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A Report

on

The Effect of 2,3,7,8-Tetrachloro-dibenzo-p-dioxin (TCDD) on Growth Curves of *Drosophila melanogaster* and Selected Nonpathogenic Bacteria

Prepared for

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Summer Research Project

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INTRODUCTION

The work outlined in this report is a follow-on to the efforts of Captain Alvin L. Young, Ph.D., and other members of the Environmental Pollution Control and Monitoring System Task Team whose report appears as Technical Report AFATL-TR-74-12, entitled "Ecological Studies on a Herbicide-Equipment Test Area (TA C-52A), Eglin AFB Reservation, Florida." Their report discusses the results so far perceived of repeated massive application of herbicides (346,117 pounds) over a period of eight years (1962-1970) onto a one square mile section of a larger test grid, documenting the "persistance, degradation, and/or disappearance of the herbicides from the soils and drainage waters of TA C-52A, and the subsequent effects (direct or indirect) of the herbicides upon the vegetative, faunal, and microbial communities."[1]

One of the herbicides used extensively, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) contains the highly teratogenic (fetus deforming) contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). This report outlines several initial experiments in which this team for the first time utilized TCDD in pure form in a controlled laboratory environment. The quantities of TCDD used in the laboratory (up to 25.7 parts per million (ppm)) were considerably greater than any encountered in the field, 710 parts per trillion (pptr) being the largest observed. Resultantly, due to TCDD's extreme toxicity and action as a skin irritant, great care was taken to avoid all contact

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[1] A. L. Young. "Ecological Studies on a Herbicide-Equipment Test Area (TA C-52A), Eglin AFB Reservation, Florida." AFATL-TR-72-31, January 1974. Unclassified.

in handling the pure TCDD and the various nutrient medias prepared with it. All experiments were conducted in an isolated area of the laboratory inaccessable to unauthorized personnel. The room was well ventilated and all personnel within were required to wear safety gloves and coats. All glassware was autoclaved and rinsed with chloroform, an excellent TCDD solvent, to remove any residue. All excess media, used media, and highly contaminated articles such as pipets used in direct TCDD transfer were secured in special waste containers and later incinerated at approximately 2000°C.

Two major biological systems, fruit flies and bacteria, were utilized in this project. They were chosen for their ease in handling and observation and their rapid and controllable growth rates. Both organisms were grown on nutrient media containing various amounts of TCDD, ranging from 25.7 parts per billion (ppb) to 25.7 parts per trillion (pptr) for the flies and .257 parts per million (ppm) to .257 ppb for the bacteria, to determine TCDD toxicity and growth curve characteristics.

MATERIALS AND METHODS

Equipment and Reagants

Drosophila.

Media (liter quantity)

- Agar
- Sucrose
- Yeast
- Potassium phosphate monobasic
- Potassium sodium tartrate
- Calcium chloride
- Sodium chloride
- Manganous chloride
- Ferrous sulfate
- Propionic adid (99%)
- TCDD stock solution* (25.7 ppb, 2.57 ppb, .257 ppb, 25.7 pptr)

Autoclave
140 fly bottles
Oregon-S wild type Drosophila melanogaster-obtained from Dr. J. Bayliss U. of West Florida, Pensacola, Florida.
Erlenmeyer flasks
Ether
Etherizers
Acetone
Fly morgue
Fly brushes
Spatulas

Bacteria.

Stock cultures of bacteria (Enterobacter aerogenes, Serratia marcescans, Esherichia coli, and Sarcina sp.) - obtained from US Army Natick Laboratory
Trypticase Soy Broth, 31 (30 g/l) - manufactured by BBL, Division of Becton, Dickinson, and Co.
Trypticase Soy Agar, 31 (40 g/l) - manufactured by BBL
Purified distilled water, 15l
TCDD stock solutions* (.257 ppm, 25.7ppb, 2.57 ppb, .257 ppb)
Acetone
Autoclave
Spatulas
Incubator
Pipets (10 ul, 100 ul, 1 ml, 5ml, 10 ml)
Erlenmeyer flasks
Artek colony counter
Coleman universal spectrophotometer
Petri dishes
Wire loop
Culture tubes
Dilution tubes

*Stock solution is TCDD dissolved in acetone

Procedures

Culturing of Drosophila. The fruit flies were grown in pint jars containing approximately 40 ml of nutrient media prepared as described below. The jars were maintained at an approximate temperature of 25°C ± 2° (optimum temperature for zygote development) and humidity of 70% ± 5%.

Experimental methods-Drosophila.

1. 5 sets of media were prepared. The control group contained only the agar and essential nutrients. The other four groups contained varying amounts of TCDD- 25.7 ppb, 2.57 ppb, .257 ppb, 25.7 pptr. The TCDD was added to each media following autoclaving and just prior to pouring into the fly jars.

2. 40 ml of media was poured into 5 groups of 28 fly bottles for a total of 140 bottles.

3. One male and one female fly were placed into each bottle.

4. After 6 days of incubation, 2 bottles of each treatment group were sacrificed each day for the next 14 days. The total number of flies and the male-female ratio in each jar was noted, along with room temperature and humidity.

5. A graph was then prepared of the number of flies counted per day over the 14 day period. (Figure 5)

Culturing of Bacteria. The bacteria were transferred from their shipment vials to 100 ml of Trypticase Soy Broth prepared as prescribed by BBL and then incubated at 30°C for 24 hours to establish stock cultures.

Experimental methods-bacteria.

1. It was determined that a turbidimetric analysis utilizing the Coleman spectrophotometer would be sufficiently accurate and convenient for the measurement of growth rates in this study.

2. A standard curve was therefore prepared correlating bacteria colony count and U.V. absorbance at 525 nm.

a. Serial dilutions were made of each bacterial stock solution from $1:4^0$ to $1:4^{15}$.

b. Each dilution was plated on Trypticase Soy Agar and incubated for 20 hours at 30°C.

c. At the same time each dilution was also inoculated into tube flasks of Trypticase Soy Broth and incubated for 20 hours at 30°C.

d. After 20 hours an absorbance reading and a colony count were made for each dilution of the four bacteria.

e. A standard curve was then prepared. (Figure 6)

3. 10 tube flasks were prepared for each bacteria consisting of one blank containing 100 ml of Trypticase Say Broth and 1 ml of Acetone, one control containing 100 ml of broth, 1 ml of Acetone, and 1 ml of bacteria, and eight experimentals(2 for each TCDD concentration) containing 100 ml broth, 1 ml Acetone, 1 ml bacteria, and TCDD(.257 ppm, 25.7 ppb, 2.57 ppb, and .257 ppb)

4. The flasks were incubated at 30°C for 24 hours.

5. Absorbance readings were taken at 0,2,4,5,6,7, 8 and 12 hour intervals for all bacteria with the following addenda.

a. A 24 hour reading was also taken for Enterobacter a.

b. Sarcina sp. was read at 5½ rather than 5 and at 11½ rather than 12 hours due to problems encoutered in arriving at the laboratory area on time.

c. No 4 hour reading was taken on Serratia m. due to time problems, but an 18 hour reading was made.

d. No 7 hour reading was made on E. Coli due to time problems.

6. Using the standard curve the absorbance readings were correlated with colony count and the result graphed against time for each bacteria. (Figures 1,2,3,4)

RESULTS

See Figures 1,2,3,4,5,and 6.

DISCUSSION

The results for Drosophila melanogaster were for the most part statistically inconclusive. It can readily be seen that 25.7 ppb is a lethal dose and therefore an upper toxicity limit; however, no conclusions can be drawn for the other three concentrations. In general the data was far too erratic, due to several factors: too short an experimental time span, multiple generation flies(resulting in the death of some older flies before reproduction could take place), use of several different cultures of flies, use of non-virgin females, and inability to insure equal environmental conditions for all fly cultures. The following recommendations are made for future experiments:

1. Extend the experimental time span to at least 30 days, as in Bodenheimer's experiments (1938)[2], and preferably to 56 or more days.

2. Use only one culture of Drosophila, all of the same generation, with virgin females.

3. Monitor the experimental environment closely and rotate the fly bottles occasionally to insure equal exposure

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[2]Paul Colinvaux, Introduction to Ecology(New York: John Wiley and Sons, Inc., 1973), pp. 316-320.

The results for all four bacteria were also statistically inconclusive, demonstrating that the toxicity range used was not broad enough. The data,however, was smooth, consistent, and accurate; therefore the methods employed were probably valid. The following recommendations are made for future experiments:

1. Extend the experimental time span to allow for the development of a full growth curve.

2. Use larger concentrations of TCDD-from .257 ppb to 25.7 ppb.

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FIGURE 1
ENTEROBACTER AEROGENES

GROWTH ON TCDD ENRICHED MEDIA
8 JUL 74

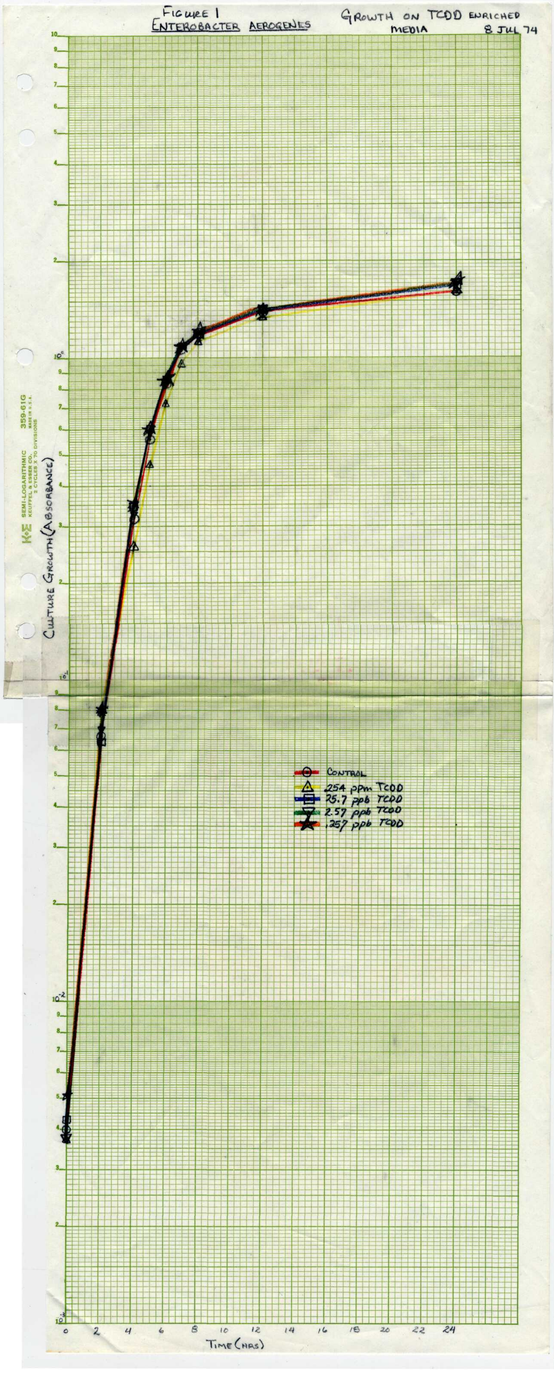


FIGURE 2

SERRATIA MARCESCANS

GROWTH ON TCDD MEDIA

10 JUL 74

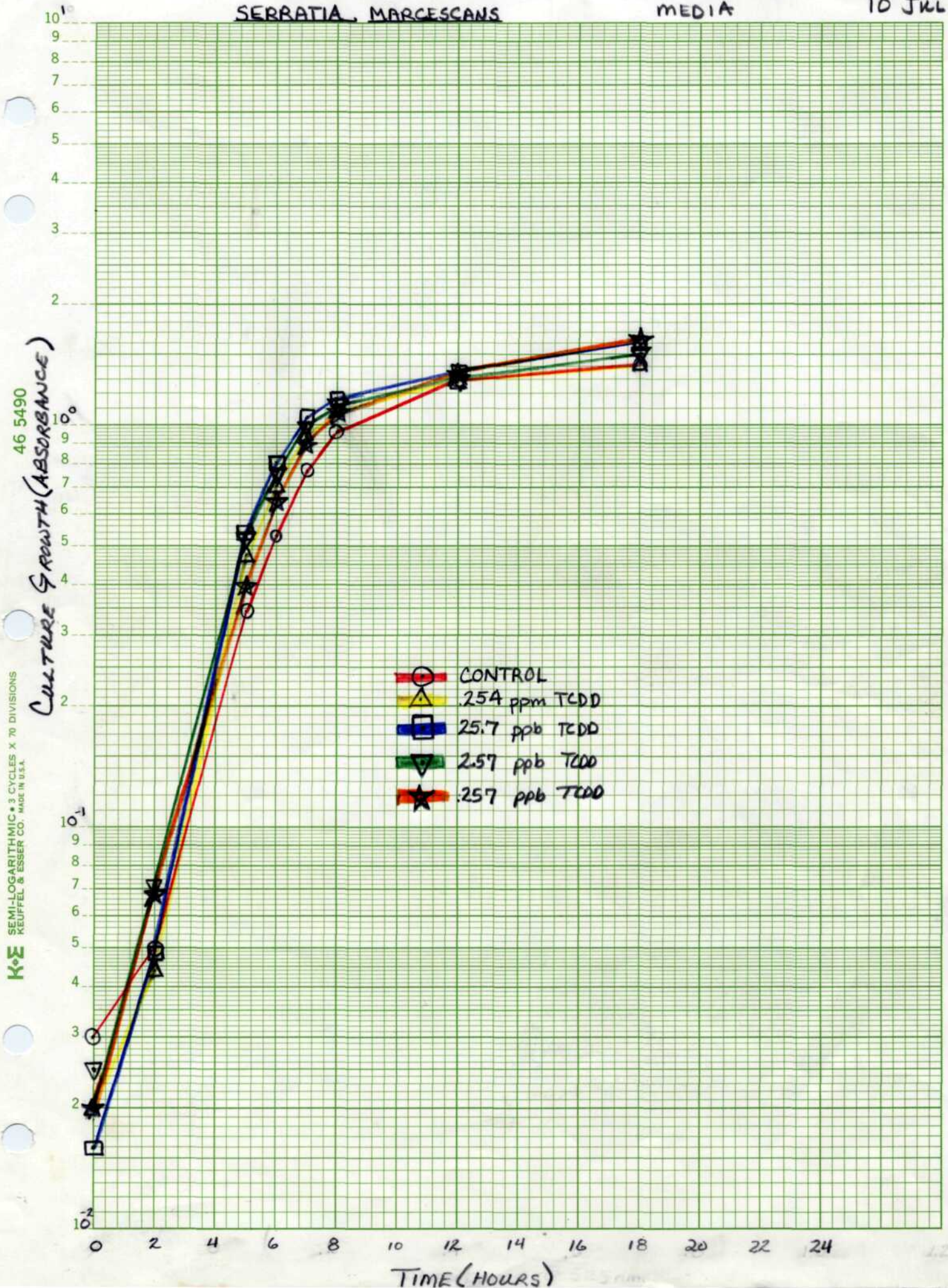


FIGURE 3 — GROWTH ON TCDD MEDIA — 9 JUL 74

SARCINA SP.

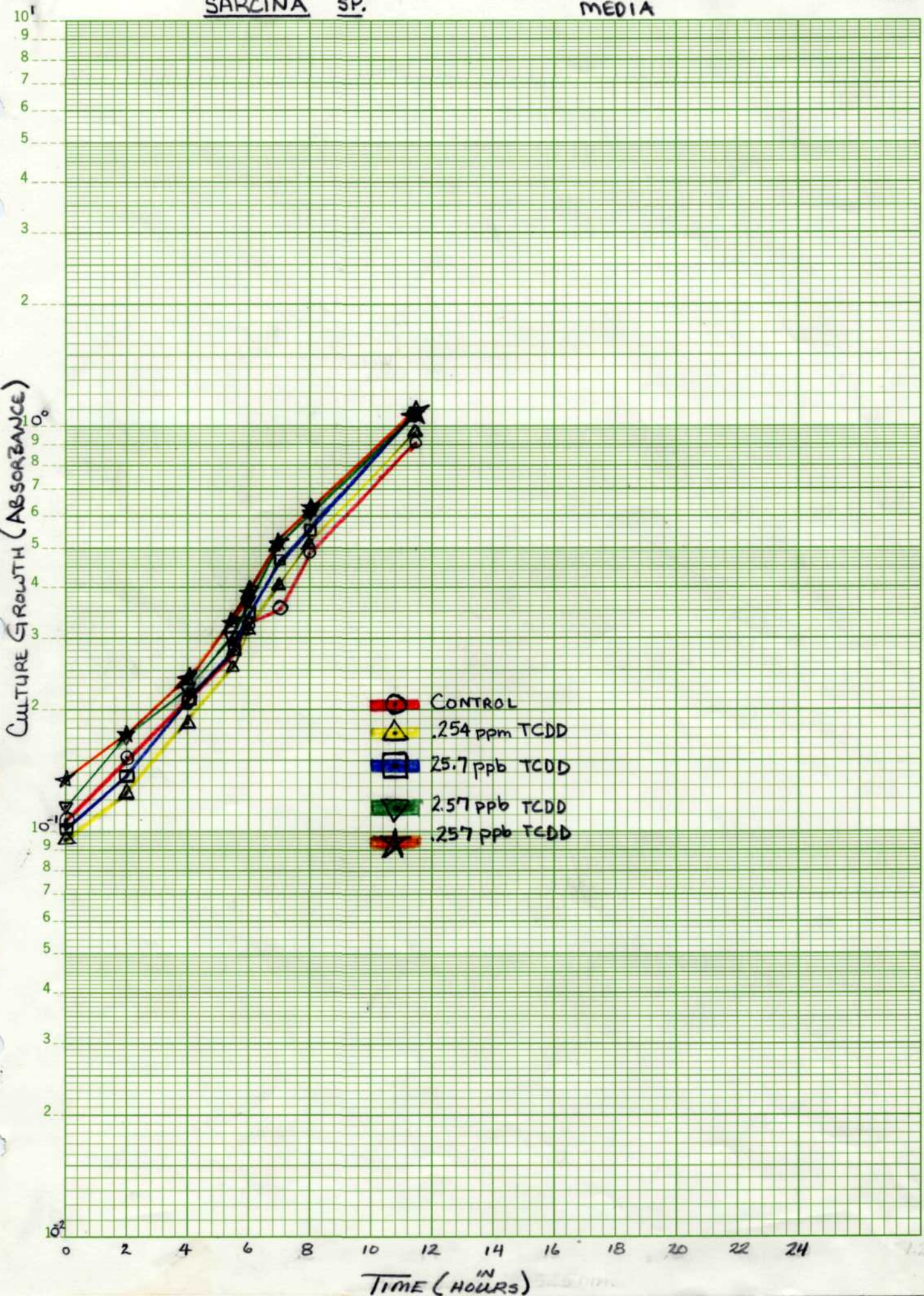


FIGURE 4

ECHERICHIA COLI

GROWTH ON TCDD MEDIA

11 JUL 74

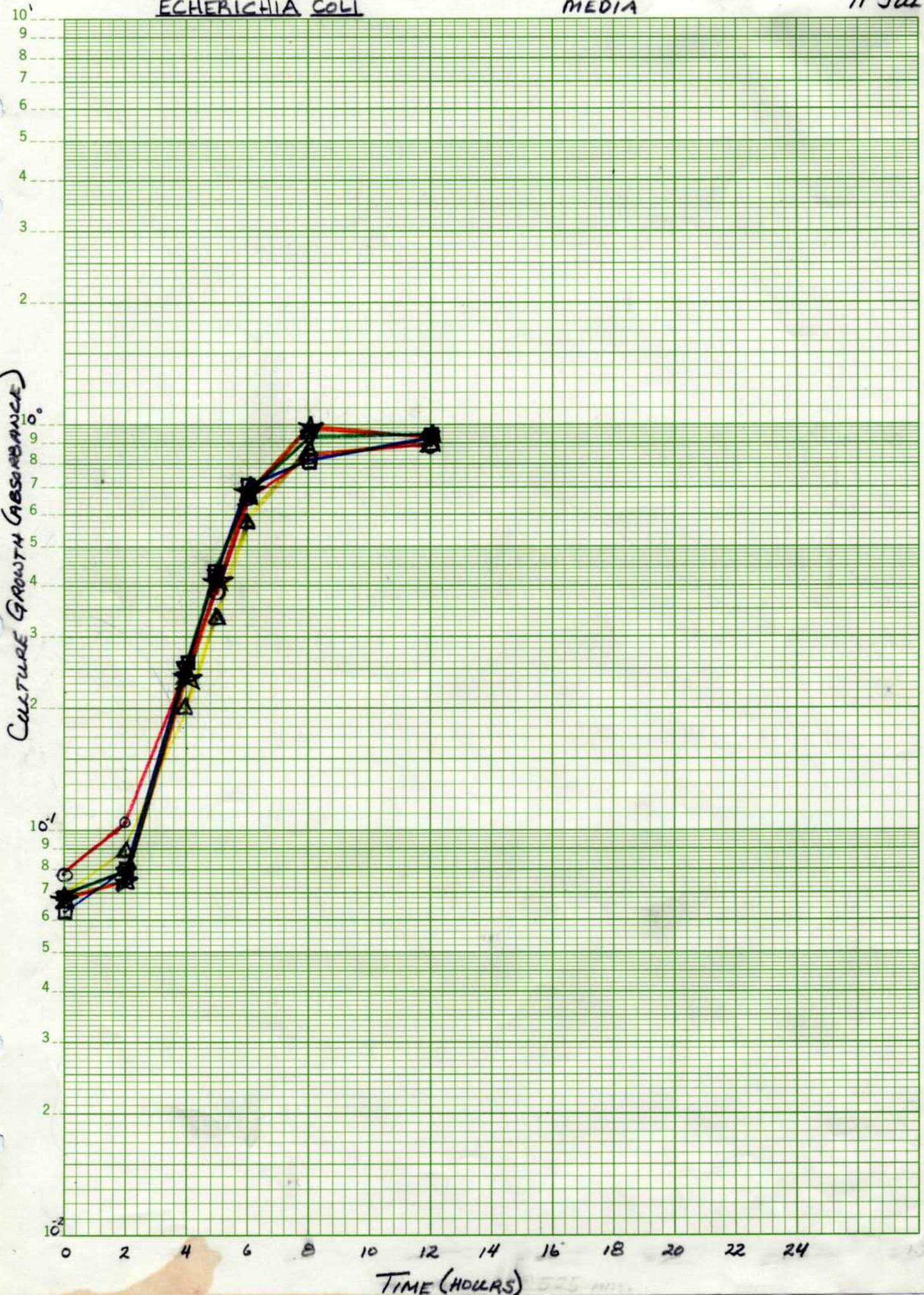
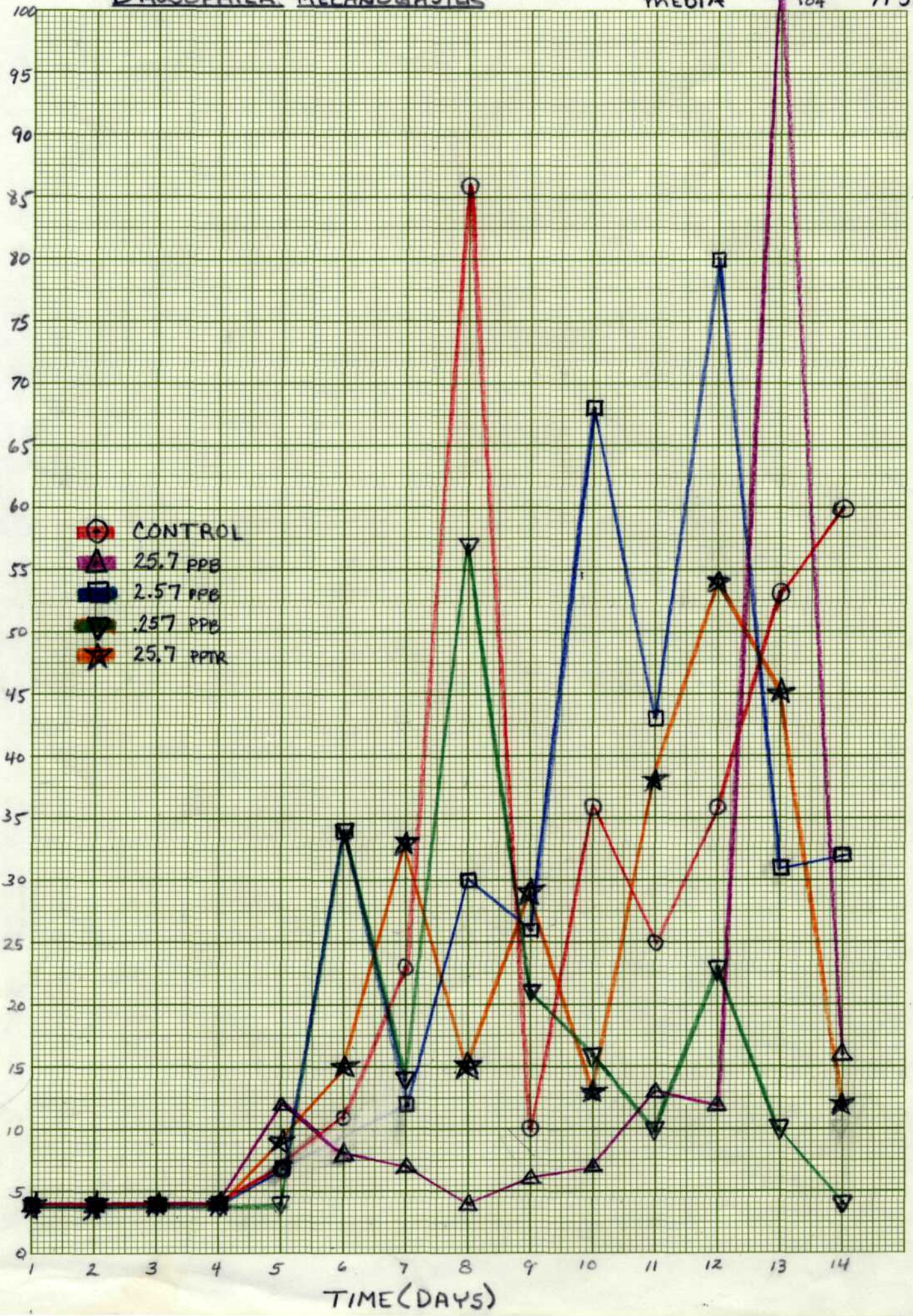


FIGURE 5

DROSOPHILA MELANOGASTER

GROWTH ON TCDD MEDIA 104

28 JUN-11 JUL 74



BEE 20x20 TO INCH

FIGURE 6

STANDARD CURVE (GROWTH COLONY COUNT VS. ABSORBANCE)