A STUDY OF THE EFFECT OF DDT ON *EUGLENA GRACILIS*

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Submitted to the Graduate College of
Appalachian State University
partial fulfillment of the requirement for the degree of

MASTER OF SCIENCE

August 1972

Major Subject: Biology

ABSTRACT

A Study of the Effect of DDT on Euglena gracilis Klebs

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The purpose of this study was to determine the effects of DDT on Euglena gracilis Klebs. The metabolism of DDT by E. gracilis was also investigated. Manometric procedures were carried out on cultures of E. gracilis under conditions of reduced temperature, axenic culture and in the presence of bacteria. DDT produced a reduction in photosynthesis at dosages as low as 1×10^{-12} g/ml. E. gracilis grown with bacteria showed reduction in photosynthesis down to 1×10^{-10} g/ml. Axenic cultures of E. gracilis were affected least by DDT and showed inhibition of photosynthesis at 1×10^{-8} g/ml. Graphs of response to dosage showed typical S-shaped curves. The rate of photosynthesis was reduced to a level of 50% to 60% of the control value, and stayed at this level with increasing dosage. All manometric techniques were carried out after 24 hours of exposure to the pesticide.

Experimentation was carried out in order to determine if the organism metabolized the DDT. Euglena was treated with DDT and incubated for 14 days. No metabolites were detected using thin layer chromatographic techniques. More sensitive techniques using radioisotopes are required for further study.

Citations in this thesis follow the style of Plant Physiology.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Insects have been, and will continue to be a source of problems for man. Most insects, however, do not cause harm to man. The role of the honeybee is a well known example of the necessary role of insects. There are only a small number of insects that cause problems for man. Only about three thousand species of insects out of a total of three billion are detrimental to man. Well known examples of undesirable insects are the rat flea which carries the bubonic plague, and the termite which destroys mans dwellings (28).

In order to combat the problem of insect pests, man has had to turn to the expedient of chemical insecticides. Insecticides have become necessary to protect mans food supply, prevent destruction of his dwellings, and maintain his health. Insecticides might be classified into first and second generation preparations. The first generation is characterized by the use of natural organic compounds and inorganic poisons. The second generation of pesticides would be characterized by the well known insecticide DDT, which is an example of the numerous synthetic organic compounds that are used to control insects.

Pyrethrum was probably the earliest of the insecticides. The origin of the use of the compound is not known. In 1763 ground tobacco was used in France to kill aphids. The active ingredient nicotine in the ground tobacco was discovered in 1809. The natives of South America have used preparations of the plant sabadilla to kill lice for many centuries (24).

As early as 1848 plant preparations containing rotenone were used. In the eighteenth century, petroleum, kerosene, creosote and turpentine were used as insecticides. The problem with petroleum derivatives was that the preparations were highly toxic to plant life as well as insects. Despite their toxicity, petroleum oils came into general use as a larvicide for mosquitos. Because of reduced toxicity to plant life, highly refined oils in emulsion preparations came into general use late in the eighteenth century (24).

Inorganic compounds were brought into use early in the history of insecticides. Paris green, an arsenical, was used in 1865 to combat the potato beetle in the western United States. In California, lime sulfur washes and hydrogen cyanide were used in 1886. In 1892, lead arsenate was used as an insecticide against the Gypsy moth in Massachusetts. Other metalic salts such as those of mercury, selenium, copper, zinc, chromium and thallium were used. The drawback of the highly poisonous inorganic compounds was the persistence of the compounds in the soil and in the plants (24).

Early in the twentieth century some organic compounds were used for insecticidal purposes. The use of napthalene, paradiclorobenzene, thiocyanates and cyclohexamine was somewhat limited. In the middle of the twentieth century, the use of organic insecticides became widespread. The use of DDT for insecticidal purposes did not take place until 1939 (20) (24).

The name DDT is indicative of the first letters in the old name of Dichloro-Diphenyl-Trichloroethane.

The IUPAC name of the compound is (1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane. DDT was first described in 1874 by the German Chemist Othmar Zeidler, but the usefulness of the compound for controlling insects remained undiscovered until 1939. Paul Muller working for the Geigy Company in Switzerland discovered the potency of the compound in controlling the Colorado Potato Beetle, and other insects. In 1942, the Geigy Company sent samples of the compound to Orlando, Florida for testing at the Laboratory of the U. S. Department of Agriculture. The compound was found to be very useful in the control of insect pests of public health importance. In 1948, the Nobel Prize in medicine was awarded to Paul Muller for his discovery of the insecticidal properties of DDT (20).

When World War II began, the supplies of pyrethrum which had been used for insect control were cut off. The situation soon developed to nearly the critical stage, and a cheap substitute had to be found. This situation brought on the large scale use of DDT for military purposes. In 1943 DDT was used to halt an epidemic of typhus in Italy (24). DDT was so effective in halting the spread of insect borne diseases that other methods of insect control were not used. With the saving of so many human lives, it was generally felt that all insect pests would be brought under control.

Late in the 1940's it was discovered that insects were developing an increasing resistance to DDT. Efforts were directed toward finding new compounds to replace DDT. Other organochlorine insecticides were synthesized but again insect resistance was a problem.

Organophosphates were used to replace the organochlorine pesticides, but the insects rapidly developed the required resistance. Additional problems were high toxicity to mammals. A U. S. Government report states that in 1969 there were over nine hundred pesticidal chemicals that were formulated into over sixty thousand marketed products (24).

The production and use of pesticidal chemicals in the United States is continually growing. Synthetic organic insecticide production in the United States is estimated to exceed six hundred million dollars by the year 1975. The rate of growth is estimated at fifteen percent per year in the pesticide industry. In 1967 the production of DDT was 103 million pounds, and production declined 27% since the year 1966. Production of DDT has declined since the peak production years of 1960-1963. Although production of DDT has declined, large quantities are still exported for use in foreign countries in malaria eradication programs by U. S. governmental agencies (24). The low cost of DDT makes malaria control economically feasible in underdeveloped countries. Were DDT banned or placed under severe restriction, malaria control and other health programs would have to be drastically reduced because of the high insecticide cost.

Due to various factors such as low cost (17.5 cents per pound), extreme persistence and ease of manufacture, large quantities of DDT are continuing to be released into the environment (24). DDT is probably one of the most widely distributed of all synthetic organic compounds. With large quantities of the compound being manufactured and used, it is not hard to imagine that DDT would begin to accumulate in the tissues of large numbers of animals, and even man himself.

The means of application of DDT is, for the most part, by spraying or dusting. The DDT would find its way from the foliage, or other surface, to the soil and the surface water. By streams and rivers, the compound would find its way to lakes and the oceans of the world. With the transmission of energy through the food chain, the DDT would tend to accumulate in the consumers high in the food chain.

Sladen, et al. (21) discovered DDT residues in animals from the most remote part of the globe. Samples of liver and fat from Adelie Penguins and a Crabeater seal were analyzed for DDT residues. These species are considered to be true residents of the Antarctic because their range seldom extends beyond the ice pack. By using electron capture gas chromatography, and thin layer chromatography, DDT and metabolites were detected from 13 to 15 parts per billion in liver, and from 24 to 152 parts per billion in the fat.

Woodwell, Wurster and Isaacson (30) have analyzed soils and animals for DDT, DDE, and DDD. The results were that the pesticide was nearing toxic levels. DDT has been found in the eggs of sea birds (Moore and Tatton, (15), and DDT has been found to be related to declining reproduction in the Bermuda Petrel by Wurster (32). The pesticide averaged 6.4 p.p.m. in the chicks and eggs of the Bermuda Petrel.

Abbot et al. (1) found atmospheric contamination. Antonmarie, Corn and DeMaio (2) also found that DDT was associated with airborne particulate matter. The presence of DDT was postulated to be from codistillation with water which was previously demonstrated to be

significant. In London, England, Wheatley and Hardman (27) derived pesticide residues from rain water. It can be seen that DDT and its metabolites seem to be ubiquitous.

In recent years, it has become apparent that DDT produces an effect on photosynthetic oxygen production and carbon uptake (14) (31). Work has been done concerning marine algae but no work has been done concerning the effect of DDT on fresh water algae. There are two methods for the oxygenation of fresh water in lakes and ponds. The methods are diffusion from the atmosphere and photosynthesis, primarily algal photosynthesis. The effect of DDT on photosynthetic oxygen production in fresh water algae has not been elucidated. I propose to investigate the results of DDT contamination of fresh water algae. The experimental organism chosen was Euglena gracilis. It was thought that the animal and plant characteristics of the organism might give a significantly different response to the pesticide.

REVIEW OF THE LITERATURE

DDT and Photosynthesis

In 1959 it was found by Hayes (10) that DDT had variable effects on two separate strains of barley. When DDT was applied according to specifications, chlorosis developed in four to ten days in susceptible strains, but not in resistant strains. The susceptable strain was Rika and the resistant strain was Proctor. Lawler and Rogers (13) studied photosynthesis and respiration as effected by DDT in the Rika strain. The determination was made by using the Warburg apparatus and excised leaf tips. The susceptible strains of barley showed a decrease in photosynthetic activity within twelve hours of treatment with DDT. The untreated strain reached its maximum photosynthetic activity as the seed stores were used up, and the chloroplasts matured. The treated susceptible strain never reached the photosynthetic maximum.

Lawler and Rogers (13) also did experimental work on the isolated chloroplasts of the Rika strain of barley. Four days after spraying susceptible plants with DDT, the chloroplasts isolated from treated barley showed a photosynthetic activity equivalent to only twenty-five percent of the untreated control chloroplasts. Chloroplasts from the resistant strains of barley did not show a reduction in photosynthetic activity after treatment with DDT. It was also found that the effect of DDT on the susceptible strain of barley could be significantly reduced by prior suspension in a solution of DDE. It may be that the prior treatment with DDE blocked some receptor site without producing any toxic effects.

Wurster (31) studied the effects of DDT on four marine species of phytoplankton. This work was done as a result of reports that DDT was starting to appear in the marine environment in significant and increasing amounts. It is known that a large portion of the oxygen produced in the world comes from the marine phytoplankton. It is a logical conclusion that interference with the photosynthetic process in the marine phytoplankton could have extensive consequences.

Wurster (31) grew cultures of algae in sea water medium for periods of twenty-four hours. The cultures were then treated with one or two microliters of pure ethanolic p,p'-DDT to reach the desired concentration. Control cultures received only ethanol. After culturing for twenty to twenty-four hours, ^{14}C-bicarbonate was added and the flasks were incubated for an additional four to five hours. Cultures were then collected on millipore filters with slightly reduced pressure and washed with sea water medium. The residue was then dried and counted. The rate of photosynthesis was determined by the amount of carbon fixed.

Results of the experiment were plotted as pesticide concentration versus percent of control photosynthesis. The curves of counts per minute versus concentration gave a typical dose-response shape. The maximum inhibition was displayed with Pyramimonas which was only about fifteen percent of the control value. The conclusion that photosynthesis is inhibited by DDT is well substantiated.

Menzel, Anderson and Randtke (14) have explored the action of DDT on cell division. Cultures of 1×10^4 cells per ml. were exposed to increasing quantities of DDT each day. Each flask containing 50 ml.

of culture received 100 ppb each day for seven days. There was variable effect on the algae noted. Increased supression of cell division was noted in all species of algae except Dunaliella tertiolecta. The photosynthetic activity as reflected by ^{14}C uptake was reduced in Skeletonema costatum, Coccolithus huxleyi and Cyclotella nana. It was found, however, that Dunaliella tertiolecta showed no reduction of photosynthetic activity with concentrations of up to 1000 ppb. D. tertiolecta also showed no photosynthetic degradation with two other insecticides dieldrin and endrin. Some of the algae showed an increased response to concentrations of DDT above the solubility limit of DDT in water. The solubility of DDT is 1.2 ppb in water (3). This phenomena indicates that the algae are capable of concentrating the pesticide from solution while the saturation of the medium is maintained. The remaining DDT is likely to be either absorbed to surfaces or in suspension.

DDT Metabolism

Although DDT is an extremely persistent pesticide, the compound is eventually degraded to less toxic or nontoxic residue by several means. Mosier et al. (16) states that insecticidal properties of DDT were reduced when the compound was sprayed on surfaces and subsequently exposed to sunlight. Mosier et al. found that DDT exposed to ultraviolet radiation at 253.7 nm decomposed. DDT deposited in a thin layer on the inside of quartz tubing was exposed to ultraviolet light in a helium atmosphere. The products of decomposition were recovered by gas-liquid and thin layer chromatography. The products of decomposition were DDD and DDE with small quantities of DBP and DDA. Carbon 14 was used to make quantitative determinations of amounts converted.

In mammals the degradation was worked out by Peterson and Robison (18). Rats fed DDT were used as experimental animals. Liver and kidneys were removed and extracted with ethyl ether and petroleum ether. Residues were cleaned up in a chromatographic column, and subsequently identified by paper chromatography. The metabolites that were found were DDE, DDD, DDMU, DDMS, DDNU, DDOH and DDA. Peterson and Robison suggested that the pathway for the degradation of DDT was DDT-DDD-DDMU-DDMS-DDNU-DDOH-DDA.

P. R. Datta (7) also carried out experiments on rats in an attempt to elucidate the pathway for DDT via DDE. The pathway elucidated was DDT-DDE-DDMU-DDNU-DDOH-DDA. Carbon 14 DDT was used and methods of detection of metabolites were paper chromatography and infrared spectroscopy. This pathway seems to be the one that is operative in the human.

Wedemeyer (26) has shown that DDT is degraded to DDD by Escherechia coli and Aerobacter aerogenes. The appearance of DDT in soil where only DDT had been applied pointed to a possible degradation by microorganisms. Experimentation with ^{14}C-DDT demonstrated DDD when incubation took place under anaerobic conditions. In other work, Guenzi and Beard (9) have demonstrated that the degradation of DDT to DDD occurs anaerobically by soil microorganisms. Additional degradation products that were found in small quantities were DDE and DDA. This accounts for the appearance of DDD in soil after DDT had been applied.

The ability of actinomycetes and fungi to degrade DDT was experimentally tested by Chacko and Lockwood (5). Actinomycetes were found to be capable of converting DDT to DDD but none of the fungi tested possessed the capability. There are additional works (11,19,17) (26) on the degradation of DDT to other compounds, but the metabolites found were the same as those mentioned previously. The aspect of DDT metabolism by algae has not been explored.

TABLE 1

ABBREVIATIONS OF METABOLITES

DDT-	1,1,1,-trichloro-2,2-bis(p-chlorophenyl)ethane
DDE-	1,1-dichloro-2,2,-bis(p-chlorophenyl)ethylene
DDD-	1,1-dichloro-2,2-bis(p-chlorophenyl)ethane
DDMU-	1-chloro-2,2-bis(p-chlorophenyl)ethylene
DDMS-	1-chloro-2,2-bis(p-chlorophenyl)ethane
DDNU-	unsym-bis(p-chlorophenyl)ethylene
DDOH-	2,2-bis(p-chlorophenyl)ethanol
DDA-	bis(p-chlorophenyl)acetic acid

A slurry of acetone was prepared and filtered through number one filter paper in order to obtain a clear filtrate of commercial DDT and acetone. The commercial mixture of DDT was recovered by evaporating the acetone in a gentle stream of air. Isomeric forms of DDT were dissolved in normal propanol with gentle heating at a ratio of five milliliters per gram. The test tube was permitted to stand overnight. Recrystallization from normal propanol was followed by vacuum filtration with a buchner funnel on number one filter paper. The needle-like crystals were washed three times with distilled water in order to remove traces of normal propanol. The crystals were dried in a vacuum for twenty-four hours.

Preparation of DDE

In order to prepare a standard for use in conjunction with p,p'-DDT on thin layer plates, DDE was prepared in the following manner. Purified DDT was dissolved in 20 ml. of 95% ethanol and excess sodium hydroxide (0.2g) was added to a 50 ml flask. Excess DDT was used to determine when the reaction had gone to completion. When the excess p,p'-DDT precipitate disappeared from the flask, the reaction was determined to be complete. The disappearance of the DDT crystals coinsided with the change of the solution from clear to slightly opaque. The solution was then added to 100 ml. of distilled water in order to precipitate the DDE. The liquid remained in an emulsion and sodium cloride was added to aid in the precipitation of DDE. The emulsion was then placed in a side arm flask and vacuum was applied

MATERIALS AND METHODS

The Organism of Study

An axenic culture of Euglena gracilis Klebs was obtained from the culture collection of Indiana University. The culture was number 369 isolated by Vischner. The culture is the same strain as number 1224/5C at the Botany School of Cambridge, England. The cultures grown in liquid media could be harvested by centrifugation in two weeks. The culture media is described by Starr (22). Stock cultures of the strain were maintained on agar slants.

Subsequent to harvesting from the heterotrophic media, the Euglena were transferred to autotrophic media and allowed to remain there for at least four days before experimentation. The autotrophic Bristol's Solution consists of only inorganic salts and distilled water (22).

Culture Illumination

Rapid growth in Euglena media was obtained with continuous lighting. Stock cultures were maintained in a photoperiod of twelve hours light and twelve hours dark. Continuous illumination was provided by one sixty watt incandescent bulb and two flourescent tubes of fifteen watts each. During incubation with pesticide the cultures were continuously illuminated by a sixty watt incandescent bulb.

Preparation of Pesticide

A commercial preparation of fifty percent wetable DDT powder was obtained from Southern Agricultural Insecticides, Boone, North Carolina.

to remove the ethanol. The precipitate formed, leaving a clear supernate. The white powder was dissolved in 95% ethanol and chromatographed on alumina thin layer chromatography sheet. Peterson and Robison (18) used a similar method for synthesis of DDE.

Chlorophyll Extraction Procedure

One hundred milliliters of Euglena gracilis culture that had been grown autotrophically in Bristol's Solution was centrifuged at 5,000 x G. The supernate was discarded, eight milliliters of 80% ethanol was added, and the cells resuspended. The alcoholic suspension of Euglena was placed in a ground glass tissue homogenizer. After fifteen minutes of homogenation, a small drop of the suspension was placed on a microscope slide and examined microscopically in order to determine if the cells had been disrupted.

The homogenate was then centrifuged at 5,000 x G and 2 ml. of the supernate was pipetted into a second test tube. Eight milliliters of acetone was added to the test tube with agitation. A reference solution was prepared of 20% ethanol and 80% acetone. The reference solution was placed in cuvettes and used to calibrate a Beckman DBG spectrophotometer. The chlorophyll extract was run on the spectrophotometer with the acetone-alcohol solution as a reference. The transmittance of the chlorophyll extract was determined at 10 nm intervals and the resultant information plotted. Peaks of maximum absorption occurred at 662 nm and 432 nm. The absorption at 660 nm was used for purposes of simplification to determine the graph of cell population versus percent transmittance. The spectrophotometric absorption spectrum is found in figure 1.

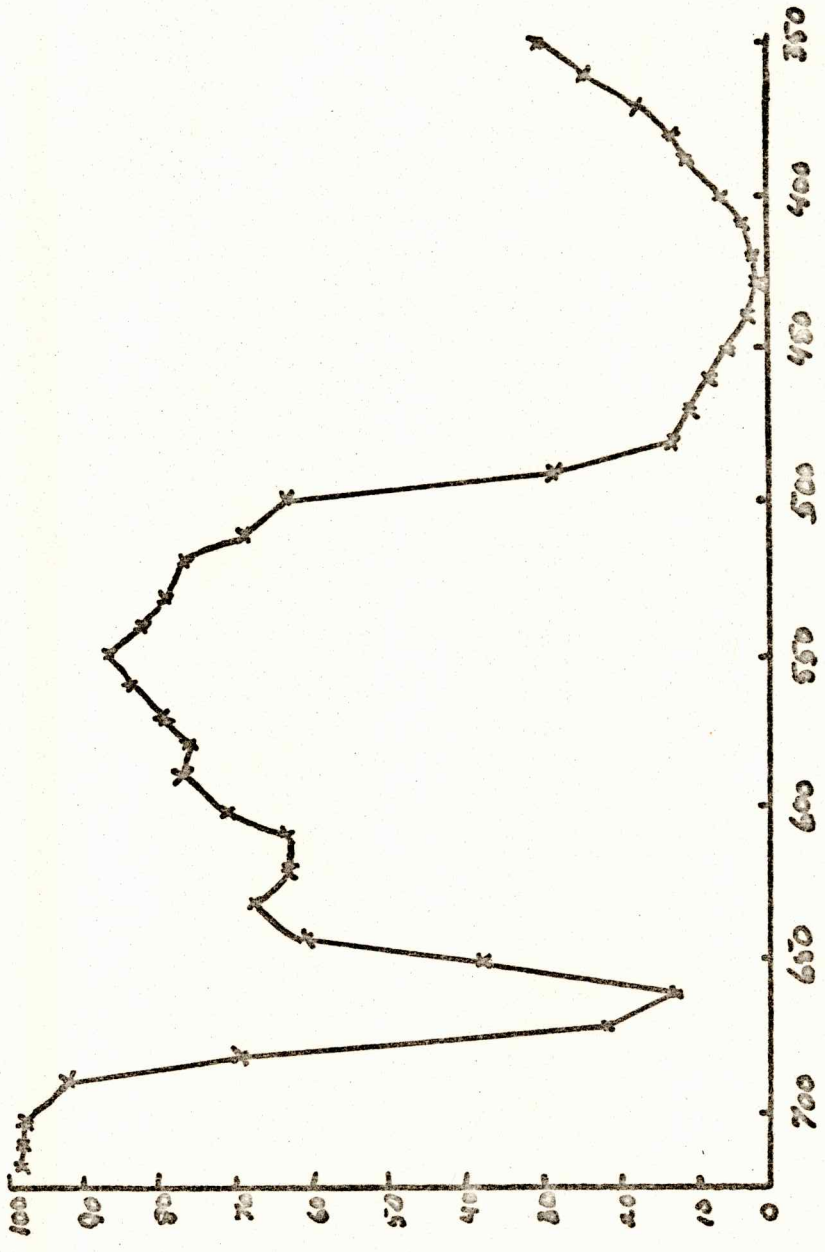

Figure 1. Absorption Spectrum of Euglena Chlorophyll

Spectrophotometric Population Determination

Spectrophotometric population determination was found to be a rapid method for obtaining a reasonable estimate of the number of cells per milliliter. It was necessary to make direct counts of cells in a culture in order to construct the required graph. The required graph would convert the percent transmittance to the population of algal cells.

The Bausch and Lomb Spectronic 20 was used to determine the percent transmittance before samples were taken for counting. After taking a reading of percent transmittance, 4 microliter samples were removed from the cuvette and placed on a microscope slide where direct counts were taken. Several samples were taken for counting in order to obtain more points on the graph. Dilution of the culture was carried out by the addition of sterile Bristol's Solution. The reference solution for the procedure was sterile Bristol's Solution and the calibration of the spectrophotometer was checked frequently.

The data were plotted on a semilogarithmic graph. It was found that if the cuvette was allowed to stand in the spectrophotometer for more than fifteen seconds, the percent of transmittance would increase due to the settling of the Euglena. In order to eliminate this error, the cuvette was inverted and the transmittance read immediately. The data derived from this procedure are present in figures 2 and 3.

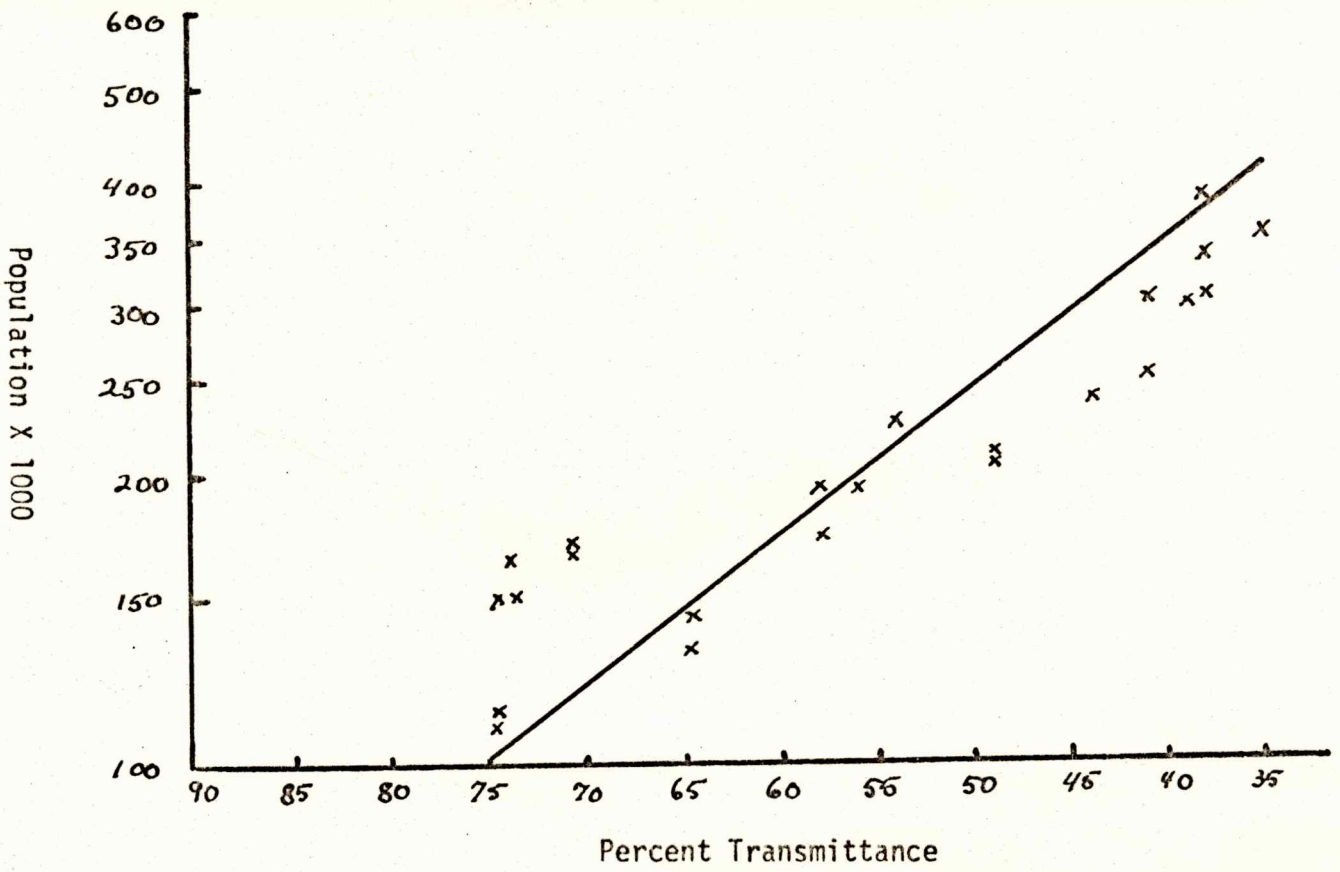

Figure 2. Population Versus Percent Transmittance (Part One)

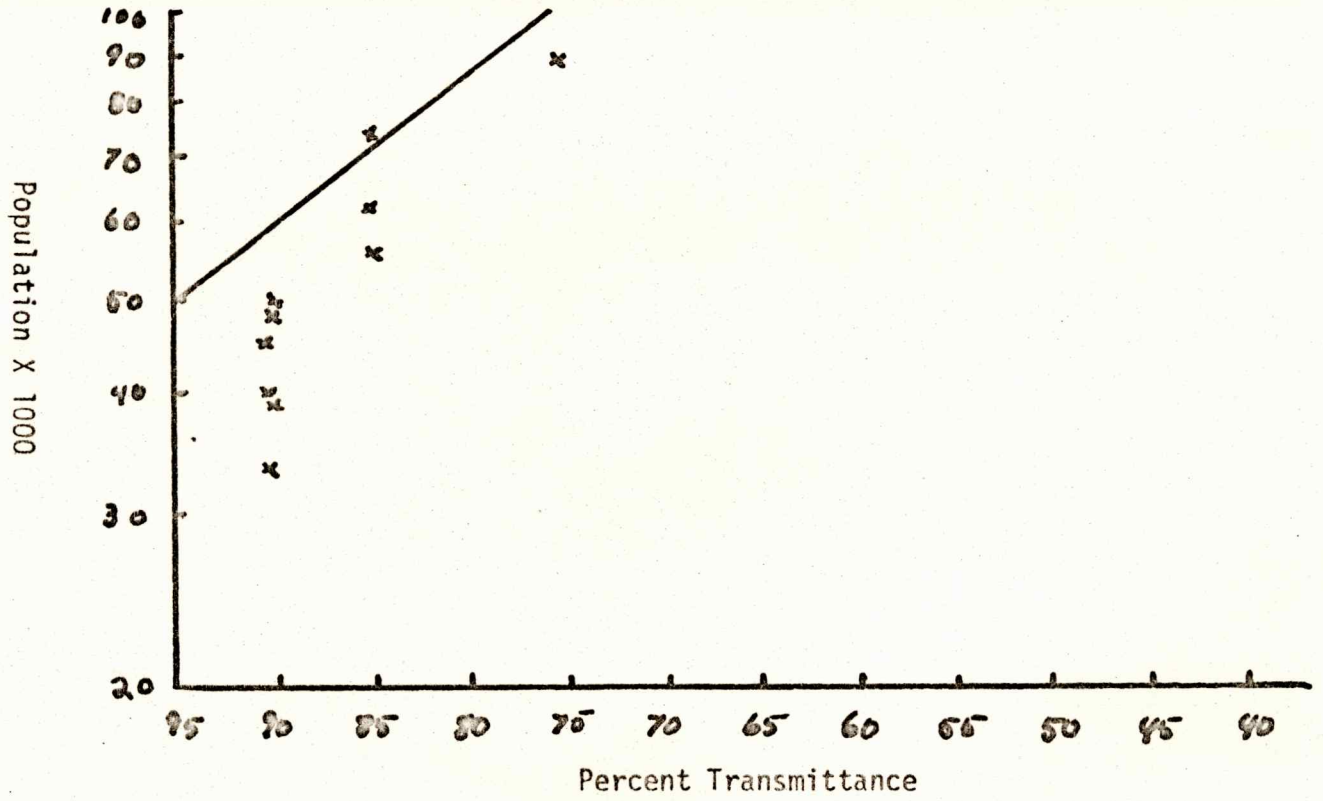

Figure 3. Population Versus Percent Transmittance (Part Two)

Temperature Graph Determination

In order to carry out manometric procedures at a maximum rate and therefore increase the accuracy of the procedures, a graph of oxygen production versus temperature was plotted. The photosynthetic oxygen production was determined at 16°C, 20°C, 25°C, 30°C, 35°C and 40°C. It was not possible to lower the reaction temperature below 16°C because of equipment limitations. The photosynthetic output of oxygen in microliters was plotted versus the reaction temperature. The resultant graph coinsided with those used by Devlin (8). The graph is typical of enzyme reactions in that the production of oxygen rises linearly and then levels off at a maximum rate. Figure 4 gives the graph of the experimental results. As a result of this experimentation the reaction temperature of 20°C was used.

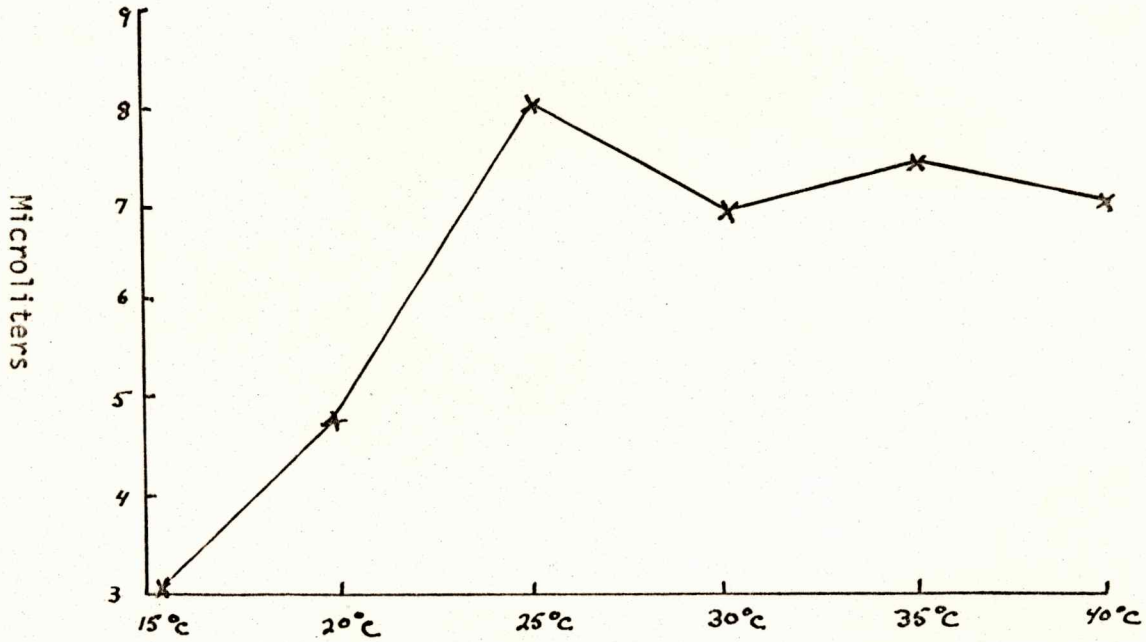

Figure 4. Oxygen Production Versus Temperature

Manometric Procedures

Manometric procedures were carried out on a Gilson Differential Respirometer. Cultures previously incubated on a shaking metabolic incubator were maintained at the required temperature. The cultures were illuminated during the twenty-four hour incubation procedure, and remained illuminated during the period of transfer to the respirometer. Fifty milliliter flasks containing the cultures of Euglena gracilis were shaken vigorously and the population determined by the previously described spectrophotometric method.

One milliliter of 0.4 M sodium bicarbonate was pipetted into each of four respirometer flasks. A small piece of filter paper was added to the center well of the flask in order to provide extra surface area for the absorption of carbon dioxide. Five-tenths milliliter of 6.0 N potassium hydroxide was added to the center wall of the respirometer flask. The Euglena culture was then agitated to insure even distribution of cells and three milliliters of culture were placed into each respirometer flask. The reference flasks contained distilled water.

The reaction vessels were closed to the atmosphere after five minutes of shaking in the water bath. After ten minutes of shaking in the water bath, the valves between the reaction and control flasks were closed. It was discovered that leaks could be detected by watching for a rise in the manometer fluid while turning the valve between the reaction and control flasks. The check for leaks was used at the end of the fifteen minute equilibration period. At the end of the equilibration period all valves were checked to see that they were closed and the manometer fluid meniscus was brought to the reference line.

The reaction was run for a period of one hour. Oxygen output was checked every five minutes on each of the four volumometers and subsequently recorded. The resultant data were corrected for differences in the cell population by use of the population graph and then plotted as a percentage of the control value.

It was found that a temperature of 26°C could be maintained without the use of cooling water. When the reactions were run at 20°C, cooling water was required. Cold tap water was usually around 17°C and was found to be sufficient to maintain the temperature. Requirements for maintaining cultures at low temperatures necessitated the use of an additional water bath. The overflow water from the respirometer was used to maintain the temperature of the pneumatic trough at 20°C.

Treatment of Cultures with DDT for Metabolites

Cultures of Euglena gracilis were treated with DDT in order to determine if metabolism of the insecticide occurs. Sterile test tubes were treated with 1.0 mg of DDT in ethanol. The ethanol was allowed to evaporate leaving a thin film of insecticide on the test tube. Twenty-five milliliters of axenic Euglena gracilis in heterotrophic media were added to the test tubes and incubated under constant illumination for fourteen days. Controls received an equal amount of DDT and sterile nutrient media. This would eliminate the possibility that metabolites were the result of photodegradation (16).

At the end of the incubation period, the culture was placed in a ground glass homogenizer and the cells disrupted. The homogenate

extracted with ether and the ether fraction concentrated to approximately 0.5 ml. The residue was then spotted on thin layer chromatography sheets and run against known standards.

Thin Layer Chromatography

After extraction of the algal culture, the ether extract was spotted on thin layer chromatography sheets. In order to provide reference standards, the unknown was run with p,p'-DDT and p,p'-DDE. The developing chamber was saturated with normal hexane prior to use. It was found that results were more reproduceable when this method was used.

The thin layer chromatography sheets used were Eastman number 6061 for silica gel and number 6063 for aluminum oxide. The sheets were cut into 7.5 cm x 20 cm pieces for use. Activation was accomplished by heating. After activation, the sheet was then spotted with DDT, DDE and the unknown. DDT and DDE reference standards were dissolved in 95% ethyl alcohol. The sheet was then placed in the developing tank and front was allowed to travel 15 cm.

The sheet was then removed from the developing tank and dried with a blower. Visualization of the compounds was done with short wavelength ultraviolet light. An alternative method for visualization utilized 2-phenoxyethanol and silver nitrate in a chromogenic spray followed by illumination with short wave ultraviolet light. The formulation of the chromogenic reagent and a similar chromatographic procedure are listed in an analytical manual (23).

R_f values for DDT and DDE were found to be .36 and .43 respectively on silical gel. On alumina sheets the R_f values for DDT was .58 and .65 for DDE.

DISCUSSION

It was thought that measurement of the photosynthetic output of Euglena could be made without determining the number of cells per milliliter. It was reasoned that a measure of productivity could be obtained by using the photosynthetic output of oxygen in microliters divided by the respiratory uptake of oxygen in microliters. Wolken (29) used a similar method to determine when Euglena gracilis had reached normal productivity during light adaptation.

Experiments were carried out in order to determine the practicality of the photosynthesis-respiration index. According to the index, cultures that had received greater dosages of DDT were more productive. The output of oxygen was less with increase in DDT dosage, but the respiration also decreased at a greater relative rate. It was suspected that DDT was having an effect on bacterial respiration, and thus leading to incorrect conclusions. Collins and Langlois (6) demonstrated a depression in growth of several bacteria. Ko and Lockwood (12) found that DDT in concentrations of 10 ppm inhibited the growth of several bacteria and a number of actinomycetes.

It was thought that with the use of antibiotics, the bacterial respiration could be reduced to near zero and the algae would be uneffected. By using stock cultures of bacteria obtained from cultures of Euglena gracilis, experiments were carried out with the use of antibiotics. Bacteria were innoculated into sterile tubes of nutrient broth and incubated at 37°C for twenty-four hours.

Three milliliters of bacterial culture were placed in each of two respirometer flasks. Respirometric procedures were carried out and the oxygen uptake of the cultures was measured. Bacterial cultures were then treated with antibiotic and the rate of oxygen uptake was again measured. The bacterial respiration rate decreased to 27% of the previous value.

The next step was to experimentally determine if the use of antibiotic would change the rate of oxygen production in Euglena gracilis. Three mililiter aliquots of axenic Euglena gracilis were checked for effect on photosynthetic oxygen production. It was found that the oxygen production of the algae decreased an average of 23%. This reduction in the oxygen production was large enough to exclude the use of antibiotic in manometric procedures.

A procedure utilizing filtration was tried in order to separate the algae from the bacteria. The respiration rate of the bacteria could be added to the photosynthetic output and the actual photosynthesis could be determined. By experimentation it was found that the process removed a large percentage of the bacterial cells. The process was not used because of inaccuracy.

Sterilization by dilution was tried and found not to be satisfactory. Since the cells were motile, many cells were lost when decanting the supernate. There were still large numbers of bacteria in the culture. The bacteria were not sufficiently separated unless the process was repeated at least ten times.

In order to make a spectrophotometric determination of the cell population, it was necessary to ascertain the absorption spectrum of Euglena chlorophyll. Peaks of maximum absorption would determine the wavelength to be used in the population determination. A maximum absorption peak was found at 660 nm and 430 nm. The peak of 660 mn was used because there would be less interference at the higher wavelength. The lower wavelength would be subject to more bacterial interference.

When the 660 nm wavelength was found, it was used in all of the population determinations. Small samples were pipetted onto a glass slide and counted by means of a mechanical stage microscope and a hand counter. The resultant data were tabulated and plotted on semi-logarithmic paper. With this graph, it was possible to relate oxygen productivity directly to cell density.

DDT used in the experiment was derived from a commerical dust containing 50% practical grade DDT. The powder was extracted with acetone and recrystallized from n-propanol. The purity of the compound was checked by infrared spectrophotometer, melting point determination and thin layer chromatography. All tests gave indication of the pure compound.

One of the metabolites, DDE, was prepared by basic reaction with sodium hydroxide at room temperature. The compound was recrystallized from water and ethanol. The compound was compared to DDT by thin layer chromatography on aluminum oxide plates. The reference data were derived from a pesticide analytical manual (23). DDT and DDE were used in chromatographic method for the identification of metabolites.

Euglena gracilis was used as an organism of study for several reasons. Most of the work that has been done with the genus Euglena has been done with Euglena gracilis. The organism has been characterized by much experimental work. Buetow (4) and Wolken (29) have reviewed much of the literature. Euglena lends itself to rapid culture in the laboratory and large numbers of cells can be obtained in a short period of time by heterotrophic growth in nutrient media. Euglena gracilis may also be grown in autotrophic media, but the time required to reach cell populations of 400,000 per milliliter is prohibitive. In heterotrophic media, the organism will reach the required level in two weeks or less. Previous work with DDT had been done with marine phytoplankton so the study of an organism with a fresh water habitat was needed.

The culture of Euglena was carried out in an undefined heterotrophic media. When the desired cell concentrations were reached, the cells were harvested and transferred to autotrophic media. The cells remained in the autotrophic media for at least four days in order to permit adjustment to autotrophic conditions. This was done to insure that the organism was fully photosynthetic. Graphs derived by Wolken (29) indicate that three days were required for adaptation from a dark to a light environment. Although organisms were not grown in the dark, it was felt that this was a suitable adaptation period.

After four days of adaptation time, the cultures were used for experimentation. Sterile 50 ml. flasks were previously treated with the required amount of DDT in ethanol. The ethanol was allowed to evaporate so that it would not be a factor in the reaction. Fifteen milliliters of the culture were added to the 50 ml. flasks. The

cultures were shaken for twenty-four hours at 26°C under constant illumination. This was to insure that the cells were exposed to the DDT under photosynthetic conditions.

The cultures were separately removed from the incubator and placed in the spectrophotometer to make the population determination. The oxygen output of the cultures was then found by manometric procedures. The cultures were illuminated during the transfer to the respirometer in order to prevent artifacts from appearing in the data of oxygen production. The time required for transfer was kept to a minimum and the average time required was twelve minutes. Fifteen minutes were allowed for equilibration so that the oxygen production would stabilize. The equilibration also removed most of the changes in volume caused by changes in the temperature of the equipment. The gas volume was checked at five minute intervals and the data recorded. The total reaction time was one hour and the data used for computation was derived from the last thirty minutes. This also allowed for additional stabilization of oxygen production.

The source of carbon for the algae was sodium bicarbonate. It was possible to use a buffer system to maintain a 3% atmosphere of CO_2, but this was discarded for the sake of simplicity. One milliliter of sodium bicarbonate was capable of providing an adequate carbon source for periods in excess of three hours. When using reduced temperatures, the flasks were incubated at 20°C and held at that temperature during all procedures. The flasks were prepared at the same temperature of incubation.

Isolation and identification procedures for metabolites of DDT were carried out on E. gracilis in heterotrophic media. Heterotrophic media was used to enable the organisms to obtain nutrient material without reliance on photosynthesis. It is logical to assume that if photosynthetic activity is reduced due to the pesticide, there would be less energy available for the metabolism of the same pesticide.

The treatment of Euglena with DDT was carried out under aseptic conditions. DDT treated control test tubes received sterile media and were incubated at the same time as the Euglena cultures. The controls were necessary to eliminate the possibility of photodegradation.

RESULTS, CONCLUSIONS AND LIMITATIONS

The experimental data show that the insecticide DDT inhibits photosynthesis significantly. Table 5, Figure 9, and Figure 10 show experimental results at reduced temperature. The reduction in photosynthetic rate by DDT occurs at reduced temperature down to concentrations of 1×10^{-12} g/ml. With increasing dosage, the oxygen production does not decrease below about 60% of the control rate. In unialgal cultures the rate of photosynthetic oxygen production is reduced by concentrations of DDT as low as 1×10^{-11} g/ml. Experimental data are listed in table 3 and plotted in figures 5 and 6.

In axenic culture, photosynthesis is not effected when the DDT concentration reaches 1×10^{-9} g/ml. At reduced temperatures, DDT experimentally produced a greater effect on the algae. The oxygen evolution was not reduced below 60% of the control value. The effect was extended to lower concentrations of the pesticide. Experimental results are shown in table 4, figure 7, and figure 8.

Axenic cultures were least effected by DDT. Unialgal cultures were found to be effected in an intermediate manner. The greatest effect was found in cultures incubated at a reduced temperature. Photosynthesis was found to decrease to a level of about 50% to 60% of the control and then level off. It must be remembered that the experimentation explored only the short term effect of the pesticide on photosynthesis. The effect of the DDT may be much more severe under long term exposure.

Within the limits of detection, no metabolites were found. It may be, however, that metabolism occurs but not in quantities detectable by thin layer chromatography. The inability of Euglena gracilis to metabolize DDT to less toxic or nontoxic residues suggests that any effect produced would be permanent. The metabolism of DDT might depend only on bacterial action in the environment, however more sensitive techniques using radioisotopes are needed to explore the metabolism of DDT by Euglena.

The reduction of photosynthesis by DDT is of ecological significance in the food chain. The reduction of algal photosynthesis would produce severe effects throughout the food chain. In cold, fresh water, photosynthesis is marginal at times. With DDT contamination, production of oxygen in ice covered lakes may be reduced to levels that result in large fish kills. In this manner, DDT would not have to be present in the water in amounts toxic to fish in order to be lethal.

In order to eliminate the problem of contamination, the use of DDT and other persistent pesticides must be eliminated. In order to eliminate DDT, a suitable cheap substitute must be found. One possible answer to the problem could be the use of insect juvenile hormones. Williams (28) suggests that the hormones are the answer to the problem. The hormones are not toxic substances as such, but simply prevent development which results in death. Resistance to the hormones could not be developed by the insect without committing suicide. The hormones are also very specific in that they produce their effect on only one family or one genus of insects. The quantity required for

the treating of large areas of land would be very small. The problem of synthesis of large amounts of these hormones could prevent practical use of these compounds for long periods of time. Biological control is another possible approach to the problem of insect pests. The concept of biological control requires extensive research like that of hormone control. The concept of biological control requires that the controlling organism not cause any harm to man if the prey is eliminated. For this reason, extensive use of biological control is not likely to occur in the near future. At the present, insecticides that degrade rapidly seem to be the best answer.

TABLE 2

LEGEND OF TABLES 4, 5, 6

1. DDT concentration in grams per milliliter.

2. Control oxygen production per 600,000 cells per hour in microliters.

3. Average oxygen production per 600,000 cells per hour in microliters. of experimental group.

4. Percent of control average of experimental group.

5. Oxygen production of individual experiments in microliters per 600,000 cells per hour.

6. Oxygen production of individual experiments expressed in per cent of control output.

TABLE 3

Data from Oxygen Evolution by Euglena in Unialgal Culture with DDT

1	2	3	4	5	6
1×10^{-10}	38.9	37.6	97.0	37.4	96.0
				38.6	99.0
				37.0	95.0
				37.4	95.0
1×10^{-9}	32.3	31.4	85.8	29.0	90.0
				32.0	99.0
				33.6	104.0
				30.8	95.5
1×10^{-8}	32.3	25.8	80.0	25.2	77.5
				26.2	81.0
				26.8	83.0
				25.0	77.5
1×10^{-7}	32.3	20.5	63.0	20.4	63.0
				20.4	63.0
				21.4	66.0
				20.0	62.0
1×10^{-6}	33.7	18.8	61.5	20.7	61.5
				18.7	55.5
				22.2	66.0
				21.4	62.5

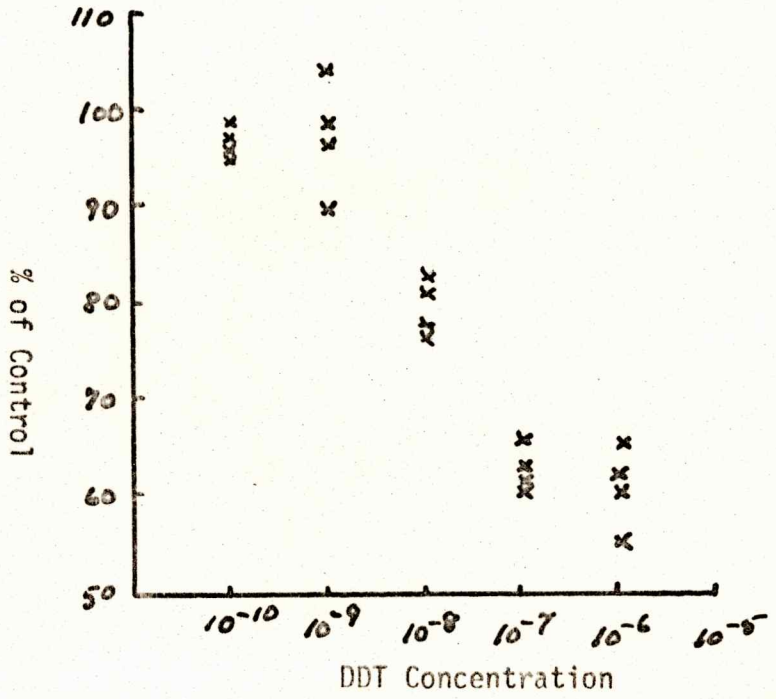

Figure 5. Oxygen Production of Euglena gracilis Versus DDT Concentration in Unialgal Culture

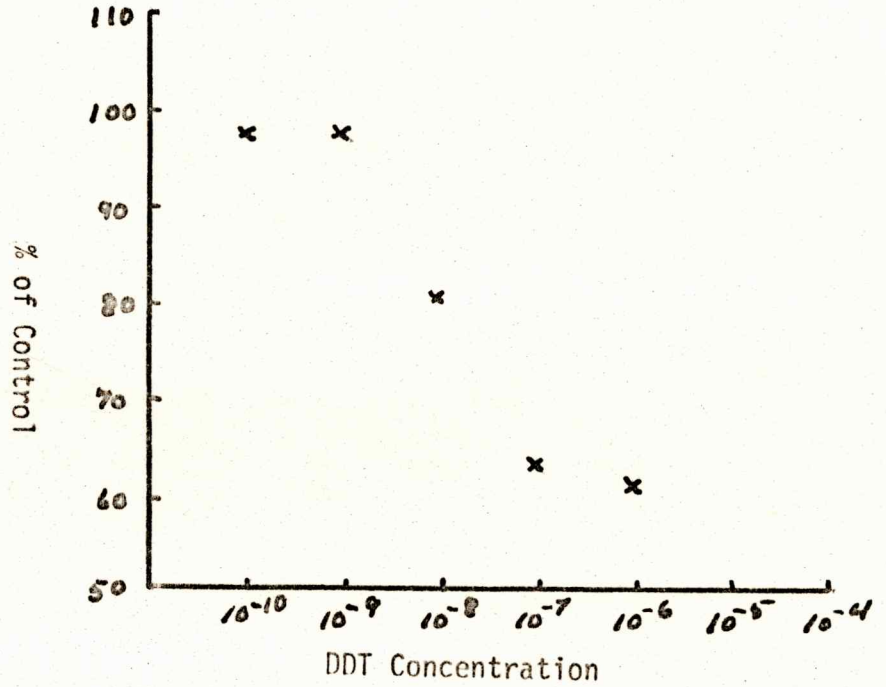

Figure 6. Average Oxygen Production of Euglena gracilis Versus DDT Concentration in Unialgal Culture

TABLE 4

Data from Oxygen Evolution by Euglena in Axenic Culture with DDT

1	2	3	4	5	6
1×10^{-10}	38.9	37.6	97	37.4	96.0
				38.6	99.0
				37.0	95.0
				37.4	96.0
1×10^{-9}	32.3	31.4	85.8	29.0	90.0
				32.0	99.0
				33.6	104.
				30.8	95.5
1×10^{-8}	32.3	25.8	80.0	25.2	77.5
				26.2	81.0
				26.8	83.0
				25.0	77.5
1×10^{-7}	32.3	20.5	63.0	20.4	63.0
				20.4	63.0
				21.4	66.0
				20.0	62.0
1×10^{-6}	33.7	18.8	61.5	20.7	61.5
				18.7	55.5
				22.2	66.0
				21.4	62.5

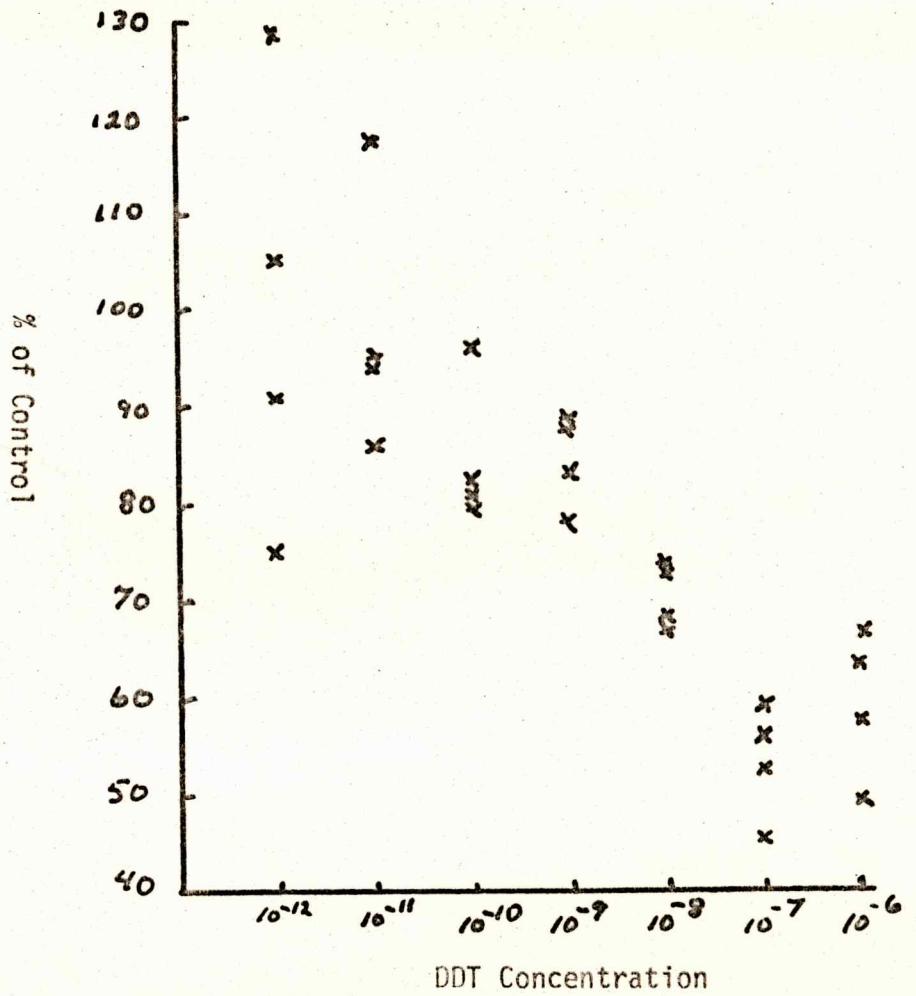

Figure 7. Oxygen Production of Euglena gracilis Versus DDT Concentration in Axenic Culture

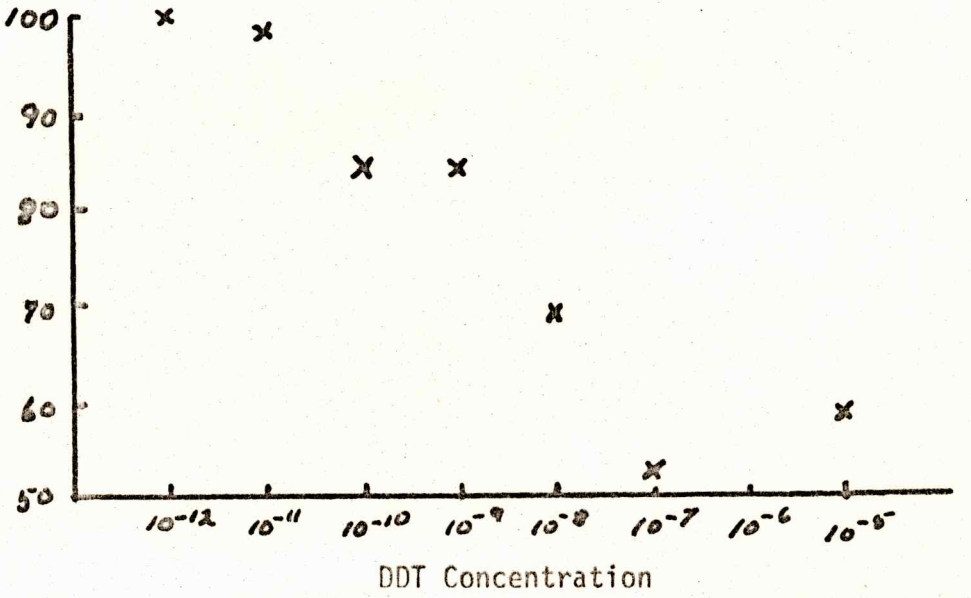

Figure 8. Average Oxygen Production of Euglena gracilis Versus DDT Concentration in Axenic Culture

TABLE 5

Data from Oxygen Evolution by Euglena in Axenic Culture at Reduced Temperature

1	2	3	4	5	6
1×10^{-13}	9.1	9.6	105	9.6	105
				10.0	109
				9.6	105
				9.2	101
1×10^{-12}	11.1	8.4	75.6	8.2	73.8
				8.6	77.4
				7.8	70.2
				9.0	81.8
1×10^{-11}	11.1	5.9	53.1	6.4	57.6
				6.0	54.0
				6.0	54.0
				5.2	46.0
1×10^{-10}	11.1	5.5	49.0	5.3	47.7
				5.3	47.7
				5.7	51.3
				5.7	51.3
1×10^{-9}	11.1	6.6	59.6	6.5	58.7
				6.5	58.7
				6.5	58.7
				6.9	62.3
1×10^{-8}	11.1	5.7	51.8	6.6	59.4
				5.6	50.4
				5.6	50.4
				5.2	46.8
1×10^{-7}	9.1	5.0	54.9	5.4	57.1
				4.4	48.3
				4.8	52.7
				5.6	61.0

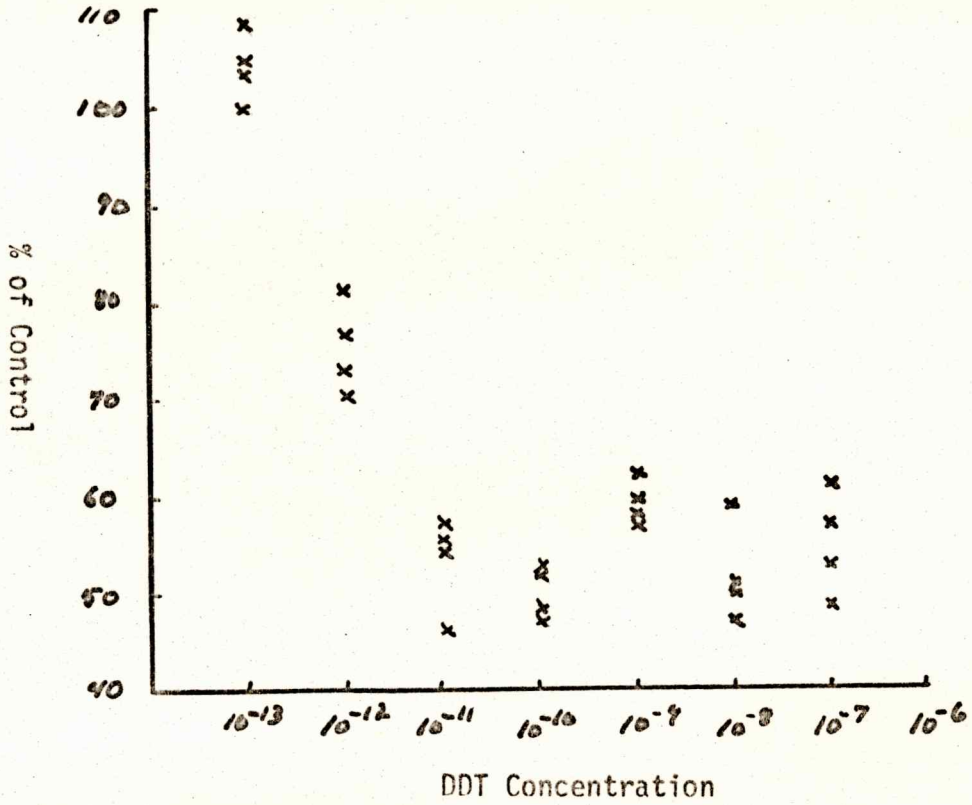

Figure 9. Oxygen Production of Euglena gracilis Versus DDT Concentration at Reduced Temperature

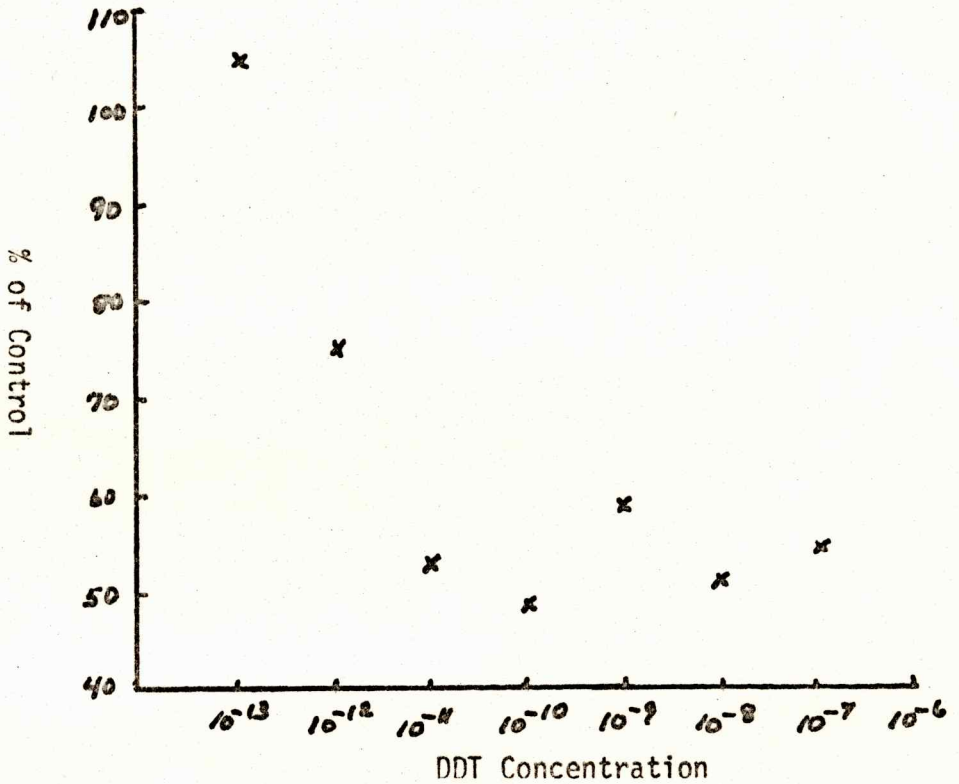

Figure 10. Average Oxygen Production of Euglena gracilis Versus DDT Concentration at Reduced Temperature

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VITA

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Upon completion of college, he enlisted in the United States Navy and was comissioned on August 25, 1967. Naval duties included a tour aboard the USS Montrose (LPA-212). He served aboard the USS Montrose as electronics officer and held many other collateral duties. Upon completion of duty aboard the USS Montrose he volunteered for advisor duty in the Republic of Vietnam. He completed advisor training, which included a course in Vietnamese, in Coronado, California. He arrived in Vietnam in January of 1970 for a one year tour of duty.

He was released from active duty in January of 1971 and began work toward a Master's degree in March. He married Greer Tarrant on June 4, 1971.

The typist for this thesis was Janice Ashley.