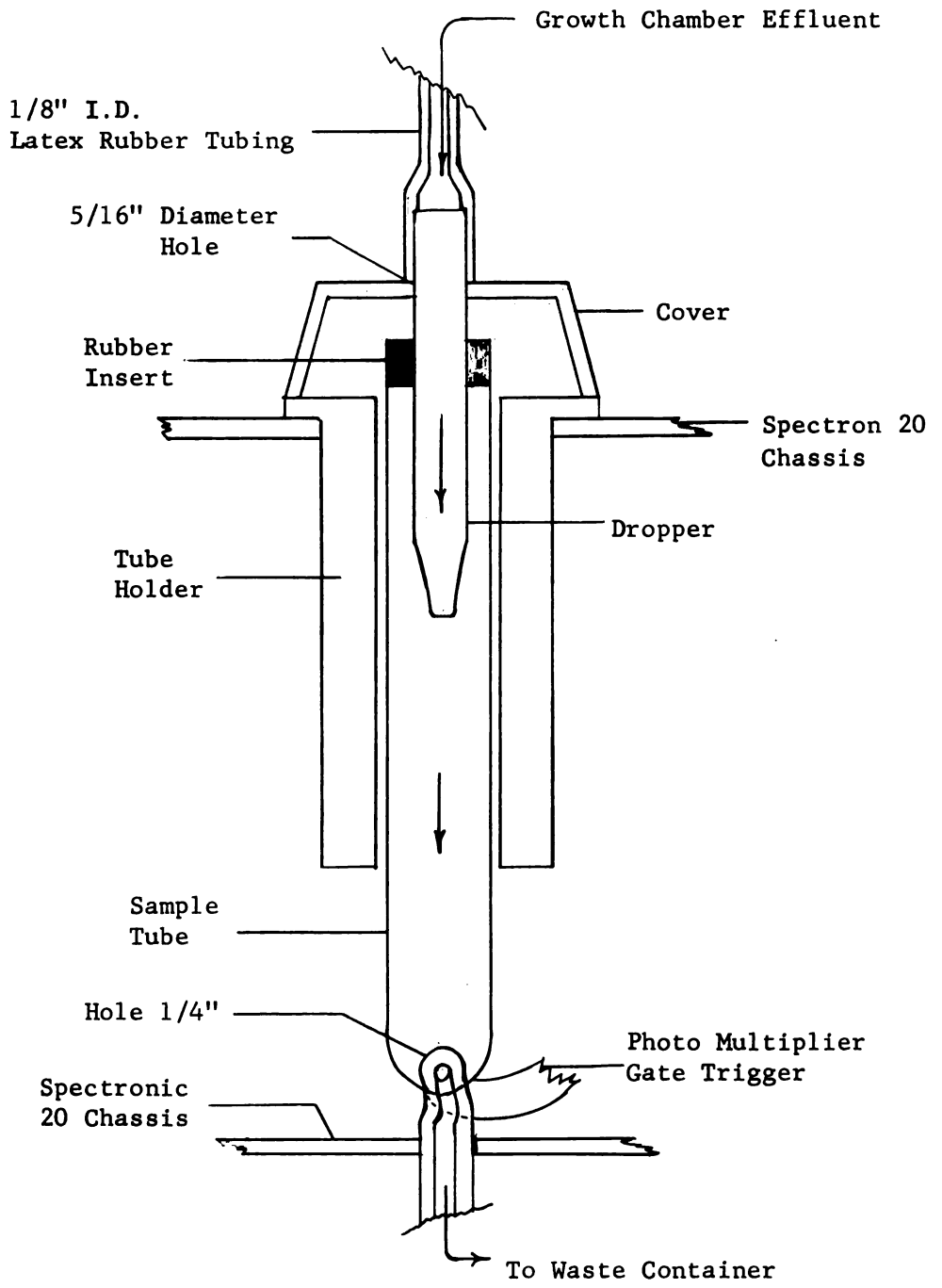


Fig. 3.--Spectronic 20 Spectophotometer Cuvette Holder modified for flow through operation.

Growth Chambers

The growth chambers designed were thin pyrex glass cylinders (Figs. 4, 5) of approximately 850 ml. capacity. They had an effective range of operating volumes between 150-500 ml. Aeration, mixing and chamber wall scouring were obtained by forcing sterile air through the inverted funnel which had a medium frited glass base. The funnel was suspended 2 mm. from the chamber walls and 3 mm. from the bottom, which caused the gas to rise in a ring of fine bubbles along the chamber walls. Venting was accomplished through a drying tube filled with plugging cotton and wrapped with a heating tape. The tape was kept hot to the touch in order to keep the cotton and drying tube free of condensation. Constant growth chamber volume was maintained by the height of the final effluent discharge point and its siphoning action.

Spectrophotometer Flow-Through Assembly

To modify the Spectronic 20 for the flow-through assembly (Fig. 3) it was necessary to drill a 5/16 inch hole through the cover of the sample tube holder so that the hole was directly over the center of the sample tube when it was in place. A hole in the bottom of the chassis below the sample tube was enlarged to slide the 3/16" O.D. rubber tubing in or out.

The flow-through assembly consisted of a sample tube, a rubber insert, and a 2-ft. piece of rubber tubing. A hole slightly smaller than the O.D. of the rubber tubing was melted through the lower angle of the sample tube. The rubber insert was made from the bulb of a medicine dropper and is inserted into the top of the sample tube. When assembled and in position in the spectrophotometer the glass portion of a medicine dropper was connected to the end of the growth chamber

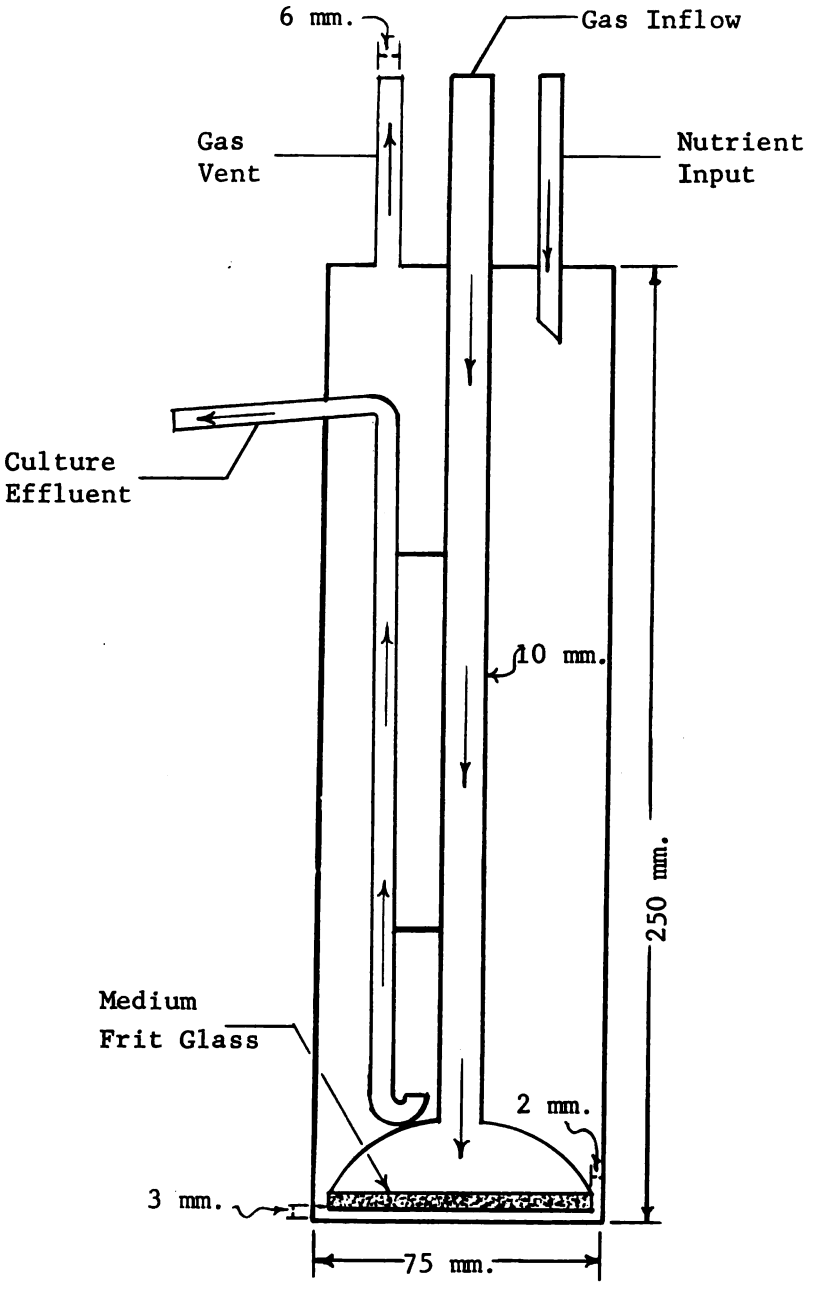


Fig. 4.--Growth Chamber.

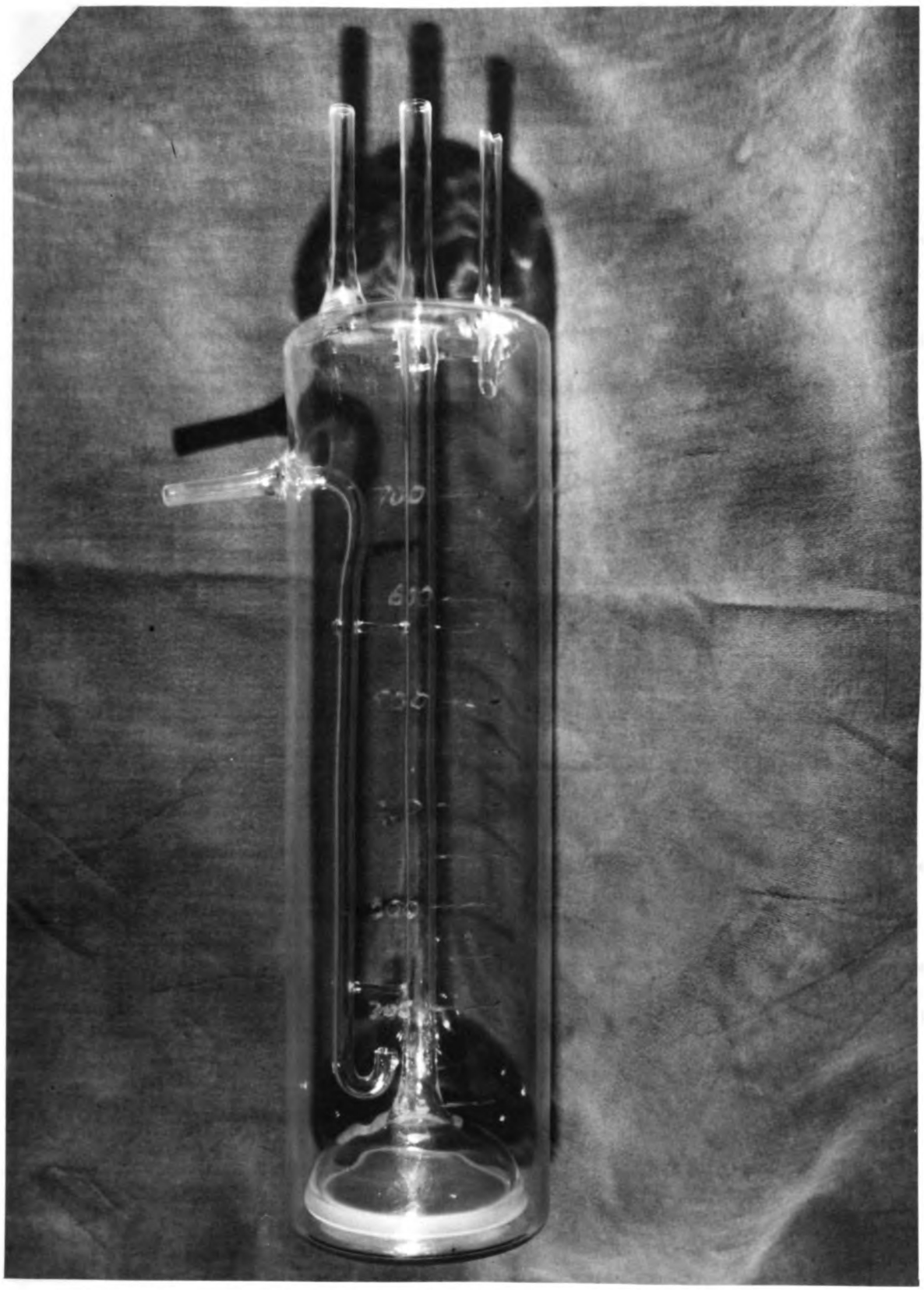


Fig. 5.--Growth chamber.

effluent tubing, and pushed through the sample tube cover into the rubber insert. This gave an airtight connection and maintained siphoning action.

Media

Anderson's minimum salt media was used for the experiments (Schoenhard 1961). This media consists of the following chemicals per liter. 1 gm. NH_4Cl; 6 gm. Na_2HPo_4, anhydrous; 3 gm. KH_2Po_4; 0.2 gm. $MgSO_4$; 5 gm. NaCl; and 4 gm. glucose. Glucose was varied from 0.1 gm. - 4.0 gm. per liter. This media is well buffered with the pH varying between 7.01 - 7.03.

Sterilization

Air passing into the growth chamber was sterilized by passing it through sterile plugging cotton. The growth chamber, tubing, plugging cotton and outlet assemblies were sterilized at 120° C. and 20 lb. pressure for 45 minutes to one hour in an autoclave. The media and media bottles were sterilized by peracetic acid which forms water, acetic acid, and oxygen at room temperature in the presence of light or heat. The 40% peracetic acid stock solution was kept refrigerated to prevent deterioration, which can occur at a rate of 2% per month. To sterilize 40 liters of media, 5 ml. of 40% stock solution was added to 25 ml. of distilled water, and this solution was added to the media, giving the recommended sterilization concentration of 50 parts per million (Greenspan 1955). Neutralization of this toxic solution occurred after five days.

Apparatus Preparation

All glassware was washed in Alconox and rinsed in distilled water

before assembly. Scum which formed on the interior of the growth chamber was removed by rinsing with 95% ethyl alcohol; 2-3 ml. were left in the growth chamber which was placed under a vented hood. At least 5 ml. of concentrated nitric acid was then poured into the growth chamber. A rapid oxidation reaction occurred, resulting in NO_2 gas formation which scoured the fritted glass. After the reaction was complete, the usual glass washing procedure was followed.

The nutrient bottle outlet assemblies were joined to the three-way stop cock which in turn was connected to the growth chamber by 1/8" I.D. latex rubber tubing. The effluent tubing was attached with a medicine dropper glass at the distal end. The vents on the growth chamber and nutrient outlet assemblies were filled with plugging cotton, as is the drying tube attached to the gas input port. Two strips of plugging cotton 4" wide and three feet long were rolled and wrapped. This part of the apparatus was then placed in the autoclave.

While the apparatus was being sterilized, a 40 liter batch of liquid nutrient media was prepared in the larger bottle. Some of the salts in the nutrient formula do not go into solution readily, but heating a large beaker of water with the salts included solved the problem. In mixing, the glucose was added last to prevent any precipitation of the salts.

After removal of the apparatus from the sterilizer the growth chamber was clamped into position in the water bath and the nutrient outlet assemblies were inserted into their respective bottles. Before wrapping and tying the outlet assembly of the large nutrient bottle in position, a solution of 5 ml. of 40% peracetic acid and 25 ml. of distilled water was poured into the nutrient media. The toxic nutrient

was agitated until the entire inner surface of the bottle and rubber stopper were wetted. A milder but toxic solution of peracetic acid was used to wipe the top of the bottle and outlet assembly. Then the assembly was inserted into position, wrapped with one of the rolls of sterile plugging cotton and tied into position with rubber tubing.

Five milliliters of peracetic acid solution was poured into the smaller bottle, into which the pesticide was eventually injected. The peracetic wetted the interior of the bottle before a large polyethylene bag, serving as a liner, was inserted. The bag greatly facilitated the pesticide clean-up procedure of the smaller bottle. The 3-way stopcock was adjusted to allow media to flow only from the larger media bottle to the smaller bottle. The siphoning action was started by applying suction to the end of the smaller nutrient bottle outlet assembly. After 1-2 liters of nutrient had siphoned into the smaller bottle, it was agitated to wet the inner surface of the liner and the outlet assembly. The top of the liner was pulled out a short distance before it was washed with a mild peracetic acid solution, as was the top of the outlet assembly. This also was wrapped with the other roll of sterile plugging cotton and tied into position. The two bottles were then placed on the same level and due to the siphoning action the resulting volumes were approximately 15 liters in the smaller bottle and 25 liters in the larger when in balance.

The gas supply is connected to the growth chamber by way of the sterile cotton filter and the growth chamber was filled to approximately two-thirds of its total volume with the toxic nutrient solution. Suction was applied to the chamber effluent tube. A slight amount of nutrient was allowed to flow out and the tube was clamped. The pump was attached

to the tubing between the three-way stopcock and the growth chamber.

When the volume of nutrient in the smaller bottle was sufficient the three-way stopcock was turned off. If the pesticide selected for experimentation was not changed by the peracetic acid, it was injected into the nutrient while it was still toxic, reducing the possibility of contamination. Pesticides were injected in an acetone solution using sterile techniques. In the experiments conducted the amount of pesticide injected was sufficient to give a saturated aqueous solution.

Inoculation and Cultures

After five days the growth chamber was inoculated with the selected microorganisms.

The microorganism used in these experiments was the bacteria, Escherichia coli, Strain B. The cultures were obtained from the Michigan State University Microbiology and Public Health Department.

Cultures were kept on agar slants in a refrigerator at temperatures between 5-10° C. Cultures were transferred to new agar slants every thirty days. To prepare a culture for inoculation, a loop of the microorganism was transferred from an agar slant to a culture tube of sterile Anderson's liquid nutrient media. When growth was observable, 2-4 ml. of the culture was injected, using sterile techniques, through the rubber tubing at the air vent port into the growth chamber. Compressed air was turned on and bubbled through the culture at a rate which was non-limiting to cell growth. When growth had proceeded to a point where it was very evident, the continuous culturing of the microorganism proceeded by starting the perstaltic pump and adjusting the siphoning effluent point to obtain the desired chamber operating volume.

Continuous Operation

Cell density was regulated by one of two methods. In the internally regulated system, the nutrient supplies all growth substances in excess. Growth proceeds at the maximum inherent rate of the microorganism in that nutrient and the cell density of the continuously growing culture was regulated by increasing or decreasing the dilution rate of the culture with sterile nutrient.

The externally regulated system relies on a limiting growth factor (LGF) in the nutrient. The cell density is held constant over a wide range of chamber volumes and will only decrease when the dilution rate exceeds the growth rate. The cell density will not increase unless selection, mutations or more of the limiting growth factor or another energy source is added, such as insecticides in these experiments. When the maximum dilution rate is exceeded in either control system, "wash out" occurs.

Cell Density Monitoring

Cell densities were monitored in two ways, manually or automatically, by a B & L Spectronic 20 at 600 μm., depending on the nature of the experiment. Manual monitoring was used for static cultures because only a small portion of the culture sample was necessary to obtain an optical density reading. Automatic monitoring and recording were obtained by calibrating the Spectronic 20 with distilled water which had an optical density which did not differ significantly from the sterile liquid nutrient. The flow-through assembly was filled with distilled water, the spectrophotometer sample cover closed and the chamber effluent tubing was clamped and inserted into the rubber insert in the flow-through assembly. The height of the end of the flow-through assembly

unit then became the volume control point of the growth chamber. Care had to be taken so that no air bubbles occurred in the effluent tubing because of the adverse effects on the siphoning action and the spectrophotometer readings.

Experiments - Static Growth

After a culture had been inoculated into the growth chamber the usual logrythmic growth response curve was ascertained for E. coli with the non-pesticide, non-limiting nutrient media. By draining the growth chamber and filling it with fresh nutrient, a closely corresponding curve was obtained. This was repeated three times. The same procedure was used with the pesticide nutrient to determine if the slope or height of the growth response curve changed.

Experiments - Continuous Growth

After a constant baseline reading of cell densities was obtained on the recorder a 25 ml. check sample was taken and then pesticide nutrient was metered in via the three-way stopcock.

The total effluent was collected after the pesticide nutrient started to flow into the growth chamber. For the first two hours, a 25 ml. portion of the effluent for each 15 minute time interval was saved and analyzed. A 25 ml. portion was saved from the effluent from each 30-minute time period for the next two hours. A 25 ml. sample was collected hourly thereafter.

The cells were separated from the liquid portion of the culture by a Sietz filter. The pesticide was extracted from the cells by pouring 50 ml. of n-Hexane onto the filter. The pesticide remaining in the liquid portion of the sample was extracted with a triple extraction

using 20 ml., 15 ml. and 15 ml. of n-Hexane. The extractant was evaporated to near dryness over steam. Complete drying was by compressed air. The dried sample was redissolved in n-Hexane and analyzed by gas-liquid chromatography.

The gas chromatograph columns were 4 mm. I.D. 6 ft. all glass. The liquid phase used was 5% DC-200 on 60/80 mesh gas-chrom. The temperature of the columns was 200° C., and the analyses were by electron capture detectors using tritium foils.

RESULTS

The apparatus was first operated as an unlimited or internal control growth system. The cell density was regulated solely by the dilution and wash-out rate of the culture in the growth chamber. Two models of variable rate metering pumps were used but did not give the accurate nutrient metering rates necessary for constant cell densities. A fixed rate (2 ml./min.) metering pump was purchased which gave more consistent nutrient input. Flow rates varied less than $\pm$ 0.1 ml./min. over 15-minute periods at metering rates of 2.0-2.5 ml./min. The resulting optical density (O.D.) of the E. coli B culture with the chamber volume at approximately 150 ml. and a flow rate within the above range was from .65-.45 O.D. units. Fluctuations of 5 units in the O.D. of the culture were noted which could not be accounted for as a mechanical variable. Cells adhered to the chamber interior in 5-7 days under the unlimited growth conditions. Inoculations from a chamber exhibiting wall growth to another sterile chamber resulted in wall growth in less than two days. A constant cell O.D. was difficult to maintain with this system of unlimited or internal control.

The apparatus was used as a continuous batch or static culture apparatus to determine growth rates with the regular media and media containing a selected insecticide. After inoculation, and when growth was observed in the growth chamber, the growth chamber was drained to the lowest possible level and rapidly filled with media. Hourly optical

density readings were manually recorded to determine the growth rate of a particular culture of E. coli. This procedure was repeated three times in most cases before media containing the selected insecticide was used following the same procedures.

The system of external control using a limiting growth factor of glucose at 0.1 gm./liter of media gave O.D. readings of approximately 25 units. This method of continuous culture extended the time before limited cell wall growth appeared. Constant culture O.D. readings over long periods of time were easily maintained and daily cell density fluctuations were not evident when the dilution rate was near the culture wash-out point. This method, coupled with static culturing using limiting growth factor media was most amenable to pesticide-microorganism research.

Technique development and experiments with the continuous growth apparatus required 34 runs with a total duration in excess of 4000 hours. Lindane was used in 12 runs, DDT 11, malathion 5, carbaryl 4, aldrin 1, and apholate 1. No conclusive results were obtained on pesticide microorganism interactions other than those given. No negative growth factors seemed evident.

A series of static or batch cultures were run using lindane, DDT, carbaryl, and aldrin with Anderson's minimum salt nutrient media. No deviation from the culture growth rate, established before each insecticide was introduced, could be ascertained when batch culturing E. coli on media containing a selected insecticide (Fig. 6 and Appendix I).

Continuous culture uptake experiments using the external or limiting growth factor control system with DDT, lindane and malathion were completed. The uptake of DDT was not determined because the filter

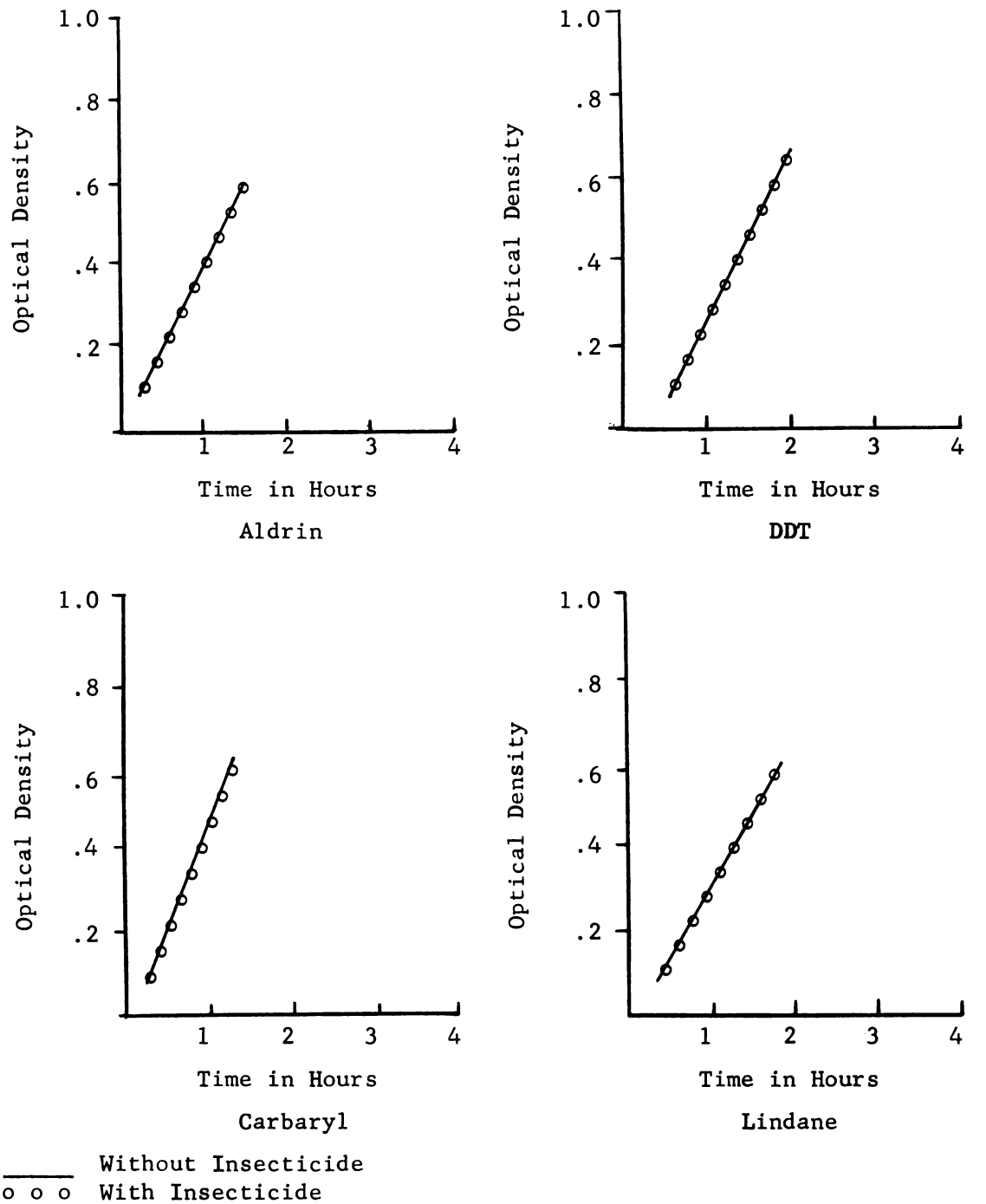
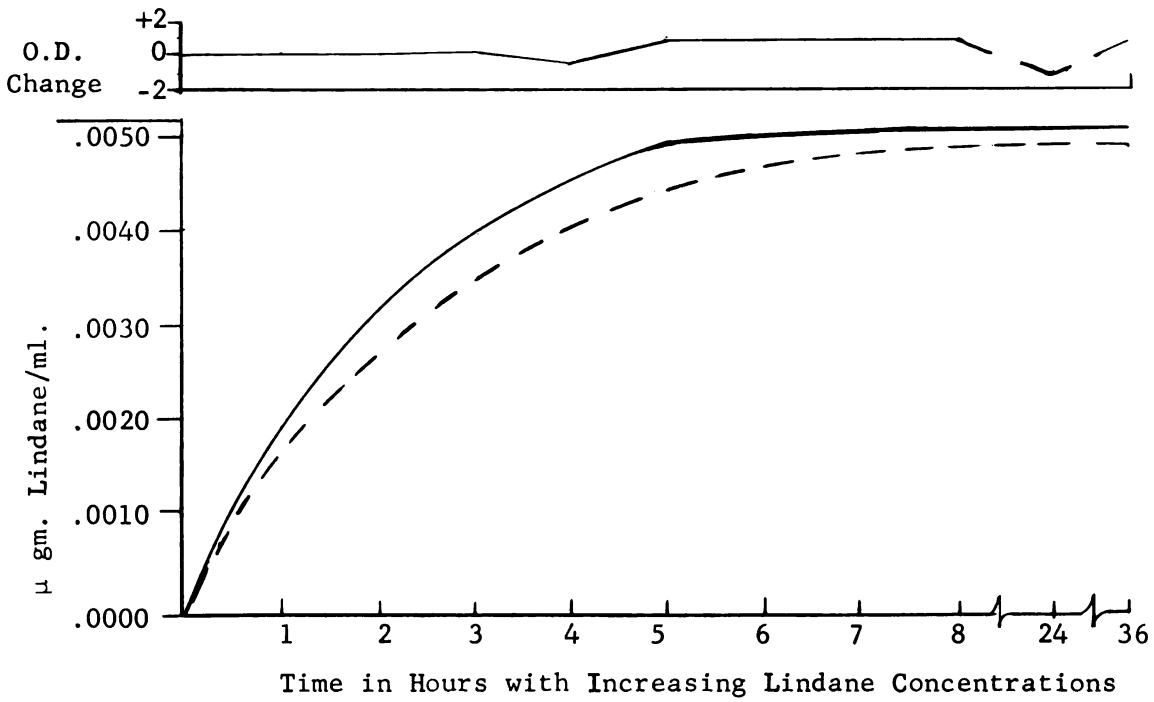
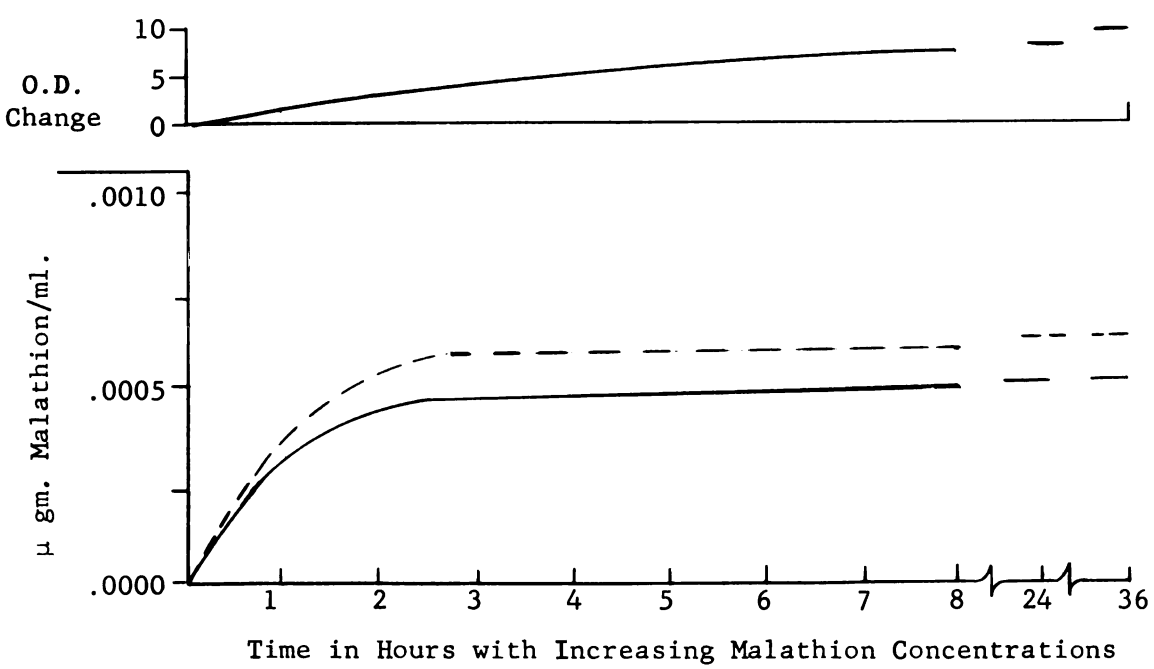


Fig. 6.--Growth rates of E. coli in static culture with and without selected insecticides.

used when separating the cells from the liquid phase of the culture absorbed all the DDT from the sample. The recorded O.D. readings indicate that E. coli was not affected by DDT, using cell density as the indicator of growth changes.

Figure 7 indicates that from an initial zero concentration of lindane to the maximum concentration of lindane in the culture during the experimental runs, no change in cell growth could be ascertained, as the culture optical density recordings are almost constant. The amounts of lindane found in the media and in the cell fractions were similar. As with lindane, the amounts of malathion removed from the media by the bacterial cells and the amount left in the media were approximately equal. The increased cell densities of 6 O.D. units in 8 hours and 9.5 units in 100 hours indicated that malathion served as an energy source for E. coli in the absence of sufficient glucose. See Appendix II for data pertaining to Figure 7.





----- Media Insecticide
_____ Cellular Insecticide

Fig. 7.--Optical density changes and increasing insecticide concentrations in the liquid and cellular fractions of growth limited continuously cultured Escherichia coli.

CONCLUSIONS AND DISCUSSION

The use of the apparatus as a continuous, self-inoculating, batch culture apparatus for the determination of growth rates of Escherichia coli B strain, using Anderson's minimum salt nutrient media, with and without a selected insecticide, proved feasible. The results of the replicated experiments regarding the growth rates of E. coli in these experiments during the logrhythmic growth phase were similar.

The culture growth rates eventually decreased, due to the increased metabolites and decreased nutrients. The resultant growth curves leveled off and then decreased slightly giving the generalized bacterial growth curve. The high point of these curves, determined from the manually recorded cell optical density readings, were plotted over time. A high degree of variability existed in the maximum height of these curves.

If, as in the case of malathion, the insecticide was metabolized and used as an energy source, the variability in maximum curve heights would not allow any assumptions to be made from the plotted curves of cell optical density readings. Effects on total cell growth or metabolism of a selected test substance could not be ascertained by optical density readings. The tendency of maximum curve heights was to become lower with each seceeding batch culture. Formation of a gradually increasing scum layer of cells on the inner walls of the growth chamber and the resultant increased effect of this layer of cells on the available nutrients would in part explain the decreased maximum curve

heights, while showing no change in the growth rate or curve ascent. While this method is useful in ascertaining the growth rate of a microorganism, the response of the microorganism to a selected experimental substance could more easily and accurately be determined over long periods of time by using the continuous limiting growth factor culture method because metabolism of an experimental substance would result in increased cell densities which are readily observed.

Under static growth conditions, aldrin, carbaryl, DDT and lindane did not change the log phase growth rates of E. coli. In later experiments, under continuous culture conditions, no change in the culture optical density readings were recorded when DDT and lindane were used in the nutrient media.

Continuous culture of microorganisms using the internal control method for constant culture densities required that all the necessary nutrients for cell growth be supplied in excess and in continuous and precise amounts. The internal control method of continuous culture has the inherent problem of cell growth on the inner surfaces of the culture chamber. This problem has been partially solved by various devices such as "windshield wipers" (Fox), G. E. Drifilm (Novick), magnetic stirrers or scouring by air agitation. This growth was always evident in 5-7 days, and was a consequence of selecting out those E. coli cells having a thick slime layer. This caused them to adhere to inner surface of the culture chamber. This "take-over" of the culture was observed when by a series of inoculations from one culture chamber to another resulted in cell wall growth in less than two days. Munson and Bridges (1964) also reported this selection process with E. coli. With the possible exception of the cyclone column (Dawson) 1963, this problem occurs when

culturing is carried on over extended periods of time. If any type of uptake experiments are to be carried on, erroneous assumptions can result after several days when this culture method is used. The alternative is to conduct short-term experiments if this method is used.

The regular fluctuations in cell densities of approximately 5 optical density units when using the internal control method resulted from "over shoot" (Fenn 1954). This was a consequence of metabolite accumulation and pH change which reduced the growth rate, resulting in a lag growth phase and decreased cell densities. Continuous inflow of fresh media, dilution of the culture, and loss of cells and accumulated metabolites allowed the culture to recover and exceed the cell density that would have existed in a steady state culture system. "Over shoot" can be corrected by growing the culture at one-tenth the maximum growth potential of the nutrient or near the culture "wash-out" point (Novick 1955).

Under continuous unlimited growth conditions, only negative growth effects could have been observed as is the case in the static cultures. At no time could negative or positive growth responses be substantiated due to addition of insecticides in these experiments under these conditions. There were no apparent advantages in using this system of internally controlled culture regulation in pesticide research unless continuous cell production is the prime objective. Adhesion of bacterial cells to the growth chamber walls and the problem of maintaining constant cell densities by precise media input and culture outflow with this relatively unsophisticated growth apparatus, using the internal method of cell density control, hindered the use of the apparatus as a reliable laboratory tool.

Continuous culturing with external control required that a nutrile be present in growth limiting amounts, which was glucose at 0.1 gm. per liter in the experiments. This nutrile as the Limiting Growth Factor (LGF) controlled the rate of growth and cell densities at any chamber volume or dilution rate that did not exceed the culture "wash out" point. The effects of decreased culture dilution rates and the concomitant increased mean cell growth chamber duration times on the cell biochemical activities were not explored during these experiments.

In the determination of the sequential steps in biochemical degradation or assimilation of a substance, the mean age of the culture at which a particular reaction occurs could be easily ascertained or varied by the dilution rate. The effluent could than be passed into any number of growth chambers in which additional nutrients or substances could be added or another biochemical reaction could occur.

Continuous culture using external control solved the problems of chamber wall cell growth and widely fluctuating culture cell densities. Cells did not adhere as readily nor did thick cell growth occur because of the starvation of the adhering cells due to the rapid absorption of the limiting growth factor by the cells in suspension (Novick 1955). Constant cell densities were easily maintained as cell densities were not dependent on the constant culture dilution by fresh nutrient and culture out-flow, but on the concentration of the Limiting Growth Factor. When constant cell densities are maintained, the physiological conditions are constant. Any selected constant and continuous physiological condition could be more accurately measured than possible when using other culture methods. Under these conditions, any negative or positive growth factor could easily be noted, whereas in the system of internal

control the response could be only an increase in the uptake of one or more of the nutrients in excess and no increase or decrease could be noted when the test substance was added. Increasing culture cell densities, upon the addition of any additional substance, would indicate that the substance was being utilized by the microorganisms. Since these are the criteria used in hydrocarbon metabolism research (Zobell, 1946; Trecanni, 1963) it also seemed applicable in these experiments. No changes occurred in the recorded cell density readings as lindane and DDT were gradually metered into the culture chamber, indicating that neither of these insecticides were metabolized under these conditions, nor were they detrimental to growth. The marked increase in cell densities when malathion was added was due to E. coli metabolizing the insecticide as a supplemental nutrient source. The uptake curves for the removal of lindane and malathion by bacterial cells from the culture media indicated that the bacterial cells removed an amount almost equal to that left in the liquid phase of the continuous culture. The values for cellular insecticide are higher than are the true values because of the amount retained by the filture during cell separation and through which the cells were extracted. A gradual increase in the concentration of the insecticide continued for several days at a decreasing rate. This continuous concentration change could have been avoided by injecting a sufficient quantity of the insecticide, in acetone, directly into the growth chamber so that the concentration of the insecticide in the growth chamber and in the insecticide media bottle were equal. Uptake of the insecticides by the filter pad, during cell separation, made the amounts of insecticide retained by the bacterial cells appear higher than the actual amounts retained, as later research with C_{14} labeled malathion

and DDT indicated. Further investigations using different microorganisms and anaerobic growth conditions should be undertaken.

Biological systems in general are continuous cultures proceeding in sequential biochemical steps under continually changing environmental and ecological conditions. A continuous culture apparatus will allow an investigator to ascertain and maintain the physiological conditions under which selected microorganisms in pure or mixed populations are cultured and interact. This has been well demonstrated by rumen microbiologists (Stewart 1961, Hobson 1962, Quinn 1962, Rufner, Slyter 1964).

The use of continuous culture methods for studying microbial ecosystems has barely begun. The opportunities are almost unlimited for the interested investigators.

SUMMARY

This research was undertaken to develop an apparatus with which pesticide-microorganism interactions could be ascertained with accuracy. A constant recording, continuous microbial culture apparatus was devised.

The B strain of Escherichia coli was grown under continuous aerobic culture conditions using Anderson's minimum salt liquid nutrient media. Two culture cell density control methods were used. The internal control method used the inherent cell growth rate and a constant dilution or culture wash-out rate to maintain constant cell densities when all nutrients were present in excess. The second method, that of external control, used a Limiting Growth Factor of glucose to maintain constant cell densities. This method of external control was applicable to this research, since constant cell densities were maintained over a wide range of growth chamber volumes and for long periods of time without growth occurring on the inner surfaces of the growth chamber.

A series of static or batch cultures, using the apparatus as a batch culture apparatus, indicated that the growth rate of E. coli was not influenced by lindane, DDT, carbaryl and aldrin. Under continuous culture using a glucose growth limiting factor, neither DDT nor lindane significantly changed the recorded cell densities when added to the growing culture. Malathion increased the recorded cell culture O.D. readings 6 units in 8 hours, and 9 units in 36 hours. This indicated that malathion was being metabolized and used as an energy source to

supplement the glucose deficiency of the media.

Uptake of lindane and malathion by E. coli was determined during the continuous culture experiments, demonstrating that only a small amount of insecticide was removed by the bacterial cells from the culture with an almost equal amount remaining in the liquid phase of the culture. This research indicated that this approach to pesticide research can provide important information as to the conditions and pathways of pesticide degradation and uptake and their effects on microorganisms. This research substantiates the general theory that insecticides have little direct negative effect on the growth of bacterial populations and that certain insecticides are degraded by microorganisms.

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APPENDIX I

STATIC CULTURES

Hours	Check	Check	Check	Lindane			Check	Lindane		
				I	II	III		I	II	III
0	.12	.04	.06	.02	.04	.06	.05	.04	.10	.06
1	.28		.11	.04		.10	.12	.07		.07
2	.67		.24			.19	.27	.15		.10
3	.81		.54	.09	.48	.42	.41	.26		.15
4	.85		.75			.61	.51	.33		.19
5			.81	.30	.72	.70	.54	.37	.28	.21
6			.84			.76	.58	.38		
7				.75		.81	.61	.38		.21
8										
9							.59			
10										.20
11										
12	.98	.93								
13										
14										.20
15				.87						
16										
17										
18									.30	.20
19					.83					
20										
21										
22										
23						.74				
24										

Hours	Check	Carbaryl	Check	Check	Check	Check	Check	Check	Check	Carbaryl		
		I								I	II	III
0	.10	.10	.07	.03	.03	.12	.14	.02	.03	.03	.02	.01
1		.25					.36			.05		
2		.45					.60	.15		.12		
3	.62	.62	.52			.60	.71				.35	
4	.73	.65				.76	.85			.64	.67	
5		.66				.80		.62		.66	.74	
6										.70	.75	
7								.72		.77		
8			.87					.77		.79	.84	
9								.80				.91
10		.78										
11					.75				.81			
12									.83			
13												
14		.85										
15												
16	.83											
17				.77								
18											.90	
19												
20												
21												
22												
23								.79				
24						.80			.83			

Hours	Check	Check	DDT	Check	Check	DDT	
			I			II	III
0	.15	.025	.05	.15	.02	.08	.05
1	.17	.03	.06	.17	.03		.06
2	.30		.07	.30	.07		.07
3	.45	.065	.10	.45	.13		.10
4	.50	.13		.50		.11	
5	.53			.53	.40	.13	
6	.55	.41		.55	.49		
7		.46	.33	.55			.33
8	.55			.54		.15	
9							
10			.35				
11							.35
12							
13			.33				
14							.34
15							
16							
17							
18		.51					
19							
20							
21							
22							
23							
24	.54		.34				

Hours	Check	Check	DDT					
			I	II	III	I	II	III
0	.17	.19	.14	.05	.08	.10	.01	.05
1	.19	.35	.27	.10	.12	.16		.15
2	.35	.60	.42			.28		.29
3	.60	.75	.55	.35	.29	.50		
4	.75		.58	.41	.30	.74	.29	.62
5		.77	.58				.56	.62
6	.78							
7	.69					.90	.75	
8					.31			
9								
10								
11								
12								
13								
14						.89	.82	
15				.40				
16								
17								
18								
19							.76	
20								
21								
22								
23								
24								

Hours	Check	Aldrin		
		I	II	III
0	.17	.08	.05	.04
1	.32	.15	.09	.06
2	.45	.28	.21	.12
3	.56	.45	.35	.23
4		.60	.50	.42
5		.68	.64	.58
6			.69	.62
7				
8				
9				
10				
11				.61
12				
13				
14				
15		.79		
16				
17				
18				
19				
20				
21				
22				
23				
24				

APPENDIX II

CONTINUOUS CULTURES

Time	μ gm. Lindane per Unit					O.D. Change Ml.		
	I		II	III				
	Cells	Media	Cells	Cells	Media	I	II	III
	.0001	.0003	.00006	.0004	.00018			
	.00006	.0014		.0015	.00043			
	.0011	.0022	.00016	.0011	.00028			
1				.0030	.0018	0	0	0
	.0020	.0028	.00013	.0037	.00147			
			.00005	.0039	.00130			
	.0005	.0023	.00021	.0032	.00175			
2			.00018	.0049	.0035	0	0	-2
	.0038	.0042		.0030	.0038			
			.00003	.0038	.0004			
	.0030	.0028		.0040	.0023			
3	.0019	.0002	.00019	.0049	.0012	+2	0	-2
	.0038	.0000	.00027					
4	.0046	.0033	.00010	.0063	.0023	-2	+2	-2
5	.0007	.0058	.00016	.0039	.0034	+2	+2	0
6	.0042	.0053	.00016	.0049	.0021	+2	+4	0
7	.0046	.0000		.0051	.0002	+2	+4	+2
8				.0072	.0032	+2	+4	0
12						-2		
24	.0047	.0012	.00019	.0052	.0021	0	-2	0

Time	μ gm. Malathion per Unit						O.D. Change Ml.			
	I		II		III					
	Cells	Media	Cells	Media	Cells	Media	I	II	III	IV
	.00024	.00024							0	
	.0005	.00028							0	
	.0006	.00027	.0004	.00033	.00025	.00055		0	0	
1	.0008	.00064	.0003	.0005	.00035	.00055	0	+1		+3
	.00075	.0006	.0003	.0005			0			
	.0009	.0005	.0002	.00055					+2	
	.0009	.0008	.0005	.0005	.00046	.0006		+3		
2	.0000	.0000	.0000	.0006	.00034	.00065		+4		+2
	.0018	.0010	.00043	.0006						
									+4	
					.00048	.00038				
3					.00021			+5		+2
			.00043	.0007	.00043	.00065		+2	+5	
4					.00043	.00055				+3
			.00043	.0007					+8	
5					.00037	.00042		+4	+7	+4
			.00041	.0006				+4		
6					.0006	.00065		+2	+7	+4
			.00045	.00055				+4		
7									+6	+4
			.00044	.0006						
8									+7	+6
			.00055	.0006						
24			.00043	.00065	.0005	.0006	+6	+6	+10	+8
30					.0002	.00055				+8
36					.00048	.00046				
48										+9
60										+9
96										+8
168										+8