

An Investigation of Tolerance to Three Triazine Herbicides in
Chlamydomonas geitleri Ettl.

by

Derek Louis François

A thesis

presented to the University of Manitoba

in partial fulfillment of the

requirements for the degree of

Master of Science

in

The Faculty of Graduate Studies

Winnipeg, Manitoba

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' When I applied my mind to know wisdom and to observe man's labor on earth - his eyes not seeing sleep day or night - then I saw all that God has done. No one can comprehend what goes on under the sun. Despite all his efforts to search it out, man cannot discover its meaning. Even if a wise man claims he knows, he cannot really comprehend it. '

Ecclesiastes 8: 16-17.

ABSTRACT

The toxicity of three triazine herbicides (atrazine, simazine and terbutryn) was examined in unialgal batch cultures of Chlamydomonas geitleri Ettl. Changes in growth and chlorophyll accumulation were used as determinants. Herbicide-induced chlorophyll fluorescence was also used to differentiate the primary effect of the three triazines. The subsequent effect on inhibition of CO₂ assimilation was also examined. Cultures were pre-treated with 2.46 μM atrazine, and subsequently treated with varying concentrations of atrazine as a possible means of inducing increased tolerance. Photoheterotrophic potential was examined as a possible mechanism of achieving tolerance to atrazine, by supplying either glucose or acetate to growth media, and by monitoring the uptake of ¹⁴C-glucose.

Terbutryn was the greatest inhibitor of growth and CO₂ fixation, with a toxicity being 2 orders of magnitude greater than for atrazine and simazine. Its half saturation constant (K_{FRI}) for chlorophyll fluorescence was the lowest, which suggested that its affinity for binding at the active site was 32 and 50 times greater than that for atrazine and simazine respectively.

Atrazine and simazine were not significantly different in their inhibition of CO₂ fixation or at inducing chlorophyll fluorescence. However, atrazine inhibited growth 2 to 3.5 times more effectively than did simazine.

Inhibition of chlorophyll synthesis was most obvious with exposure to atrazine and terbutryn, with only a slight stimulation in chlorophyll occurring at the lowest concentrations of these herbicides. A strong

stimulation in chlorophyll synthesis, which was observed with simazine exposure, was interpreted as being a tolerance mechanism.

The obvious stimulation in growth and CO₂ fixation observed with simazine was perhaps due to the stimulatory effect of the herbicide on protein synthesis.

Pre-conditioning of cultures with 2.46 μM atrazine did not enhance tolerance as evidenced by, unchanged growth, CO₂ fixation, and chlorophyll fluorescence response upon subsequent treatment with atrazine.

Photoheterotrophic potential did not significantly contribute to growth, hence it did not provide a means of tolerance.

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INTRODUCTION

The triazine herbicides are used very extensively in agricultural practice for the control of broadleaf and grassy weeds in a wide variety of crop systems (WSSA,1983). The presence and accumulation of these triazine herbicides in the environment where they are used, may subsequently have an effect on plant communities that are not of agricultural importance, but may be of ecological significance. Like other herbicides, the triazines may accumulate in aquatic environments, by means of seepage into runoff waters (Hall et al., 1977; Glotfelty et al., 1984). In such cases, it has been suggested that the structure of the algal communities may be affected dramatically, in response to the toxicity of the herbicide, and thus alter the food chains/webs of aquatic systems. One change in community structure due to herbicide exposure, is the predominance of certain species or groups of algae, and the absence, or reduced number, of others, that occur naturally. This may be a feature of differential tolerance (DeNoyelles et al., 1982; Larsen et al., 1986). If the degree of tolerance is the means by which particular species will or will not survive herbicide exposure, then, it is valid to ask the following questions:

1)What is the tolerance of individual algal species, and how can it be measured ?

2) Do species demonstrate different levels of tolerance to different triazines?

3) Does an organism increase its tolerance with continual herbicide exposure ?

4) How does an organism acquire tolerance ?

Hence, these four aspects were investigated as they relate to tolerance. In this case, the study focused on the effects of three triazines on one alga. The triazine herbicides used were atrazine, simazine, and terbutryn. The alga used was Chlamydomonas geitleri Ettl .

The study was divided into two phases. The first of these examined the overall effects of the triazines, as indicated by changes in the organism's growth rate and chlorophyll accumulation. Secondly, since, the triazines function primarily by inhibiting a specific site in electron transport of photosynthesis, chlorophyll fluorescence response was used to detect the degree of inhibition at this site. The subsequent inhibition of CO₂ fixation was also examined as part of the investigation. In addition, the potential for utilization of organic substrates was examined as a possible mechanism of acquiring tolerance.

Hypotheses investigated were that: the degree of tolerance is different for the three herbicides tested; that pre-conditioning the organism at a sublethal concentration of herbicide will induce increased tolerance; that a degree of tolerance is achieved by increased chlorophyll synthesis; and that the degree of tolerance achieved may be related to the ability of the organism to shift to photoheterotrophic metabolism.

CHAPTER 1: LITERATURE REVIEW

1-1) Toxicology of Triazines

The s-triazines are one of the most widely used and economically important groups of herbicides. Many triazines are used as selective herbicides for control of broadleaf and grassy weeds in a wide variety of agricultural crops including corn, sorghum, and sugarcane (WSSA,1983). The most well known of these herbicides is atrazine, which has been described as the most heavily used pesticide in the U.S. (DeNoyelles et al.,1982). The triazines are characterized as having a central triazine ring of alternating nitrogen and carbon atoms, with hydrocarbon side chains, branching from two of the ring's atoms (positions 4 and 6). They are subdivided into 3 subgroups based on structure. The chloro-s-triazines, contain a chloride atom at position 2 of the triazine ring, and includes such herbicides as atrazine, simazine, propazine, cyanazine, and trietazine. The second group known as the methoxy-s-triazines, contain methoxy group on position 2, and includes such compounds as prometon, atraton, and simeton. The third subgroup, the methylthio-s-triazines, includes terbutryn, prometryn, simetryn, and ametryn, and are characterized by having a methylthio group at position 2 of the triazine ring (Fedtke,1982). In terms of relative toxicity, the methylthio-s-triazines are considered most toxic, followed by the chloro-s-triazines with the methoxy-s-triazines being the least toxic (Radosevich et al.,1979).

The triazines are classified as photosynthetic electron transport inhibitors. Their site of action is at the so called 'urea site' (or diuron site),

which is located between photosystems II and I prior to the reduction of plastoquinone (Duysens and Ames, 1962). There is evidence of a secondary electron carrier situated between Q and plastoquinone, called "R" or "B". A model envisaging the binding site as a "protein shield", that undergoes a steric change when binding occurs was first proposed by Renger (1976). This 32 kD protein exposed on the surface of the thylakoid membrane and which, is located over Q and the secondary acceptor B, could be removed by mild trypsin digestion. Associated with this removal was a loss in sensitivity to triazines and other electron transport inhibitors (Renger, 1976). Therefore, the site of action is now considered to be situated between Q and B (Moreland, 1980). The orientation of the triazine molecule at the active site may be controlled by alkylamino substitutes at ring positions 4 and 6 of the triazine ring (Moreland and Hill, 1962). Triazines (and other PS II inhibitors) act by lowering the midpoint potential of B relative to that of Q, thereby, preventing the transfer of electrons from Q to B (Moreland, 1980).

Due to the suppression of the availability of reduced ferredoxin, NADPH, and ATP, brought upon by inhibition of photosystem II, several secondary effects may occur. Loss of integrity of membranes (chloroplast, tonoplast, and plasmalemma) has been reported to occur within 2 to 4 hours following treatment with Hill reaction inhibitors (Moreland, 1980). The degradation of pigments is considered to result from photooxidation induced by the inability of chlorophyll to dissipate its absorbed excitation energy when electron transport is inhibited. A large number of energy-requiring biosynthetic reactions that are light-mediated are also inhibited. These include RNA and protein synthesis, biosynthesis of phenolic compounds, various enzymes involved in the synthesis of chlorophyll, and other

pigments and lipids, and many of the enzymes of carbon dioxide fixation pathways (Moreland,1980).

1-2) Triazine Levels in the Environment

The continued usage of triazines has led to contaminated aquatic environments resulting from herbicide seepage into agricultural runoff. Several researchers have concluded that erosional losses of these triazines from agricultural fields are dominated by movement in the water phase and not by movement of eroded soil (Hall et al.,1972; Glotfelty et al.,1984). Ritter et al. (1974) found atrazine concentrations were higher in sediment (7.35-1.77 mg/L) than in runoff water (4.91-1.17 mg/L), however, greater total losses were associated with the greater volume of water involved. Atrazine losses in runoff water and soil sediment have been reported as 2.4 and 0.16 %, respectively, of the total pre-emergent application to corn (Zea mays L.) by Hall et al. (1972).

It has been demonstrated by several researchers that the peak concentrations of triazines occurred in runoff water shortly after application. Reports by Hall et al. (1972) indicated that 1 month after atrazine application an average of 67.9 % remained in the soil, and 3 months later recoveries had decreased to 21.4 % of that applied implying that, seepage of the herbicide from sediments into runoff had occurred. Also atrazine and simazine in concentrations of 0.48 and 1.2 mg/L respectively, were present in runoff soon after application but rapidly declined with time (Triplett et al., 1978). Similarly, atrazine residues in five agricultural watersheds that

ranged in concentrations from 0.01 to 26.9 $\mu\text{g/L}$, coincided with the time of application (Muir et al., (1978). Levels of simazine even reached as high as 250 $\mu\text{g/L}$ in flowing canal water, following ditchwater application (2.25-7.43 kg/ha), but rapidly declined to less than 5 $\mu\text{g/L}$ (Anderson et al., 1978). However reports by Frank and Sirons (1979) have indicated that erosional losses of atrazine in runoff accounted for only 1% of the atrazine applied. A similar investigation by Hall et al., (1974) suggested that the amount of methoxy-s-triazines in runoff was very small (.03%). Hence it appears that peak concentrations of triazines occur only briefly in the water phase shortly after application. This is followed by accumulation in sediments, which, in some cases is preceded by a slow release from sediments back into the water phase.

Peak concentrations of triazines have also been reported following significant runoff events such as rainstorms (Hall et al., 1972; Glotfelty et al., 1984). In addition, it has been documented that significant amounts of simazine and atrazine have persisted throughout the year, following agricultural application, and have appeared in groundwater, stream water, irrigation ditches and farm wells (Smith et al., 1975; Frank et al., 1982,1987; Wilson et al., 1987).

From data compiled from the literature on concentrations of herbicides in runoff, several authors were able to formulate models for predicting maximum concentrations of herbicides in agricultural runoff (Leonard et al., 1979; Wauchope et al., 1978, 1980,1987). These models should be useful in water-quality planning for estimating the worst possible scenario of herbicide inputs from agricultural sources.

1-3) Effects of triazines on aquatic macrophytes and algae.

Many researchers, in their investigation of the effects of triazines on algal communities, have utilized in situ artificial enclosures and even artificial streams. Some of their findings have indicated a definite decline in algal biomass, with the most intense damage focusing on particularly susceptible species (DeNoyelles et al., 1982; Larsen et al., 1986). Atrazine at 10 mg/L in water was shown to cause a 27 fold decline in total biovolume of the periphyton community in a study utilizing artificial streams (Kosinski and Merkle, 1984). Using experimental ponds Larsen et al. (1986), demonstrated that a significant reduction in $^{14}\text{CO}_2$ uptake occurred at 100 and 200 $\mu\text{g/L}$ atrazine, within 2 weeks of application. $^{14}\text{CO}_2$ uptake then returned to control levels but again decreased relative to controls after another 2 weeks. A similar pattern of immediate depression followed by recovery, then depression, occurred at 500 $\mu\text{g/L}$ atrazine. Also changes were observed in phytoplankton community composition that were due to less sensitive species replacing the more sensitive species (Larsen et al., 1986). Crawford (1981) reported that within a farm pond exposed to 25 pound doses of simazine granules, all the vegetation in the pond was killed with the exception of traces of the dominant Chara vulgaris. Epiphytic algae were killed. Here the normal substrate-colonizing Rhizoclonium was replaced by a collection of benthic algae (Pediastrum, Anabaena, Mougeotia and, Spirogyra). Likewise, Herman et al. (1986) demonstrated with in situ limnocorrals that an atrazine concentration of 100 $\mu\text{g/L}$ inhibited periphyton production and altered the composition of the community. A decrease in Chlorophyte species was observed, whereas, Bacillariophyceae were the least sensitive algal group, and produced a greater biomass than in the controls.

Chlorophyll content and carbon assimilation were also shown to be reduced by >98% and >95% in periphytic algal communities exposed to 10, 100, 1000 µg/L terbutryn and 1000 and 5000 µg/L simazine respectively (Goldsborough and Robinson, 1983). Single applications of simazine at rates of 250 and 500 µg/L were very effective against blue-green algae (Anabaena , Anacystis , Aphanizomenon , and Gomphosphaeria), whereas, most flagellates and diatoms were tolerant , with the exceptions being Ceratium , Synura , Asterionella , Nitzschia , and Tabellaria (Ellis et al, 1976). Applications of 50 µg/L terbutryn to ponds, were sufficient to control filamentous green algae (Cladophora , Mougeotia , and Spirogyra), and at 100 µg/L the ponds were free of Chara but became recolonized with the more tolerant Rhizoclonium (Mackenzie et al, 1983).

Some studies were conducted on ponds where triazines were used to control nuisance algae. Tucker and Boyd (1978) demonstrated that applications of 13.4 kg/ha of simazine to the bottom of channel catfish ponds resulted in extended periods of low dissolved oxygen. This in turn resulted in a 19% reduction in catfish yield, which was possibly due to the suppression of Spirogyra , Rhizoclonium , and Pithophora . Terbutryn was also shown to reduce dissolved oxygen, and increase dissolved CO₂ and B.O.D., in ponds where it was used to control algal growth (Robson et al, 1976; Murphy et al, 1981).

The triazines have also been used to control aquatic macrophytes. Applications of 1 to 2 mg/L simazine controlled Potamogeton, Najas, and Ceratophyllum, (Walker, 1963). Terbutryn successfully controlled floating and submerged aquatic plants at concentrations of 100 µg/L (Muir, 1980). Similarly, Raymond and Davis (1981) demonstrated that atrazine at

concentrations of 80 $\mu\text{g/L}$ and 1040 $\mu\text{g/L}$ inhibited the growth of Elodea canadensis and Myriophyllum spicatum by 50% . Also, Elodea was shown to be inhibited at 3 and 5 μM simazine that caused the migration of chloroplasts to the center of the cell, where they aggregated. This was followed by the emptying of pigments into the cytoplasm, and subsequent death (Dabydeen and Leavitt, 1981). Using artificial nutrient medium, cultures of Lemna minor, Elodea canadensis, and Myriophyllum brasiliense, suffered reduced growth and oxygen evolution at simazine exposure of 0.12 to 1.0 mg/L (Sutton et al., 1968).

Investigators have utilized a number of culturing techniques for studying herbicide toxicity under laboratory conditions. Most of these include some form of batch culture and continuous culture. The batch culture technique involves growing the algae in a closed container with no addition of fresh medium or removal of catabolic waste in the experiment. The algae go through the characteristic pattern of growth cycle namely, lag, exponential, stationary, and death phases. Continuous culture systems involve a continuous supply of fresh medium to cells allowing for optimal growth and removal of catabolic waste. When nutrients are supplied at a constant rate to a growing culture, the cell population reaches "steady state" of growth and division, in which the total number of cells and the rate of cell division remain constant. To maintain this steady state the rate at which the culture medium is supplied must equal to the rate of cell division (Wong et al., 1983).

Parameters used in cultures to monitor toxicity include growth, chlorophyll content, $^{14}\text{CO}_2$ assimilation rate, and chlorophyll fluorescence. The concensus of these investigations is that, triazine toxicity (either 50%

inhibition or greater) occurs to varying degrees in the micromolar concentration range (Loeppky and Tweedy, 1969; Vance and Smith, 1969; Kratky and Warren, 1971; Ellis *et al.*, 1976; Torres and O'Flaherty, 1976; Hawxby *et al.*, 1977; Plumley and Davis, 1980; Bednarz, 1981; Vebér *et al.*, 1981; DeNoyelles *et al.*, 1982; O'Neal and Lembi, 1983; Stratton, 1984; Yee *et al.*, 1985; Larsen *et al.*, 1986). Also, some of these reports have stated that stimulatory effects on algal cultures, particularly on chlorophyll content and $^{14}\text{CO}_2$ assimilation rate, were observed with exposure to extremely low concentrations of triazines.

1-4) Triazine Tolerance and Resistance

The terms "resistance" and "tolerance" have commonly been used interchangeably and even misused, hence it is fitting to make a clear distinction between the two. According to Gressel (1982), tolerance refers to the natural or normal variability of sensitivity to pesticides or other chemicals, which exist within a species. Tolerance can be used to make distinctions between species, by saying for example, that one species is more tolerant than another to a particular pesticide. Resistance on the other hand, refers to the survival of a species or population at the usually effective dose of a pesticide.

Metabolism resulting in detoxification is an effective mechanism for reducing the toxicity of a herbicide, and increasing the organism's tolerance to the chemical (Shimabukuro, 1986). The triazines have been shown to be detoxified mainly by three metabolic pathways. One of the first known examples of detoxification was that of simazine to hydroxysimazine and later

with atrazine to hydroxyatrazine in tolerant maize (Zea mays L.) (Hamilton and Moreland, 1962; Shimabukuro, 1967). This process was via an hydroxylation reaction which was shown to be catalyzed by benzoxazinone (2,4 dihydroxy-7-methoxy-1,4-benzoxazine-3-one) and is characterized by the replacement of the chloride atom at position 2 of the triazine ring, with a hydroxy group (Shimabukuro, 1967). Another detoxification pathway is N-dealkylation which is accomplished by the removal of one or more N-alkyl side chains of the triazine ring. Sorghum was shown to completely dealkylate atrazine to 2-chloro-4,6-diamino-s-triazine, which had very little activity (Shimabukuro et al., 1973). Glutathione (GSH) conjugation is recognized as a major detoxification pathway in plants, and is a major factor in herbicide selectivity, as well as the mechanism for triazine resistance in corn (Shimabukuro, 1986). Here glutathione-s-transferase mediates the reaction that displaces the 2-chloro group, and replaces it with glutathione, thus rendering it inactive. It has been suggested that glutathione-s-transferase is specific for the 2-chloro group as well as the presence of both N-alkyl side chains, and that this mechanism was shown to be present in atrazine-tolerant grasses such as corn, sorghum, sudangrass, and sugarcane (Jensen, 1982). There is little information in the literature on the detoxification of terbutryn. Muir et al. (1981) revealed that the major degradation products of terbutryn were N-deethylated terbutryn, and hydroxy-terbutryn, detected in ponds containing heavy growths of Typha and Lemna which had been treated with 100 µg/L of terbutryn.

Many researchers have demonstrated the existence of resistance to the s-chloro-triazines in a number of weed biotypes. One of the first weeds to show triazine resistance was a biotype of Senecio vulgaris that showed

resistance to the application of 9 kg/ha atrazine (Radosevich and Appleby, 1973). Also the resistant biotype of Amarantus retroflexus was shown to survive an atrazine application of 18 kg/ha, whereas the susceptible biotype was killed at 0.6 kg/ha (Thompson et al., 1974). It was determined that there was no difference in the rate of simazine degradation between resistant and susceptible S. vulgaris biotypes (Radosevich and Appleby, 1973). Similar studies with resistant and susceptible biotypes of Amarantus sp. and Chenopodium album revealed no significant differences in the rate of atrazine degradation (Jensen et al., 1977; Radosevich, 1977). It was concluded by several authors that the plastids of these weeds were resistant, because the triazines did not bind to thylakoids (Arntzen et al. 1982; Gressel, 1985). It was also shown that this plastid-level of resistance was maternally inherited, most probably on the chloroplast genome (Souza Machado et al., 1978; Darr et al., 1981;). The resistance of the weed, Amarantus hybridus to s-triazines, has been shown to involve a change in the binding affinity of the herbicide to a chloroplast polypeptide of 32,000 daltons, which was due to a single amino acid difference in this protein (Hirschberg and McIntosh, 1983). It was suggested that the various families of herbicides had both specific and overlapping binding sites on this 32 kDalton shielding protein, and that at least two binding sites had to be occupied to induce conformational changes that would inhibit electron flow (Trebst and Draber, 1979; Pfister and Arntzen, 1979; Pfister et al., 1981; Steinback et al., 1981). Laasch et al. (1981), showed that four different herbicides (atrazine, diuron, ioxynil, and dinoseb) had specific binding sites. Included in the binding area model, are a central "essential element domain" that is common for all inhibitors bound, and a "specific domain" where different groups of PSII inhibitors occupy different overlapping regions of the outer binding

area. Mutational changes in one part of the binding area would eliminate binding and inhibition by one group of inhibitors, and affect only slightly or not at all binding and inhibition by other groups (Fedtke, 1982). The evidence for this stems from the fact that all triazine-resistant weed biotypes are susceptible to diuron (Gressel, 1986). Also, Matoo *et al.* (1984) demonstrated that alterations in the lipid composition and ultrastructure of the chloroplast of atrazine-resistant *Spirodela oligorrhiza* (Kurtz) resembled those of triazine-resistant weed biotypes and this may be a compensatory mechanism for maintenance of protein and lipid interaction of thylakoid membranes. Similarly, thylakoid membranes were more fluid in atrazine-resistance biotypes of horseweed (*Conyza canadensis*), perhaps suggesting these differences are responsible for triazine resistance (Lehoczki *et al.*, 1985). Hence it appears that the conformational changes in the binding protein and alterations in lipid and protein components of the thylakoid membrane are closely associated, and are very important in plastid functions, and dictate the onset of resistance through mutation.

Few studies have been conducted on true herbicide resistance in algae. Calvayrac *et al.* (1979) concluded that changes in conformation of PSII units were associated with DCMU resistance (2.5×10^{-5} M) in *Euglena gracilis*. Similarly in DCMU-resistant mutants of the blue green alga, *Aphanocapsa* 6714, alteration of the thylakoid membranes was suggested as a resistance mechanism (Astier *et al.*, 1979). These reports suggest a similar phenomenon of triazine-resistant that occurs in higher plants. Goldsborough and Robinson (1987) claimed that herbicide resistance can develop in lentic periphyton after short (7 day) exposure to simazine concentrations greater than 0.8 mg/L. Resistance was also verified by DeNoyelles *et al.* (1982), in species

recovery following 20 and 500 µg/L atrazine. In addition, Kruglov et al. (1970,1976), demonstrated that the decomposition of simazine in cultures of Chlorella vulgaris was an indication of some resistance.

CHAPTER 2 **THE EFFECTS OF THREE TRIAZINES ON THE
GROWTH, CHLOROPHYLL ACCUMULATION, AND
PHOTOHETEROTROPHIC POTENTIAL OF
CHLAMYDOMONAS GEITLERI**

2.1) INTRODUCTION

Several researchers have utilized bioassays with single algal species as a means of measuring herbicide toxicity and tolerance. (Wells and Chappell, 1965; Loepky and Tweedy, 1969; Vance and Smith, 1969; Pillay, 1972; Hollister and Walsh, 1973; Ellis *et al.*, 1976; Fowler, 1977; Tubea *et al.*, 1981; O'Neal and Lembi, 1983). Efforts have also been made to investigate the potential effects of triazines on whole algal communities (Johannes *et al.*, 1973; Butler *et al.*, 1975; Plumley and Davis, 1980; DeNoyelles *et al.*, 1982; Goldsborough and Robinson, 1984; Kosinski, 1984; Stratton, 1984; Herman *et al.*, 1986; Larsen *et al.*, 1986). These latter investigations have concluded that, in the recovery from triazine application, communities became dominated by relatively "resistant" or tolerant species. In general, these species were members of the Chlorophyceae and Bacillariophyceae, although, within these tolerant groups, some species were significantly more tolerant than others (Loepky and Tweedy, 1969; O'Neal and Lembi, 1983). This is an indication that different species, although similar in their community status, may express different responses to triazine applications, indicating that in depth examination of individual species is required. One of the purposes of this study was, therefore, to examine the toxicity of three triazines herbicides on a green alga in batch culture, by measuring their effects on growth rate, final yield, and chlorophyll accumulation. Since previous

exposure of an organism to sub-lethal levels of a herbicide may influence the degree of toxicity expressed. I also investigated the possible induction of tolerance by pre-adapting cells to a sub-lethal level of atrazine .

Many algae are able to incorporate certain organic compounds into cellular material in the light (Neilson and Lewin 1974). The term "photoheterotrophy", "photometabolism", and "photoassimilation", have all been used to describe such processes, which are generally restricted to situations where the organic substrates are the main source of cell carbon, during growth (Neilson and Lewin, 1974) . True heterotrophic growth may be expressed when fixation of respiratory carbon dioxide is suppressed, or by the addition of photosynthetic inhibitors (Neilson and Lewin, 1974). Therefore, a third purpose of this investigation was to determine if photoheterotrophy is a means of overcoming inhibition by triazines in a species of green alga, by measuring growth parameters of batch culture exposed to triazines and provided with an organic substrate, and by determining the uptake potential of that organic substrate .

2.2) MATERIALS AND METHODS

The green alga, Chlamydomonas geitleri Ettl, was isolated by J. Hall from Schist Lake, near Flin Flon, Manitoba (54° 45'N, 101° 50' W). The species was identified by J. Ettl .

For all growth experiments the alga was grown in artificial medium, in batch culture under a phosphorus concentration that was limiting to final yield (Appendix A) . In these experiments, WC medium (Guillard and Lorenzen,1972) was used, with the following modifications . The K_2HPO_4

concentration was reduced from 50 μM to 10 μM . The concentration of $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$ was increased from 100 μM to 800 μM and also, Tris buffer (4.1 μM) was replaced with Hepes buffer (5 μM). Both Na-silicate and Hepes were added as solids prior to autoclaving. The pH was adjusted to 7.8 with 1M NaOH. Nutrient stocks were added to the medium prior to autoclaving with the exception of Ferric-EDTA and, vitamins. These stocks were filter-sterilized through a 0.2 μm membrane filter, and added to the medium after autoclaving.

All glassware and storage containers for nutrient and herbicide stocks were emersed in an acid bath of 3N HNO_3 for at least 48 hours, after which they were thoroughly rinsed with deionized water (Barnstead Nanopure II). Deionized water was used in medium and nutrient stock preparation. Reagent grade chemicals were used for stock preparations.

Herbicide stocks for atrazine and terbutryn were made by dissolving the appropriate amount of herbicide in 100% methanol such that, the addition of 1 mL of stock to a litre of medium would give the desired final herbicide concentration. Due to its low solubility in methanol (1.98 μM), simazine stocks were prepared such that the addition of 5 mL to a litre of medium was required. To ensure that the same volume of methanol was added in each treatment, separate herbicide stocks were used for each desired final concentration.

For each growth experiment, 800 mL of medium was dispensed into each of 24 1-L Erlenmeyer flasks, fitted with silicone stoppers. In experiments where atrazine and terbutryn were used, herbicide stocks were added to the flasks at an equivalent of 1 mL/L of medium. Control flasks

contained 100% methanol (1 mL/L). In experiments with simazine, herbicide stocks were added at an equivalent of 5 mL/L of medium and controls contained 5 mL/L of methanol. Methanol-free controls were also established in all experiments. There were 8 treatments namely: 2 controls and 6 herbicide levels, with each treatment being triplicated. Each flask was inoculated with exponentially growing cells from either a batch or continuous culture, to give an initial cell density of 4000 cells/mL. All flasks were placed in a controlled environment at a temperature of 23°C with continuous illumination of $250 \mu\text{E m}^{-2} \text{sec}^{-1}$ (LI-1935SA Spherical Quantum Sensor with a LI-COR 1000 data logger) supplied by Cool White Fluorescent VHO tubes (Sylvania). Each flask was bubbled with air passed through a cotton trap, deionized water and finally a sterilized $0.45 \mu\text{m}$ membrane filter.

Initial cell counts were made on culture samples taken from each flask and subsequent cell counts were conducted twice daily on all flasks. Cell counting was performed with a Model B Coulter Counter. Experiments were terminated once stationary phase was well established, although different treatments within any experiment were not always terminated at the same time.

Where continuous cultures were used as an inoculum source each was maintained under steady state conditions of exponential growth ($100,000 \text{ cells/mL} \pm 10\%$) and a dilution rate of $0.133 \pm 5\%$. Dilution rate was maintained with a Manostat Cassette pump. All continuous cultures were grown with the modified WC medium, and the environmental conditions were identical to those used for batch cultures.

Cell counts were used to determine growth rates and final yields. Using plots of \log_{10} of the cell number against time (h), simple linear regression analysis was applied on the linear exponential growth phase of the curve (6-12 points) to determine growth rate, which was expressed as the growth constant, k' , or as generation time, G (doubling time). Final yield was taken as the mean of the last three cell counts in stationary phase .

For chlorophyll a analysis , known volumes of culture from each flask, in both exponential and final phase were filtered onto 2.4 cm Whatman GF/A filters . Duplicate filters from each flask were immediately wrapped in aluminium foil and kept frozen until extraction. Cell counts were recorded at the time of filtration. The filters were immersed in 10 mL of 90% methanol and placed in the dark for 24 hours. The chlorophyll extracts were vacuum-filtered, under low light ($0.314 \mu\text{E m}^{-2} \text{ s}^{-1}$), using 2.4 cm Whatman GF/A filters. Extracts were removed with a 5 mL glass syringe and, 4 mL dispensed into disposable polystyrene cuvettes. Absorbance was measured spectrophotometrically (LKB Biochrom Ultrospec 4050) first, at 665 and 750 nm, then samples were acidified with 0.1 mL of 4×10^{-2} N HCl and allowed to stand for 1 hour. Absorbance was again measured at 665 and 750 nm . The procedure for determination of phaeophytin corrected chlorophyll a was essentially that of Lorenzen (1967) and described by Marker et. al. (1980).

For some growth experiments , chlorophyll a was determined using a modified version of a fluorometric method by Stainton and Capel (1977) .The modifications included the following : the extraction was performed with 90% methanol instead of the prescribed 90% acetone; samples were neither macerated (tissue grinder) nor filtered, instead samples were shaken thoroughly, and the particulate material was allowed to settle, before the

extract was removed. Extracts were placed into 5 mL Pyrex cuvettes and measured with a Turner Model 111 Fluorometer equipped with a blue light source (Turner 110-853) and blue excitation (Kodak Wratten 47B) and red emission (Corning 2-64) filters. Values obtained from the fluorometer were used to calculate the amount of chlorophyll a present (Appendix B) With this method there was noticeable variation in chlorophyll a content between replicate samples. Also, there was no correction for phaeopigments. It was therefore decided to proceed with the method by Lorenzen. Fluorometrically determined chlorophyll a values were converted to values comparable with those determined spectrophotometrically with the Lorenzen method, using an established linear relationship between chlorophyll levels determined by both methods (Appendix C).

Three growth experiments were conducted with each of the three herbicides, atrazine (96.4%), simazine (99.62%), and terbutryn (99.99%) (Technical grade provided by Ciba-Geigy).

To investigate the induction of tolerance, exponentially growing cells (derived from continuous culture) were exposed to an atrazine concentration of 2.46 μ M atrazine (an approximate LD₅₀ based upon growth rate), in batch culture. After 3 days of growth (exponential phase), 800-900 mL of culture was centrifuged in sterilized 50 mL plastic centrifuge tubes, at 4000 rpm for 15 minutes. Supernatant was discarded and each cell pellet was washed with 40 mL of sterilized medium, and centrifuged again. The resulting cell pellets were resuspended in sterilized medium, and were used as the inoculum in growth experiments, which were conducted in a manner similar to those described above.

For the investigation of photoheterotrophic potential, experiments were also conducted as above, with atrazine except that, the medium was supplied with either glucose or acetate at a concentration of 1250 $\mu\text{g/L}$ (500 $\mu\text{g C/L}$). Growth rates and final yield were determined, but chlorophyll a content was not. One growth experiment was conducted for each of the two substrates.

For the determination of glucose uptake potential, 6 different pre-conditioned batch cultures were used. Each culture contained 800-900 mL of medium inoculated with exponentially growing cells from continuous culture. The two control cultures both contained methanol (1 mL/L), but only 1 contained glucose, at a concentration of 1250 $\mu\text{g/L}$ (500 $\mu\text{g C/L}$). The 4 atrazine treatments consisted of: 1.0 μM atrazine without glucose; 1.0 μM atrazine with glucose; 2.46 μM atrazine without glucose; and 2.46 μM atrazine with glucose.

Each exponentially growing culture was centrifuged at 4000 rpm for 15 minutes. The cells were washed with fresh medium and recentrifuged before being resuspended in medium. With each pre-conditioned treatment, 20 mL of resuspended culture was dispensed into each of 21 glass vials. The treatments consisted of 7 ^{14}C glucose concentrations, ranging from 4.10-844 $\mu\text{g C/L}$, with each treatment being triplicated. Each vial received an appropriate volume of standardized ^{14}C glucose stock (0.5 $\mu\text{Ci/mL}$, 20.33 $\mu\text{g glucose/mL}$). All treatments were incubated at 23°C under a light intensity of 250 $\mu\text{E m}^{-2} \text{sec}^{-1}$ supplied by Cool White VHO Fluorescent tube (Sylvania), for 1 hour. Samples were vacuum-filtered onto 0.45 μm membrane filters and washed with 25 mL of an unlabelled glucose wash solution (1250 $\mu\text{g glucose/L}$). Filters were placed into glass scintillation vials containing 5 mL

of Scinti-Verse (Fisher Scientific) scintillation cocktail , and allowed to stand for 48 hours. Specific radioactivity of samples was determined with a Beckman 4050 Scintillation Counter. Triplicate isotope standards were established by dispensing 10 μ L of C^{14} glucose stock into vials containing 5 mL of Scinti-Verse cocktail.

Glucose assimilation was calculated as follows:

$$V = \text{dpm}(\text{filter})/\text{dpm}(\text{provided}) \times \text{glucose concentration} \times 1.05,$$

where V is the velocity of uptake (μ g C/20 mL/hr); and 1.05 is a ^{14}C discrimination factor.

Cell counts were made on all 6 resuspended cultures and the rate of glucose-C assimilation was ultimately expressed as pg glucose-C/cell/hour. Chlorophyll a content (pg Chl. a /cell) was also determined on all resuspended cultures.

For each herbicide treatment within all experiments the parameters, growth rate, final yield, and chlorophyll a content, were expressed as percent inhibition of the methanol control treatments:

$$\text{Percent inhibition} = 100 \times (\text{treatment} - \text{control})/\text{control} .$$

Cell carbon of exponentially growing cells, was determined from cell volume, and based on the carbon content for algae (0.21- 0.24 pg C/ μm^3) reported by Reynolds (1984). Cell volume was determined from 21 microscopic measurements of cell radii, and by approximating the shape of cells to be that of a sphere.

2.3) RESULTS

2.3.1) Growth Rate

An ANOVA performed on growth rate inhibition, indicated that there were significant differences (at $\alpha=.05$) between triplicated experiments, and between identical treatment levels between experiments (Table 2-1). Growth rate inhibition expressed as a percent of methanol controls was plotted as a function of herbicide concentration (Figure 2-1). Probit analysis (SAS), was used for the determination of EC_{50} for all experiments. Simazine experiments A and C and terbutryn experiment A, failed to fit probit, as evident by the large chi-squared values listed in Table 2-2. EC_{50} values for these three experiments were estimated from the plots of growth rate inhibition, as a function of herbicide concentration (Figures 2-1b and 2-1c). Both derived values with 95% confidence limits, and estimated, EC_{50} values are listed in Table 2-3. The variation in EC_{50} , determined from probit analysis was greatest in atrazine experiments A and B, with a range of 2.03- 4.42 μM , and 1.67-4.45 μM , respectively. The smallest variation in EC_{50} occurred in terbutryn experiments A and C with respective ranges of 0.012-0.028 and 0.013-0.032 μM . Scheffe multiple comparison of means was applied to EC_{50} values, and is summarized in Table 2-4. Simazine treatment was shown to express the largest EC_{50} of 4.2819 μM and terbutryn the smallest with an EC_{50} of 0.0207 μM . Both were shown to be significantly different (at $\alpha=.05$) from all other groups. Atrazine, and supplementary atrazine, treatments were not significantly different (at $\alpha=.05$) from each other, although, their EC_{50} values of 2.2295 and 1.2968 μM respectively, differed by 42%. The

TABLE 2-1: Summary of ANOVA testing for differences in inhibition of growth rate of C. geitleri , between experiments, for different treatment levels, and for identical treatments, in triplicated experiments with atrazine, simazine, terbutryn, and with supplementary atrazine exposure. The analysis was performed at $\alpha=.05$ level of significance.

<u>Variable</u>	<u>P-Value</u>			
	<u>Atrazine</u>	<u>Simazine</u>	<u>Terbutryn</u>	<u>Supplementary Atrazine</u>
Experiment	0.0001	0.0001	0.0019	0.0001
Different Treatment	0.0001	0.0001	0.0001	0.0001
Identical Treatment	0.0001	0.0221	0.0004	0.0387

FIGURE 2-1: Inhibition of growth rate as a function of herbicide concentration, in C. gellleri for replicated experiments A (—), B (-----), and C (---), in cell cultures treated with atrazine (a), simazine (b), terbutryn (c), and with cell cultures pre-conditioned with 2.46 μ M atrazine, and subsequently treated with atrazine (d). Bars represent standard errors of replicates (n=3).

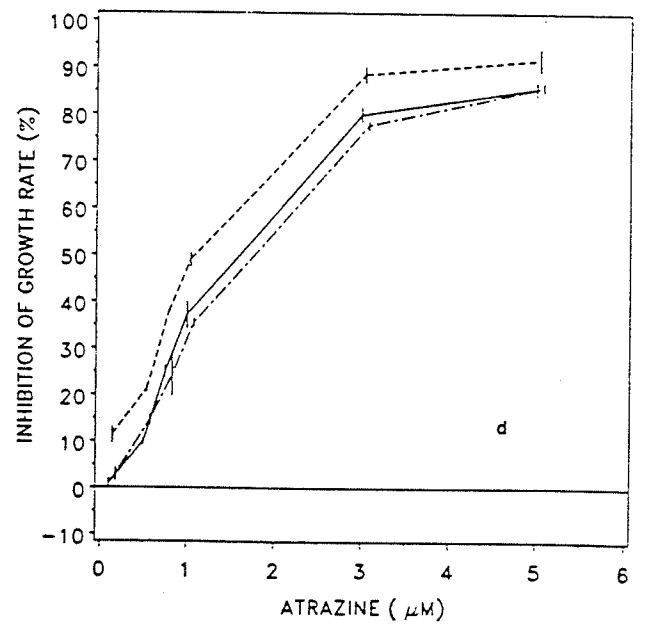
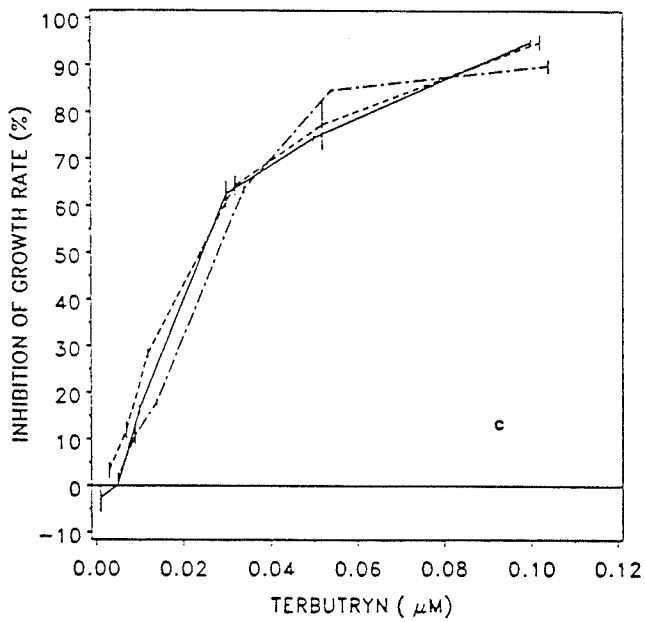
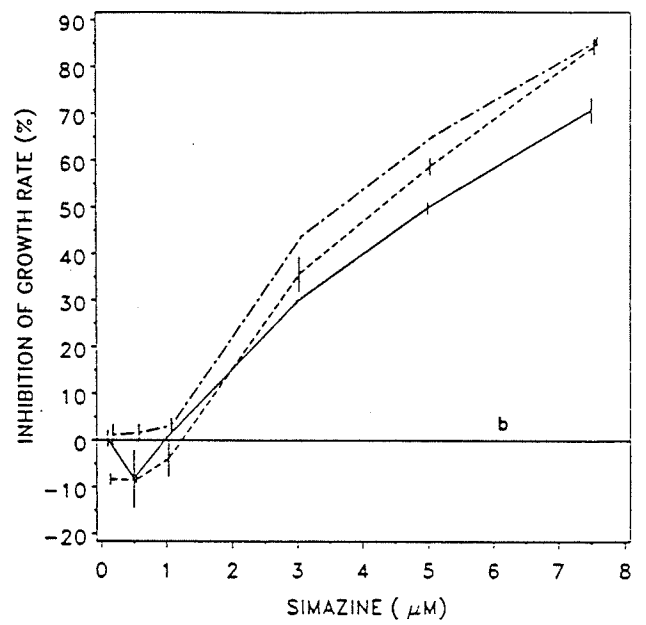
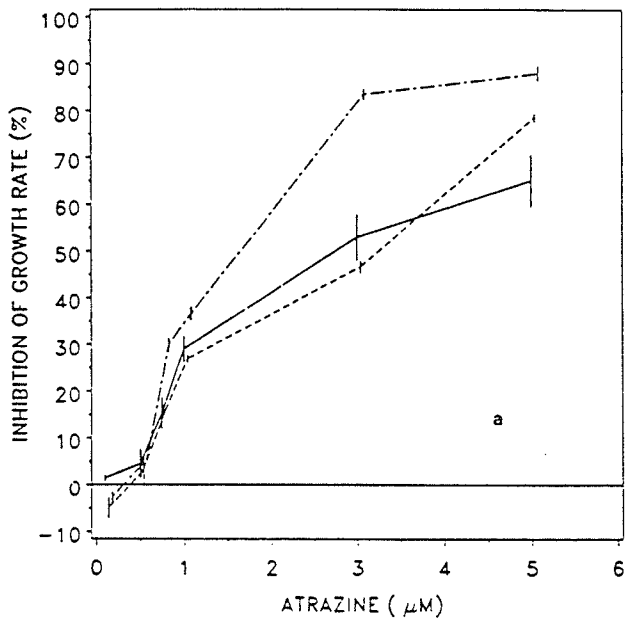


TABLE 2-2: Summary of goodness-of-fit test for probit analysis of EC₅₀ for growth rate, in C. geitleri, for triplicated experiments conducted with atrazine, simazine, terbutryn, and supplementary atrazine treatment.

<u>Experiment</u>	<u>Chi-squared value</u>			
	<u>Atrazine</u>	<u>Simazine</u>	<u>Terbutryn</u>	<u>Supplementary Atrazine</u>
A	21.4716	587.3375*	332.7997*	18.0730
B	23.0443	4.0221	39.9112	63.9551
C	27.8136	944.6743*	48.1915	18.5255

* The large chi-squared values indicate that the inhibition response did not fit probit analysis.

TABLE 2-3: Values of EC₅₀ for growth rate in *C. geitleri* determined from probit analysis (with 95% confidence limits), and estimated EC₅₀ values (* determined from inhibition response curves) for triplicated experiments with atrazine, simazine, terbutryn, and with supplementary atrazine treatment.

<u>Experiment</u>		<u>EC₅₀ (μM)</u>	<u>95% confidence limits</u>	
			<u>Lower</u>	<u>Upper</u>
Atrazine	A	2.7943	2.0295	4.4203
	B	2.4683	1.6722	4.4495
	C	1.4258	1.0043	2.0862
Simazine	A	5.12*	-	-
	B	4.0258	3.7816	4.2675
	C	3.70*	-	-
Terbutryn	A	0.0230*	-	-
	B	0.0184	0.0117	0.0281
	C	0.0207	0.0130	0.0322
Supplementary Atrazine	A	1.4871	1.1908	1.8876
	B	0.9320	0.5231	1.6362
	C	1.4712	1.1549	1.9173

TABLE 2-4: Summary of Scheffe multiple comparison of EC₅₀ means for growth rate in C. geitleri , in experiments with atrazine, simazine, terbutryn, and with supplementary atrazine treatment. Means with the same letter are not significantly different from each other. Analysis was performed at $\alpha=.05$ level of significance.

<u>Experiment</u>	<u>MeanEC50</u>
Atrazine	2.2295 a
Simazine	4.2819 b
Terbutryn	0.0207 c
Supplementary Atrazine	1.2968 a

coefficient of variation in EC_{50} for growth rate, for all experimental groups, ranged from 11.1 to 32.1 % .

A slight stimulation in growth rate was observed when atrazine, simazine, and terbutryn were applied at low concentrations (Figures 2-1a, 2-1b, and 2-1c). No experiment with supplementary atrazine treatment showed stimulation in growth rate (Figure 2-1d).

Maximum inhibition was achieved at the highest herbicide concentrations in all experiments, with values in the range of 76 to 90 % for atrazine, 76 to 87 % for simazine, 92 to 98 % for terbutryn, and 88 to 96 %, for supplementary atrazine treatments (Figure 2-1).

2.3.2) Final Yield

A single factor ANOVA (Table 2-5) indicated that significant differences (at $\alpha=.05$) in inhibition of final yield, occurred between experiments and identical treatments. Inhibition of final yield was plotted as a function of herbicide concentration for all experiments (Figure 2-2). Individual experiments were analyzed by determining EC_{50} from linear regression analysis, that followed a Woolf transformation of coded values of inhibition of final yield (Figure 2-3). In two simazine experiments, EC_{50} was not derived since in one case, 50% inhibition was not attained, and in the other, the linear relationship was poor ($r^2=.5808$). For the remaining experiments the linear relation value of r^2 ranged in value from .7541 to .9622 . The EC_{50} for these experiments were estimated from plots of inhibition of final yield as a function of simazine concentration (Figure 2-2b). A list of derived and estimated EC_{50} values is given in Table 2-6. A Scheffé

TABLE 2-5: Summary of ANOVA testing for differences in inhibition of final yield in C. geitleri, between experiments, different treatment levels, and between identical treatment levels, in triplicated experiments with atrazine, simazine, terbutryn, and supplementary atrazine treatment. Analysis was performed at $\alpha=.05$ level of significance.

<u>Variable</u>	<u>P-value</u>			
	<u>Atrazine</u>	<u>Simazine</u>	<u>Terbutryn</u>	<u>Supplementary Atrazine</u>
Experiment	.0001	.0076	.0001	.0123
Different Treatment	.0001	.0001	.0001	.0001
Identical Treatment	.4053	.0001	.0001	.4197

FIGURE 2-2: Inhibition of final yield as a function of herbicide concentration, in *C. geitleri* for replicated experiments, A (—), B (-----), and C (---), for treatments with atrazine (a), simazine (b), terbutryn (c), and supplementary atrazine treatment with cells pre-conditioned with 2.46 μ M atrazine (d). Bars represent standard errors of replicates (n=3).

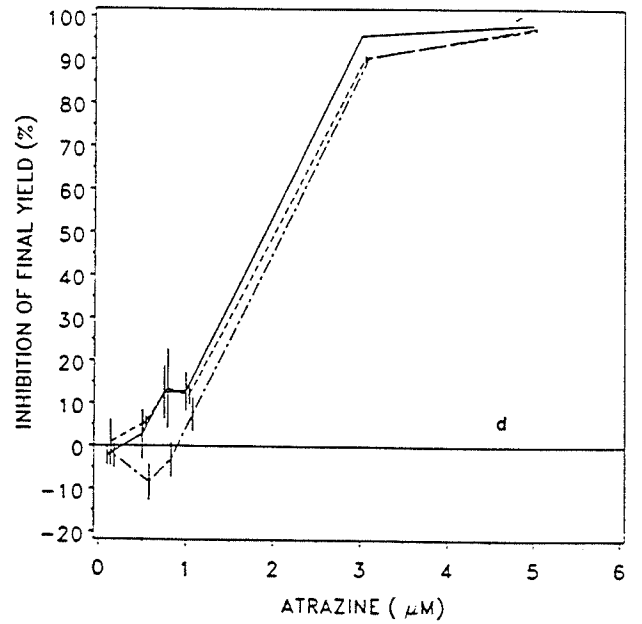
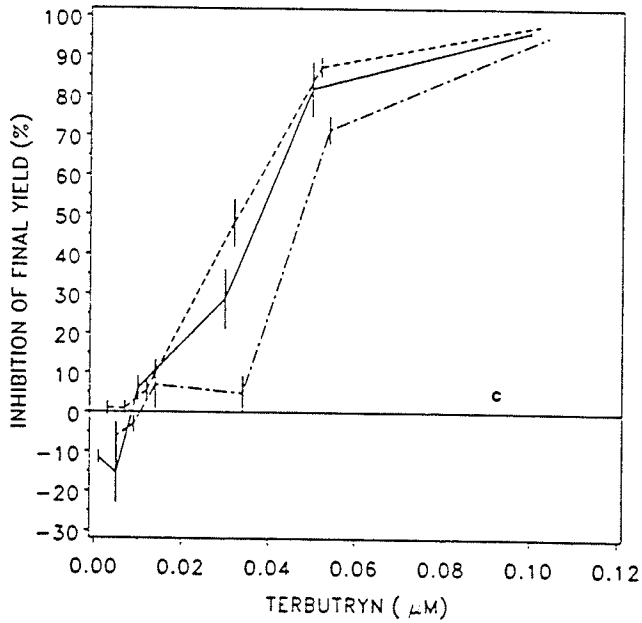
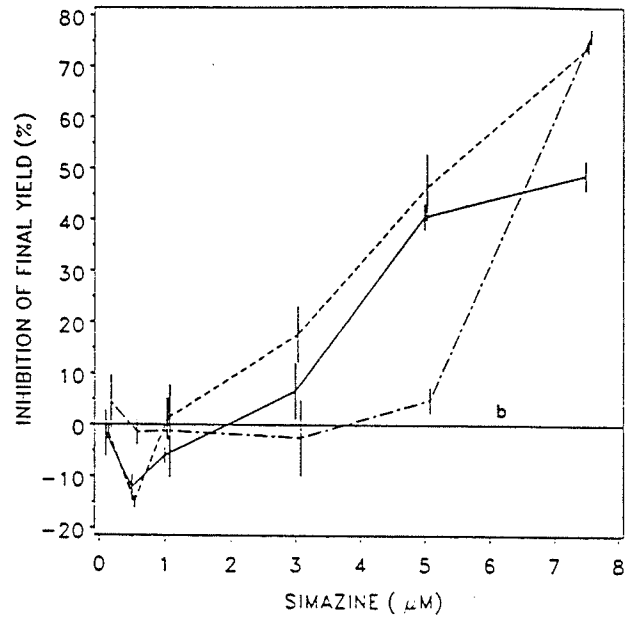
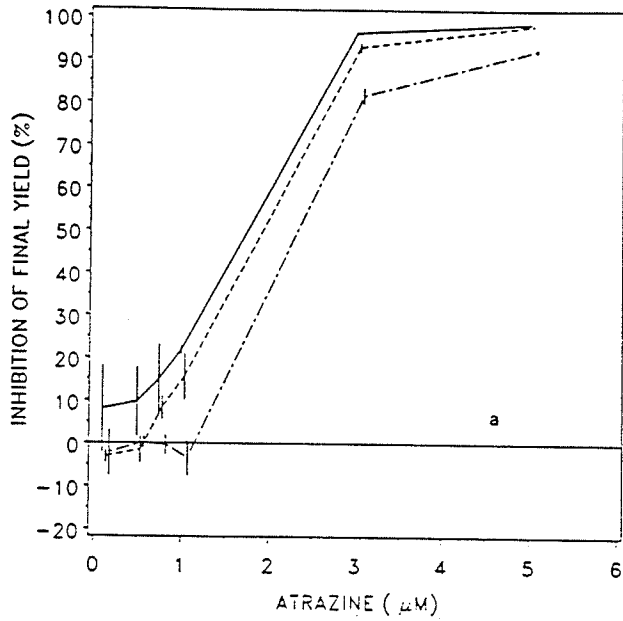


FIGURE 2-3 Regression plots of inhibition of final yield as a function of herbicide concentration, following a Woolf transformation of coded inhibition values, in experiments using Chlamydomonas geitleri exposed to atrazine (a), simazine (b), terbutryn (c), and to a supplementary atrazine treatment (d). Replicated experiments are represented as A(—), B (-----), and C (---). Symbols \circ \bullet \blacksquare represent individual values for experiments A, B, and C respectively.

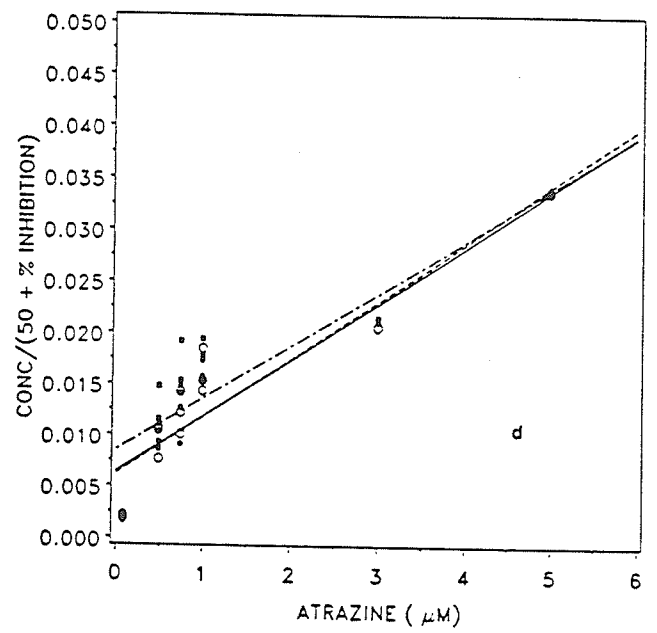
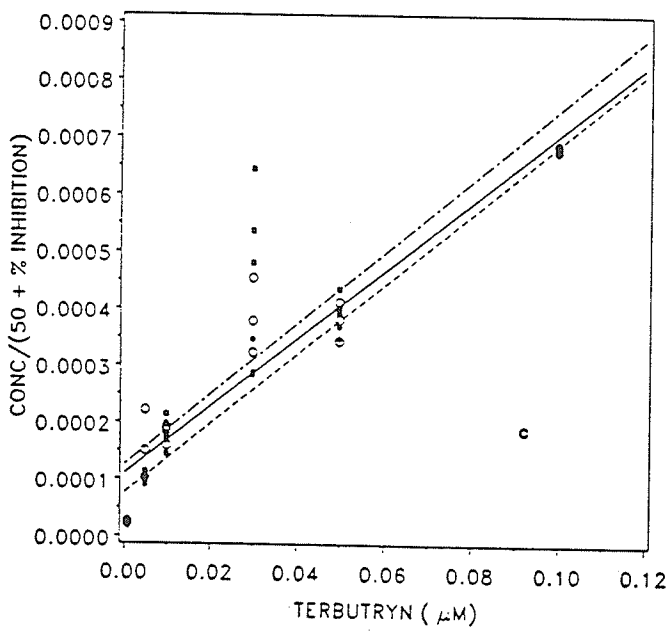
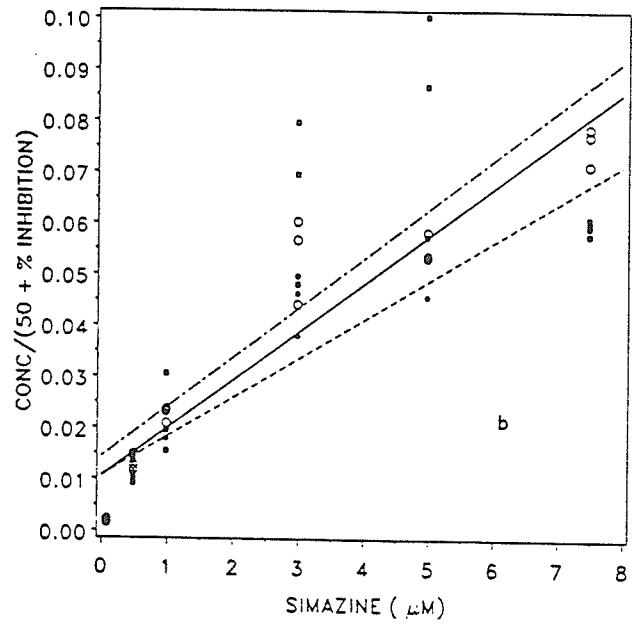
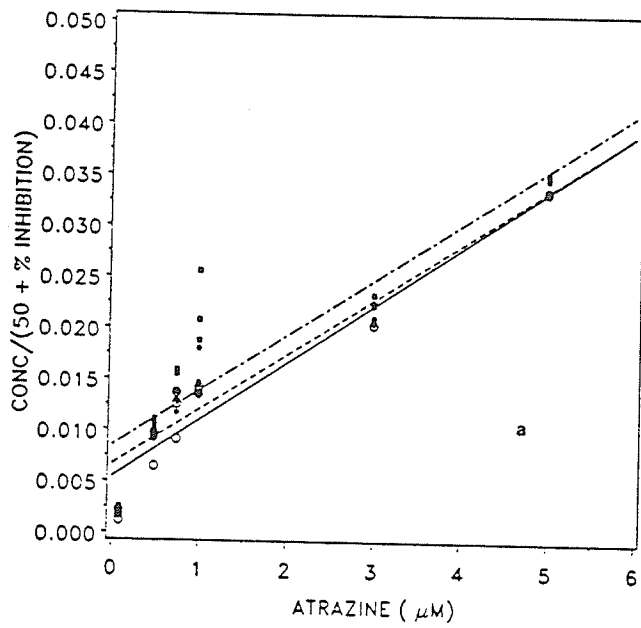


TABLE 2-6: Values of EC₅₀ for final yield in *C. geitleri* determined from linear regression analysis, and estimated EC₅₀ (* determined from inhibition response curves) for triplicated experiments with atrazine, simazine, terbutryn, and with supplementary atrazine treatment. (** EC₅₀ was not estimated since response curve did not attain 50% inhibition).

		EC ₅₀ (μM)	
		Atrazine	Simazine
Experiment	A	1.2303	**
	B	1.4417	4.4710
	C	1.8561	6.52 *
		Terbutryn	Supplementary Atrazine
Experiment	A	0.0269	1.3985
	B	0.0331	1.3020
	C	0.0194	1.7276

multiple comparison of means was applied to EC₅₀ values (Table 2-7). Simazine was shown to have the largest EC₅₀ of 5.4955 μ M, and terbutryn the smallest, with a value of 0.0265 μ M. Both simazine and terbutryn were significantly different from all other groups. Atrazine and supplementary atrazine treatments were not significantly different (at $\alpha=.05$) from each other, with respective EC₅₀ values of 1.5093 and 1.4760 μ M.

In all herbicide groups, stimulation in final yield occurred to varying extents at low herbicide concentrations (Figure 2-2). The most obvious case of stimulation was observed with simazine treatment (Figure 2-2b).

Inhibition of final yield reached a maximum at the highest herbicide concentrations in all experiments, with values in the range of 92 to 98 % for atrazine, 48 to 75 % for simazine, 94 to 97 % for terbutryn, and 96 to 98 % for supplementary atrazine treatment.

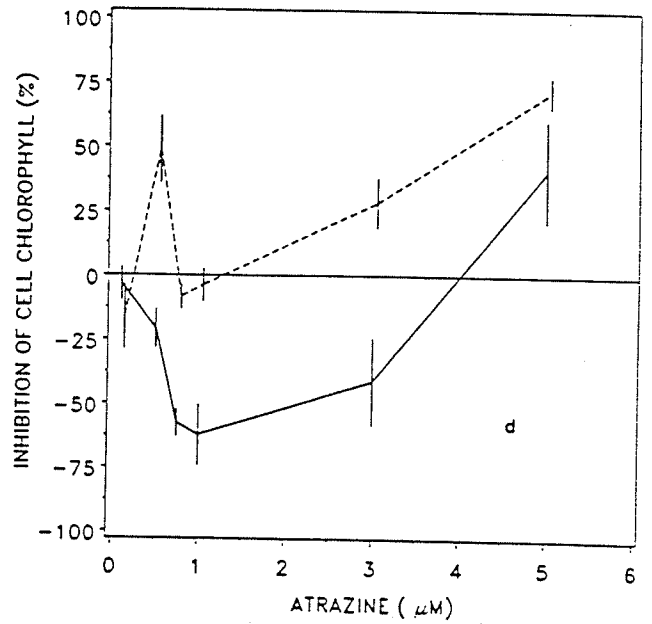
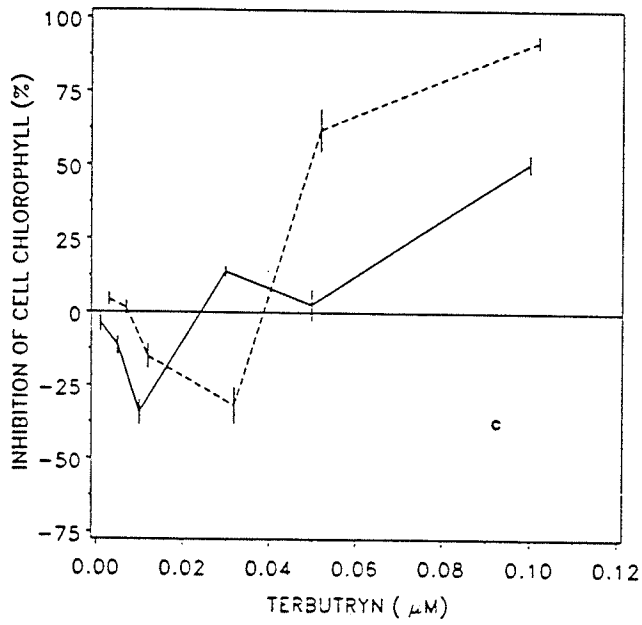
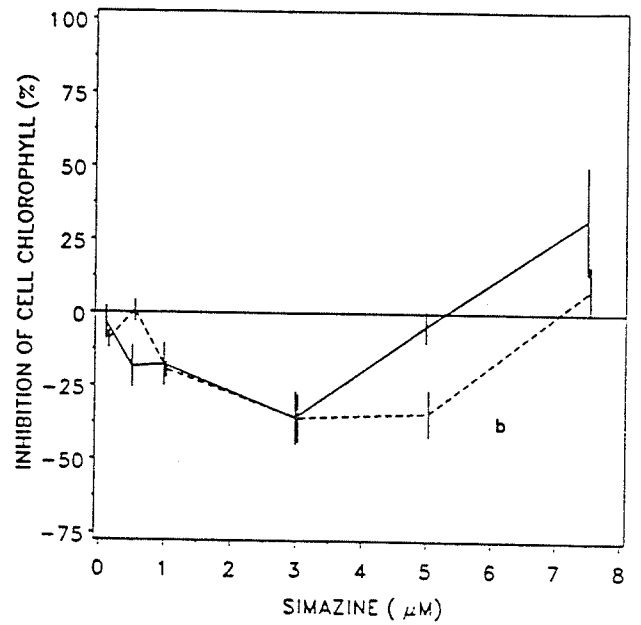
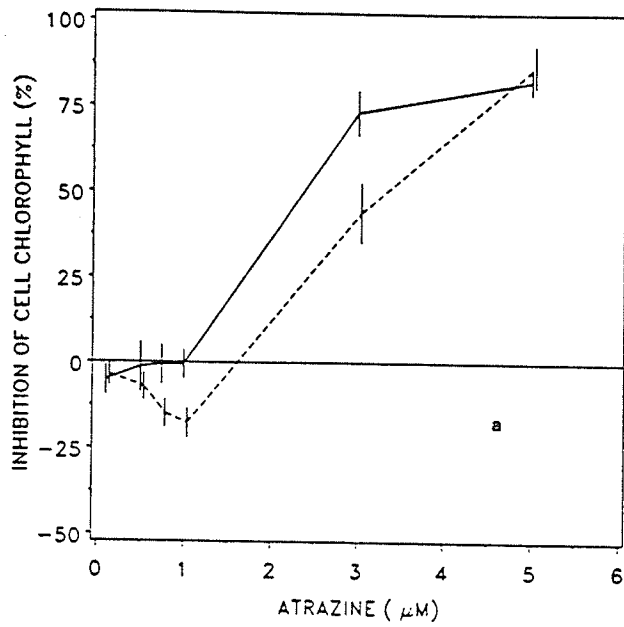
2.3.3) Chlorophyll a Accumulation

Data from triplicated experiments were combined and inhibition of chlorophyll a accumulation, expressed as a percent of methanol controls, was determined for cell cultures in both exponential and stationary phases of growth, for all experimental groups. Inhibition of cell chlorophyll a accumulation as a function of herbicide concentration is illustrated in Figure 2-4.

TABLE 2-7: Summary of Scheffe multiple comparison of means of EC₅₀ of final yield, caused by atrazine, simazine, terbutryn, and supplementary atrazine treatments. Means with the same letter are not significantly different from each other. Analysis was performed at $\alpha=0.05$ level of significance.

<u>Experiment</u>	<u>Mean EC₅₀ (μM)</u>
Atrazine	1.5093 a
Simazine	5.4955 b
Terbutryn	0.0265 c
Supplementary Atrazine	1.4760 a

FIGURE 2-4 Inhibition of chlorophyll a accumulation in C. geitleri for cells in exponential (—), and stationary (-----) phases of growth, in experiments with atrazine (a), simazine (b), terbutryn (c), and supplementary atrazine (d), treatments. Bars represent standard error of replicates (n=9).



Clearly, there appears to be greater stimulation of chlorophyll synthesis in cells at stationary phase, when compared to cells in exponential phase, at the atrazine concentrations of 0.10 to 1.0 μM (Figure 2-4a). The inhibition is less for cells at stationary phase of growth throughout the range of exposure concentrations except at 5.0 μM atrazine, where it attained a slightly higher value (87%) than for exponentially growing cells (83%). Stimulation in chlorophyll synthesis reached a maximum of 0.6% at 0.10 μM atrazine in exponentially growing cells, whereas, with cells at stationary phase the maximum stimulation was 20% achieved at 1.0 μM .

A marked increase in chlorophyll accumulation was evident with simazine treatment (Figure 2-4b), that appeared throughout from 0.10 to 5.0 μM range for cells in both exponential and stationary phases of growth. Stimulation was at a maximum at 3.0 μM in both exponential and stationary phases, with respective values of 35 and 47%. Maximal inhibition of 32 and 8% was achieved at 7.5 μM simazine at exponential and stationary phases of growth respectively.

Increasing chlorophyll accumulation was observed in exponentially growing cells with terbutryn exposure of .001 to .01 μM (Figure 2-4c), and was extended to 3.0 μM with cells in stationary phase. Stimulation was maximal at .01 μM for cells in exponential phase (37%) and at .03 μM for cells at stationary phase (36%). Maximal inhibition of 51 and 92% was achieved at 0.10 μM terbutryn in cells at exponential and stationary phases of growth, respectively.

With supplementary atrazine treatment, following pre-conditioning with atrazine, chlorophyll a accumulation was inhibited to a much greater extent for cells at stationary phase as compared to cells in exponential phase (Figure 2-4d). The stimulation in chlorophyll accumulation for exponentially growing cultures proceeded throughout most of the exposure concentrations, reaching a maximum of 71% at 0.75 μM and, attaining an inhibition maximum of 41% at 5.0 μM atrazine. Cells in stationary phase expressed a dramatic reduction in chlorophyll accumulation throughout the range of exposure concentrations with a stimulation of 26% occurring at 0.10 μM , and with a maximum inhibition of 71% achieved at 5.0 μM atrazine.

In summary, simazine expressed the most sustained stimulation of chlorophyll a accumulation, throughout most of the exposure concentrations, for cells at both stages of growth. Simazine treatment also induced the lowest maximal inhibition (32 and 8 % for exponential and stationary phases, respectively) for cells at both stages of growth. Atrazine treatment caused the highest inhibition and the lowest stimulation at both stages of growth. Supplementary atrazine treatment caused sustained chlorophyll stimulation in cells at exponential phase, whereas, at stationary phase of growth, inhibition was more pronounced. Inhibition was greater at stationary phase of growth than at exponential phase, with terbutryn treatments.

2.3.4) Organic substrate utilization

A Scheffe's multiple comparison was used to analyze the inhibition means of growth rate and final yield, for the batch culture experiments

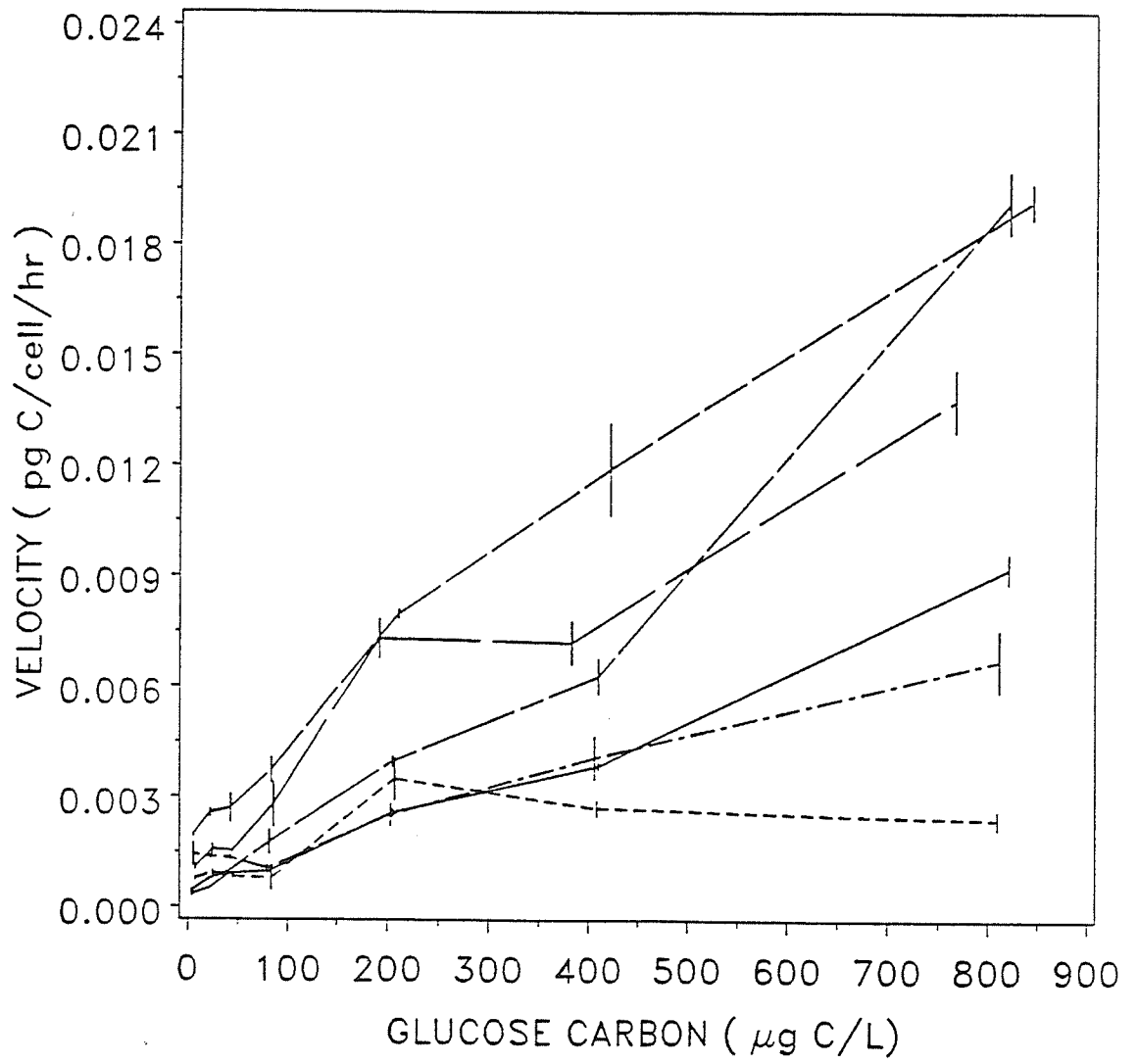
utilizing glucose and acetate (Table 2-8). The analysis indicated that neither organic substrate caused any significant (at $\alpha=.05$) reduction in inhibition.

The mean velocity of glucose uptake rate (expressed as $\text{pg C cell}^{-1} \text{ hr}^{-1}$), as a function of glucose carbon concentration, for each pre-conditioned treatment, is illustrated in Figure 2-5. The control without glucose pre-treatment, exhibited a greater uptake of glucose than the control with glucose pre-treatment, at concentrations of $300 \mu\text{g C/L}$ and above, and attained a maximum uptake rate of $.0092 \text{ pg C/cell/hr}$. This was 4 times greater than the uptake maximum for the control with glucose pre-treatment. Uptake rates were similar for both control treatments at glucose carbon concentrations less than $300 \mu\text{g C/L}$. Pre-treatment with $1.0 \mu\text{M}$ atrazine, elicited a greater rate of uptake than the control with glucose pre-treatment at concentrations of $300 \mu\text{g C/L}$ and greater. The maximum rate of uptake for $1.0 \mu\text{M}$ atrazine pre-treatment was 2.8 times greater than the control without glucose pre-treatment. Pre-treatment with $1.0 \mu\text{M}$ atrazine and glucose exhibited increasing uptake with increasing glucose carbon concentration that was significantly greater than the controls throughout most of the glucose concentration range. The maximum rate of $0.019 \text{ pg C/cell/hr}$, attained at $825 \mu\text{g C/L}$, was 8 times greater than the maximum uptake for the control with glucose pre-treatment. Atrazine treatment at $2.46 \mu\text{M}$ exhibited a rapid increase in uptake with increasing glucose concentrations up to $192 \mu\text{g C/L}$, at which point, the uptake rate did not increase, and even decreased slightly at concentrations between 192 and $383 \mu\text{g C/L}$. At $383 \mu\text{g C/L}$ and greater, the uptake rate increased to a maximum of $0.014 \text{ pg C/cell/hr}$, which is double the maximum rate of the $1.0 \mu\text{M}$ atrazine pre-treatment. In pre-treatment with $2.46 \mu\text{M}$ atrazine and

TABLE 2-8 Summary of Scheffe multiple comparison of means for inhibition of growth rate, and final yield in *C. geitleri*, in batch culture experiments conducted with atrazine and glucose, and similarly, with atrazine and acetate. Means with the same letter are not significantly different from each other. Analysis was performed at $\alpha = .05$ level of significance

<u>Treatment</u>	<u>Inhibition (%)</u>	
	<u>Growth rate</u>	<u>Final yield</u>
Atrazine(1.0 μ M) (no glucose)	24.59 a	15.71 b
Atrazine(1.0 μ M) (with glucose)	27.86 a	24.68 b
Atrazine(1.0 μ M) (no acetate)	26.21 a	20.79 b
Atrazine(1.0 μ M) (with acetate)	27.28 a	18.68 b
Atrazine(2.46 μ M) (no acetate)	68.46 c	41.47 d
Atrazine(2.46 μ M) (with acetate)	67.39 c	32.29 d

FIGURE 2-5 Velocity of glucose uptake as a function of glucose concentration, for: control cultures pre-treated with glucose (-----), and without glucose (-----); cultures pre-treated with 1.0 μM atrazine with glucose (— — —), and without glucose (————); cultures pre-treated with 2.46 μM atrazine with glucose (— — —), and without glucose (— — —). Bars represent standard error of replicates (n=3 or 6).



glucose, the uptake rate was greatest at all glucose concentrations as compared to the other treatment groups. The maximum uptake rate of 0.019 pg C/cell/hr, achieved at 844 $\mu\text{g C/L}$, is identical to the maximum uptake rate attained at a similar concentration in the pre-treatment with 1.0 μM atrazine and glucose. However, the overall uptake response was lower in 1.0 mM atrazine pre-treatment than in pre-treatment with 2.46 μM atrazine and glucose.

Hence, glucose uptake response was greater in cultures pre-conditioned with 2.46 μM atrazine than in cultures pre-conditioned with 1.0 μM atrazine. In cultures pre-conditioned with atrazine and glucose, the glucose uptake rate was also greater than with cultures pre-conditioned to atrazine alone.

The results from Table 2-9, indicated that with the control and 1.0 μM atrazine conditions, both with glucose treatment, the chlorophyll content is reduced compared to similar conditions without glucose treatment. With the 2.46 μM atrazine conditions, with and without glucose, chlorophyll content was reduced.

The volume of exponentially growing cells was estimated to be 1286 μm^3 , which is equivalent to a carbon content of 289 pg C/cell.

2.3.5) Comparison of Control treatments

Table 2-10 is a summary of a Scheffé multiple comparison of means, testing for differences in inhibition between controls with, and without

TABLE 2-9 Chlorophyll a content of exponentially growing cultures of C. geitleri that were pre-adapted to various conditions. Values of cell chlorophyll are means of 3 or 6 replicates.

<u>Condition</u>	<u>Cell chlorophyll (pg Chl.a/cell)</u>
Control	7.32
Control (with glucose)	5.56
1.0 uM atrazine	7.96
1.0 uM atrazine (with glucose)	5.48
2.46 uM atrazine	4.11
2.46 uM atrazine (with glucose)	4.58

TABLE 2-10 Summary of Scheffe comparison of inhibition means, of growth rate, final yield, and chlorophyll a accumulation (at exponential and stationary phases of growth), between controls without methanol, and controls with methanol. Inhibition values are combined means (N=9) of controls without methanol, from triplicated experiments using atrazine, simazine, terbutryn, and supplementary atrazine treatments.

<u>Experiment</u>	<u>Growth rate</u>	<u>Inhibition mean (%)</u>		
		<u>Final yield</u>	<u>Chlorophyll a</u>	
			<u>Exponential</u>	<u>Stationary</u>
Atrazine	6.687	-9.791	10.428	16.402 *
Simazine	-5.078	-10.422 *	-8.063	10.347
Terbutryn	5.039 *	-9.743 *	-14.189 *	9.342
Supplementary Atrazine	7.583 *	0.979	0.095	14.810 *

* Means are significantly different (at $\alpha=0.05$) from controls with methanol.

methanol, for growth rate, final yield, and chlorophyll accumulation. In several instances the two types of controls were significantly different (at $\alpha=.05$) from each other, but in most cases they were not.

2.4) DISCUSSION

2.4.1) Growth Parameters

Terbutryn had the greatest inhibitory effect on growth rate, and final yield, whereas, simazine had the least effect. Atrazine was intermediate in its effect. Very seldom have authors expressed the inhibitory effects of triazines on algae in terms of EC_{50} values. Although direct comparison is difficult, it does appear, however, that the EC_{50} values presented here fall within the ranges of concentrations that cause inhibition of other algal species. Several reports have indicated that $5.0 \mu\text{M}$ simazine strongly inhibited the growth of several species of diatoms and green algae (Kratky and Warren, 1971; Johannes *et al.*, 1973; O'Neal and Lembi, 1983). Similarly, the consensus of several reports indicated that $4.6 \mu\text{M}$ atrazine, strongly inhibited the growth of green and blue-green algae, as well as several other planktonic species (Kratky and Warren, 1971; Butler *et al.*, 1975; Stratton, 1984). Terbutryn was reported to strongly inhibit the growth of filamentous algae and diatoms at concentrations of 0.12 to $0.04 \mu\text{M}$ (Johannes *et al.*, 1973). Somewhat higher levels of terbutryn (0.21 to $1.7 \mu\text{M}$) were reported to control filamentous algae (Muir, 1980; Mackenzie *et al.*, 1983). Certainly the majority of these reports are in close agreement with the levels of triazines determined in this study, that caused inhibition in growth near 100%. Also, the results presented here clearly showed that tolerance

(defined as < 50% inhibition) occurred at similar concentration ranges of atrazine and simazine (1.24 to 2.48 μM) reported by Loepky and Tweedy (1969), and Ellis *et al.* (1976). However, Robson *et al.* (1976), showed that 0.10 μM terbutryn was tolerated by several diatoms and green algae, which is in disagreement with the results of this study where at this concentration, terbutryn inhibited growth to nearly 100%.

Simazine has been shown to be detoxified by several species of green algae by first being metabolized and then binding to a protein (Kruglov and Paromenskaya, 1970; Kruglov and Mikhailova, 1975). If detoxification of simazine occurred to a greater extent than atrazine and terbutryn in *C. geitleri*, then this would provide some explanation for the highest EC_{50} achieved with this herbicide, in this study.

Vance and Smith (1969), demonstrated that simazine at 0.10 μM increased the growth of *Chlamydomonas eugametos* which, is in agreement with the stimulation in growth rate observed in this study. The stimulation of growth seen particularly with simazine might be explained by the work of Ries and Wert (1972). They showed that low levels of triazine herbicides often stimulated growth, especially in plants grown on low nitrogen. Such stimulation of growth, may be explained by the enhancement of nitrate reductase or nitrite reductase activity, leading to increased protein accumulation, induced by simazine at concentrations of .05 to 0.8 μM as has been demonstrated in corn (*Zea mays* L.) by Ries *et al.*, 1967. However, Klepper (1979) demonstrated that nitrite accumulation increased with atrazine treatment (9.3×10^{-4} M) on excised leaves of wheat, indicating that nitrite reductase activity was depressed thus indicating an opposite effect with atrazine. The stimulatory effects of low levels of atrazine and terbutryn

seen in this study, were not as evident as those of simazine. Prometryn which has a similar structure to terbutryn, was shown to have no effect on growth stimulation in Chlorella and Lyngbya, when compared to some other herbicides (Tubea et al., 1981). Thus it would appear that simazine causes unique secondary effects.

The complete lack of growth rate stimulation with supplementary atrazine treatment, probably reflects the prior accumulation of atrazine within the chloroplast membrane. Subsequent exposure may then have led to greater concentrations within cells, and would also explain why the overall maximum inhibition in growth rate was higher in pre-treated cells. This is supported by the work of Bednarz (1981), who demonstrated that, the toxic effect of atrazine and simazine on several species of green algae (Ankistrodesmus, Chlorella, Scenedesmus, Dictyosphaerium, and Hormidium) was irreversible, even when the algae were transferred to herbicide-free media. It was also reported that the growth of Spirogyra was inhibited to a greater extent, at high light intensities ($400 \mu\text{E m}^{-2} \text{s}^{-1}$) than at low light intensities ($100 \mu\text{E m}^{-2} \text{s}^{-1}$) with $5.0 \mu\text{M}$ simazine (O'Neal and Lembi, 1983). Growth recovery of the algal species (345 to 616% increase in biomass) from simazine application was evident when cultures were transferred to simazine-free media for 15 days at light intensities between 15 and $100 \mu\text{E m}^{-2} \text{s}^{-1}$ but, at $400 \mu\text{E m}^{-2} \text{s}^{-1}$, the cultures showed no signs of recovery. Although, my pre-conditioned culture (treated with $2.46 \mu\text{M}$ atrazine) was not transferred to atrazine-free media, it is interesting to note, that light intensity perhaps plays an important role in the recovery mechanism. Apparently, simazine is retained within cells and once transferred to simazine-free media under high light, photooxidation continues. A similar

occurrence may be present with our pre-treated cells where further accumulation of atrazine upon subsequent exposure, leads to greater toxicity through photooxidation.

2.4.2) Chlorophyll a Accumulation

Inhibition of chlorophyll a accumulation was most obvious in experiments utilizing atrazine and terbutryn whereas, in experiments conducted with simazine, there was a strong stimulatory response. Several authors have determined the levels of triazines that have significantly inhibited chlorophyll production. Concentrations of 5.0 μM simazine, 10 μM atrazine and 0.04 μM terbutryn, were shown to inhibit chlorophyll accumulation by >90% in unialgal cultures and in periphyton (Torres and O'Flaherty, 1976; Plumley and Davis, 1980; Goldsborough and Robinson, 1983). In comparison with the results of this study, 5.0 μM atrazine was sufficient to cause appreciable inhibition (83-87%) of chlorophyll whereas, inhibition was not evident at 5.0 μM simazine, and at 0.04 μM terbutryn, there was only slight inhibition, that occurred at both stages of growth. Similar to this study, Torres and O'Flaherty (1976) also demonstrated varying degrees of chlorophyll stimulation (up to 85% stimulation) in green and blue green algae at concentrations of 2.5 and 5.0 μM simazine. That some of these reports conflict with findings of this study, may simply be a reflection of a greater tolerance expressed by C. geitleri compared to algal species used in other investigations. Also, evidence would suggest that the stimulatory response induced by simazine is a mechanism of herbicide tolerance.

It was interesting to see that chlorophyll stimulation with atrazine and simazine increased with cell ageing, but with terbutryn and

supplementary atrazine treatment, there was an increase in inhibition. Clearly then, terbutryn has a different effect on chlorophyll synthesis at early and late stages of growth, since with subsequent atrazine exposure this phenomenon is a reflection of the accumulation of atrazine. One of the secondary effects of triazines (as well as other inhibitors of photosynthesis) is the photooxidation of photosynthetic pigments, leading eventually to the destruction of chlorophyll molecules (Moreland,1980). Also, primary inhibition leads to inhibition of various energy-requiring enzymes involved in the synthesis of chlorophyll and other pigments and lipids (Moreland,1980). This is reflected in our results with the reduction in chlorophyll accumulation at high triazine concentrations. This effect is enhanced at the later stages of cell growth with terbutryn and supplementary atrazine treatment.

With supplementary atrazine treatment the reduction in chlorophyll accumulation at later phases of growth might be a reflection of damage to thylakoid membranes that had occurred in pre-treatment and at early stages of growth, and upon further exposure the penetration of atrazine became less arduous. Cells at later stages of growth were able to accumulate additional amounts of atrazine, that would perhaps result in either the photooxidation of chlorophyll or reduction in chlorophyll synthesis due to secondary inhibition of energy-requiring enzymes involved in synthesis.

Apparently, triazine-induced stimulation of chlorophyll content is influenced by light. O'Neal and Lembi (1983), stated that chlorophyll increased at $5\mu\text{M}$ simazine at a light intensity of $100\ \mu\text{E m}^{-2}\ \text{s}^{-1}$ although, photosynthesis was completely blocked at this concentration, but at $15\ \mu\text{E m}^{-2}\ \text{s}^{-1}$, chlorophyll declined significantly in Spirogyra. At higher light

intensity ($400 \mu\text{E m}^{-2} \text{s}^{-1}$) chlorophyll decreased by 100%, with $5 \mu\text{M}$ simazine exposure. This would be expected since a higher light intensity would lead to a greater photooxidative effect on pigments, due to the capture of greater amounts of light energy. Our results revealed that chlorophyll stimulation was very pronounced with simazine treatment at a light intensity of $250 \mu\text{E m}^{-2} \text{s}^{-1}$. This perhaps, reflects a similar occurrence to that reported by O'Neal and Lembi, (1983). It would be of interest to know how different light intensities influence the enhancement of chlorophyll accumulation in C. geitleri with simazine exposure as well as with atrazine and terbutryn.

2.4.3) Photoheterotrophic Potential

Results from both batch culture experiments with glucose and acetate, and ^{14}C -glucose uptake experiments, clearly indicated that glucose and acetate were not utilized to the point where they affected the growth of the organism as a means of overcoming toxicity. The maximum uptake rate of $0.020 \text{ pg C/cell/hr}$ attained with $2.46 \mu\text{M}$ atrazine and glucose pre-treatment is insignificant in terms of cell growth. Based on this rate of uptake the generation time was determined to be 14450 hours (based on a cell carbon content of 289 pg C/cell). This was compared to the generation times of 8.5 to 13.0 hours that were determined from $^{14}\text{C-HCO}_3$ experiments, with $1.0 \mu\text{M}$ atrazine treatment (Chapter 3), where the uptake rate of inorganic carbon was $22\text{-}34 \text{ pg C/cell}$, which is in close agreement with the generation time of approximately 12.0 hours, obtained from growth experiments utilizing batch culture. Similarly, in growth experiments with either glucose or acetate, the growth rate and final yield did not increase, and thus the degree of inhibition was unchanged.

Thus photoheterotrophy was not evident with exposure to atrazine. A possible explanation may be that glucose and acetate was not supplied in sufficient quantities in batch culture to allow for effective growth of the alga. Also higher ^{14}C -glucose concentrations were probably required for the detection of active uptake kinetics that were absent in the results presented here .

Hellebust et al., (1971) proposed that transport systems for glucose are controlled by light conditions. The diatom Cyclotella cryptica when grown at high light intensities possessed a low ability to take up glucose, however, when incubated at lower light intensities, or in the dark, glucose transport increased significantly (Hellebust, 1971). It was postulated that some product of non-cyclic photophosphorylation (e.g. NADPH), inhibited glucose uptake, since the action spectrum for the inactivation of the transport system is identical to that for photosynthesis (Hellebust, 1971). Therefore, at the sublethal concentrations of atrazine used in this investigation (1.0 and 2.46 μM), photosynthesis was still active, and any photosynthetic product(s) may have interfered with the transport system. Jolley et al.(1976), concluded that DCMU partially overcame the inhibition of glucose transport in the light, by blocking electron flow. Certainly, this is supported by the results presented here, in that at higher atrazine concentration (2.46 μM), photosynthesis is inhibited to a greater extent, and thus the inactivation of glucose uptake may have been partially offset, which was indicated by the enhanced rate of glucose uptake. The transport of glucose was also enhanced in cells adapted to glucose. Changsang and Cooksey(1977), concluded that glucose transport system was induced by glucose, as well as D-mannose, and D-fructose, in the diatom, Amphora coffeaeformis . Also, Hellebust (1971), concluded that

growth rate, and the rate of glucose uptake in the diatom, Cyclotella cryptica, depend on glucose concentration in the medium.

In the light, acetate was assimilated into lipids by Chlorella vulgaris (Sumida and Ueda, 1973; Neilson and Lewin, 1974). Also, Russell and Gibbs (1966), concluded that in Chlamydomonas mundana , ribulose diphosphate carboxylase, and other enzymes of the Calvin cycle, were very much lower in cells growing photoheterotrophically, with acetate than those growing photolithotrophically suggesting that acetate was exerting control of the cell from an energy standpoint. Therefore, it is likely that acetate transport, if present in C. geitleri, was not inhibited by light. However, there was no indication of acetate utilization in batch culture experiments where acetate was supplied in the medium.

The decrease in chlorophyll, observed in both, control, and atrazine treated (1.0 μM) cultures with glucose, as compared to similar cultures, without glucose, is perhaps some indication of a shift to photoheterotrophy. However, this was not evident in cultures pre-adapted to glucose and a higher atrazine concentration (2.46 μM), although with this treatment the culture did possess a greater ability for glucose uptake, and thus would suggest that there is no correlation between reduced chlorophyll content and the onset of photoheterotrophy.

2.4.4) Summary

It has been demonstrated that terbutryn was the greatest inhibitor of growth, and chlorophyll a synthesis. Simazine was the least toxic of the three herbicides. Simazine induced a marked stimulatory response in growth, and

especially, in chlorophyll accumulation. It seems that higher concentrations of simazine are required to attain a more typical inhibition curve like those expressed with atrazine and terbutryn. The stimulation in growth that might possibly be a reflection of enhanced protein synthesis and, the stimulation in chlorophyll, both induced by simazine, are tolerance mechanisms. Atrazine was intermediate in its toxicity, of the three herbicides. Increased tolerance was not acquired with pre-conditioning with atrazine, as evident by the increased inhibition of chlorophyll and the unchanged inhibition of growth. Although, there was some indication of atrazine-induced photoheterotrophy, the utilization of glucose and acetate was not sufficient to contribute to the growth of the organism, and thus did not provided an effective means of tolerance.

CHAPTER 3 TRIAZINE-INDUCED CHLOROPHYLL FLUORESCENCE IN CHLAMYDOMONAS GEITLERI

3.1) INTRODUCTION

Chlorophyll fluorescence has been proven to be a simple and rapid method for studying the kinetics of inhibition of photosynthesis by herbicides (Ali and Souza Machado,1981; Richard et al., 1983; Habash et al., 1985;). It has been used as a sensitive indicator of in situ photosynthetic energy conversion, and is believed to reflect the photochemistry of photosystem II (Arntzen et al.,1981). Studies of the kinetics of chlorophyll fluorescence in leaf tissues have been used to assess the efficiency of herbicide penetration into the leaf (Habash et al.,1985). Ali and Souza Machado (1981) developed a rapid fluorescence method for detecting differences between triazine-susceptible and resistant plants. In addition, chlorophyll fluorescence has been used to detect changes in protein-pigment complexes, that were brought about by herbicides that inhibit electron transport of photosynthesis (Grumbach,1981; Buschmann and Grumbach,1982; Laskey et al.,1983). Also, several researchers have used DCMU to induce fluorescence, as a measure of the photosynthetic capacity of phytoplankton in marine and freshwater habitats (Roy and Legendre, 1979; Cullen and Renger,1979; Prezelin and Ley, 1980; Vincent 1980,1981). Hence, not only is it a rapid and simple method, but it also has a wide range of application.

When light is absorbed by a chlorophyll molecule, an 'excited state' is created as the electron in the pigment molecule attains a higher energy level. The excited state is unstable, and the electron is either lost from the molecule or returns to its ground state. Most likely, the electron in PSII is transferred to the primary acceptor Q, which becomes reduced. When the reaction center chlorophyll is excited to a higher energy state, and Q is already reduced, the excited electron can return to its ground state by release of heat or as re-emission of a lower energy level photon, as fluorescence. The red light emitted (near 685 nm) as in vivo chlorophyll fluorescence, is no longer useful for photosynthesis, and thus is a measure of photosynthetic inefficiency (Vincent, 1981). Fluorescence is induced by blue light (Arntzen et al., 1982). Following illumination, fluorescence rises to a maximal level. The time for this rise provides some information of the electron transport steps that are occurring prior to complete reduction of Q. The addition of photosynthetic inhibitors alters the characteristics of chlorophyll fluorescence induction. Both atrazine and diuron stimulate a fast maximal fluorescence induction (Arntzen et al., 1982).

Since triazine herbicides affect the photochemical reactions of chloroplasts, an analysis of the radiant energy utilization through fluorescence is a direct measure of herbicide action. For herbicide fluorescence induction, both isolated chloroplasts and leaf discs have been used (Souza Machado, et al., 1978; Ali and Souza Machado, 1981). Microalgae have been shown to be especially advantageous for fluorescence studies, in that they have been used to analyze individual reaction steps of photosynthesis (Bohme et al., 1981). It was the intent of this study to characterize the fluorescence response of a green alga induced by three

triazines, and to determine any indication of increased tolerance (evident by reduced fluorescence) which might be achieved in algal cultures that were pre-exposed to atrazine. Several researchers have monitored fluorescence over very short time periods (msecs) following a flash of light (Souza Machado *et al.*, 1978; Habash *et al.*, 1985), in which they showed that fluorescence was resolved into a fast and a slow phase. Here fluorescence was not monitored following a brief illumination, but rather maximum fluorescence measurements were recorded over a range of herbicide concentrations, and under continuous illumination with blue light.

3.2) MATERIALS AND METHODS

The organism used was the green alga, Chlamydomonas geitleri Ettl. Conditions for the growth of the organism are described in Chapter 2.

Approximately 400 mL of exponentially growing batch culture of the organism was centrifuged at 4000 rpm for 15 minutes. The cell pellets were resuspended in an equivalent amount of sterilized medium. Five mL of resuspended culture was dispensed into each of twenty eight 5 mL Pyrex cuvettes making up 4 controls and 8 triplicated herbicide treatments. Each treatment received 100 μ L of herbicide stock dissolved in methanol, and controls received 100 μ L of methanol. At any one time, 2 treatments were measured for chlorophyll fluorescence at 2-3 minute intervals, until no further increase in fluorescence was observed. Fluorescence was measured with a Turner Model 111 Fluorometer equipped with a blue light source (Turner 110-853) and blue excitation (Kodak Wratten 47B) and red emission (Corning 2-64) filters. An aperture size of 30x was chosen for maximum

sensitivity for detection of small fluorescence differences between treatments, upon addition of herbicide. Cuvettes were kept at a constant low light intensity ($0.314 \mu\text{E m}^{-2}\text{sec}^{-1}$) throughout. Controls were measured just prior to treatments. Taking the highest fluorescence value obtained in each treatment, a fluorescence response index (FRI) was determined by:

$$\text{FRI} = 1 - (F(\text{control}) / (F(\text{treatment}))) \quad (\text{Goldsborough and Robinson, 1984}).$$

For the examination of tolerance, batch cultures containing $2.46 \mu\text{M}$ atrazine were grown to exponential phase within 4 days. About 400 mL of exposed culture was centrifuged at 4000 rpm for 15 minutes. The cells were washed once with sterilized medium and centrifuged again, before being resuspended in 200 mL of sterilized medium to be used as the test inoculum. The measurement of fluorescence and the determination of FRI over the range of atrazine concentrations were as above. In the three experiments conducted, the inocula for the pre-exposed batch cultures were taken from continuous cultures in steady state.

3.3) RESULTS

A single factor ANOVA was applied to the FRI data for all four treatment groups, which is summarized in Table 3-1. In all cases, the data from triplicated experiments were not combined, due to significant differences between experiments (at $\alpha=.05$), and significant differences

TABLE 3-1: Summary of ANOVA results testing for differences in FRI of Chlamydomonas geitleri , between experiments, for different treatment levels, and for identical treatment levels, in triplicated herbicide-induced fluorescence experiments using: cell cultures exposed to atrazine, simazine, terbutryn, and with pre-conditioned cell cultures to 2.46 μM atrazine,exposed to atrazine. Analyses was performed at $\alpha=.05$ level of significance .

<u>Variable</u>	<u>P-Value</u>			
	<u>Atrazine</u>	<u>Simazine</u>	<u>Terbutryn</u>	<u>Pre-conditioned Atrazine</u>
Experiment	.0001*	.0001*	.0001*	.0001*
Different Treatment	.0001*	.0001*	.0001*	.0001*
Identical Treatment	.0064*	.0001*	.0091*	.0001*

* Significantly different at $\alpha=.05$

between identical treatment levels in triplicated experiments (at $\alpha=.05$). Instead, individual experiments were analysed separately.

In all cases, the herbicide-treated cultures gave an asymptotic fluorescence response that permitted the determination of the parameters FRI_{max} (maximum reaction velocity) and K_{FRI} (half saturation constant).

Response curves (Figure 3-1) were linearized with a Woolf transformation (Figure 3-2), and a predictive equation was derived from regression analysis, from which FRI_{max} and K_{FRI} values were determined. The measure of linear relation was good in all experiments ($r^2=0.9869-0.9981$). FRI_{max} and K_{FRI} values for each triazine-treatment group are presented in Table 3-2. A natural log transformation of FRI_{max} and K_{FRI} was required since these values were not normally distributed. A Scheffe multiple comparison of means was applied to these transformed values of FRI_{max} and K_{FRI} for all four treatment groups (Table 3-3). The comparison clearly indicated that there was no significant difference (at $\alpha=.05$) in FRI_{max} between the four different experimental groups. There was a significant difference in K_{FRI} between terbutryn and the other three experimental groups. But there was no significant difference between these groups, namely, atrazine, simazine and pre-conditioned atrazine. The K_{FRI} values for the three similar groups were between 32 and 53 times larger than that for terbutryn. Greater variation was observed overall, in the values of K_{FRI} than in FRI_{max} values. The greatest variation in K_{FRI} was observed with pre-conditioned atrazine with a coefficient of variation equal to 90.5%. Simazine also possessed a high variation in K_{FRI} with a coefficient of

FIGURE 3-1: Fluorescence response index (FRI) in Chlamydomonas geitleri as a function of herbicide concentration, in replicated herbicide-induced fluorescence experiments, A (————), B (-----), and C (— — —), for atrazine (a), simazine (b), terbutryn (c), and atrazine with cells conditioned with 2.46 μ M atrazine (d). Bars represent standard errors of replicates (n=3).

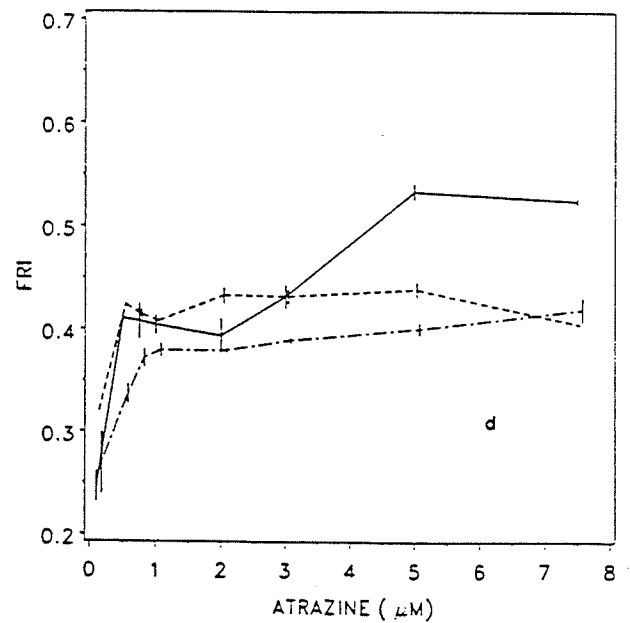
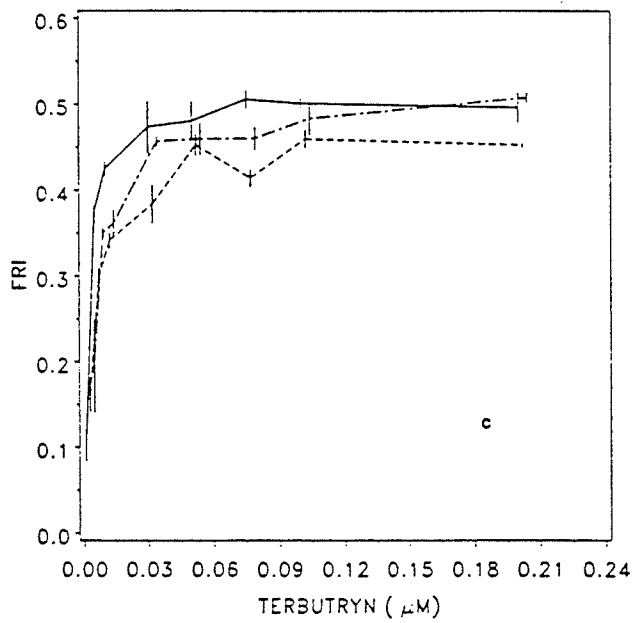
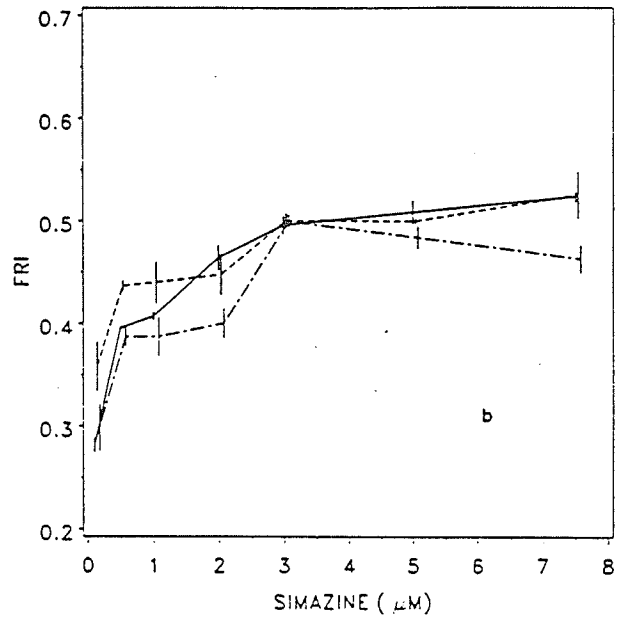
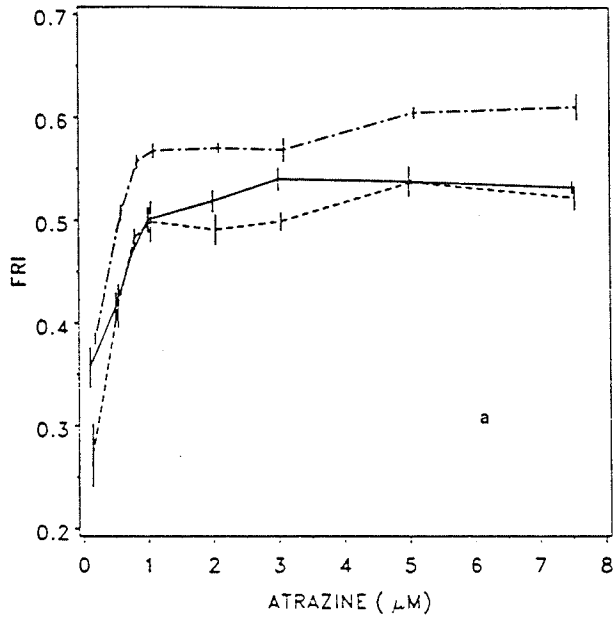


FIGURE 3-2: Regression plots of FRI as a function of herbicide concentration, following a Woolf transformation, for herbicide-induced fluorescence in Chlamydomonas geitleri using, atrazine (a), simazine (b), terbutryn (c), and with subsequent atrazine exposure to cells pre-conditioned with 2.46 mM atrazine (d). Replicated experiments are represented as, A (—), B (-----), and C (---). Symbols ○ • ■ represents individual values for experiments A, B, and C respectively.

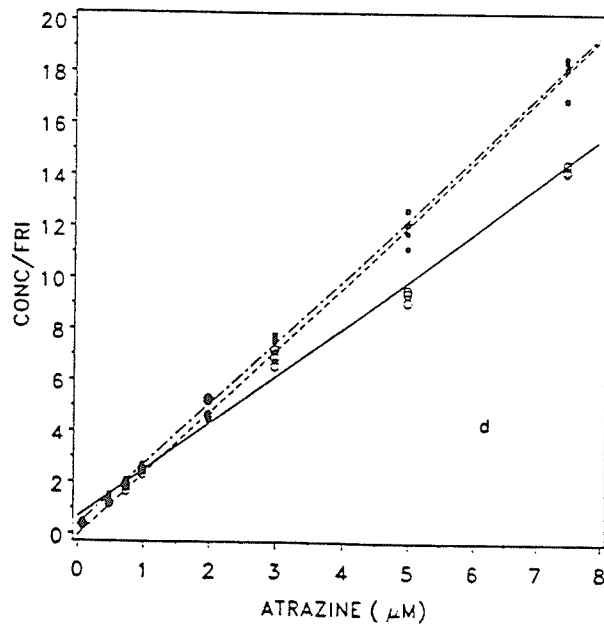
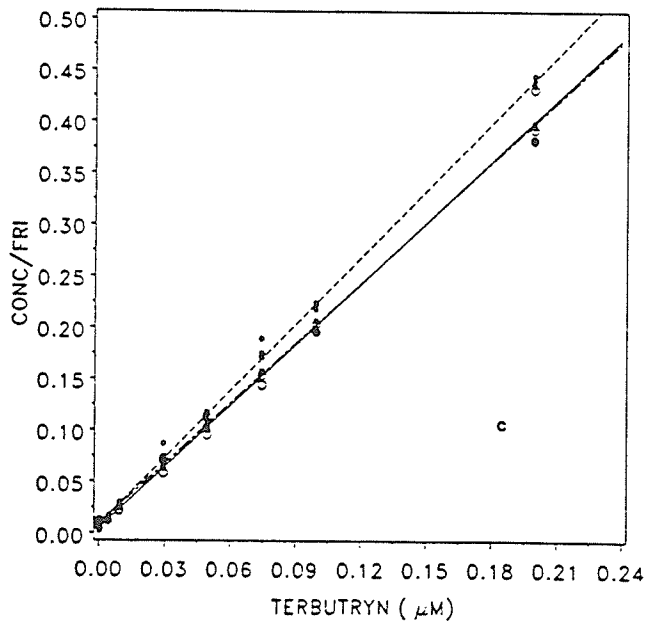
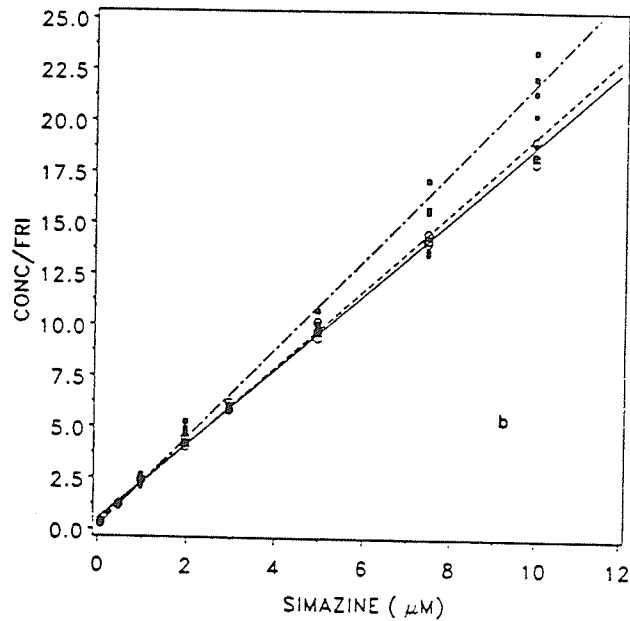
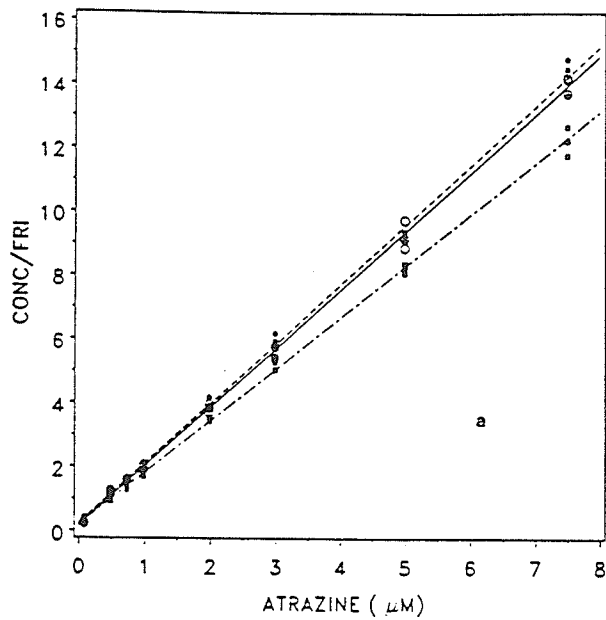


TABLE 3-2: FRI_{max} and K_{FRI} values derived from linear regression analysis for triplicated herbicide-induced fluorescence experiments (A,B,C) using cultures of Chlamydomonas geitleri exposed to atrazine, simazine, terbutryn, and in cell cultures pre-conditioned with 2.46 μ M atrazine and subsequently exposed to atrazine.

<u>Experiment</u>		<u>FRI_{max}</u>	<u>K_{FRI}</u>
Atrazine-	A	.5425	.0887
	B	.5338	.1159
	C	.6161	.1134
Simazine-	A	.5466	.2676
	B	.5279	.1776
	C	.4597	.0517
Terbutryn-	A	.5038	.00207
	B	.4605	.00343
	C	.5117	.00433
Pre-Cond. Atrazine	A	.4131	.0385
	B	.5420	.3455
	C	.4190	.1361

TABLE 3-3: Summary of Scheffe multiple comparison of means of \log_e transformed values of FRI_{max} and K_{FRI} for atrazine, simazine, terbutryn, and pre-conditioned atrazine. Means with the same letter are not significantly different from each other (at $\alpha=.05$).

<u>Experiment</u>	<u>FRI_{max}</u>	<u>Means</u>	<u>K_{FRI}</u>
Atrazine	-54755 a		-2.2515 a
Simazine	-67336 a		-2.0029 a
Terbutryn	-71035 a		-5.7659 b
Pre-Conditioned Atrazine	-78881 a		-2.1047 a

variation equalling 65.5%. Variation in FRI_{max} were not as dramatic, with the largest coefficient of variation (15.9 %) occurring in pre-conditioned atrazine.

With the pre-conditioned atrazine experiments there was a 18.8% reduction in FRI_{max} and a 39% increase in $KFRI$ compared to the atrazine experiments. Values were however, not significantly different (at $\alpha=.05$) between these two treatments.

Triplication of single concentrations for all experiments showed a mean coefficient of variation in FRI ranging from 5.7 to 11.5 % .

The saturation phase in fluorescence response was first achieved at about 1.0 μM atrazine for all atrazine experiments, A,B, and C (Figure 3-1a). Experiments B and C showed a gradual increase in fluorescence from 3.0 to 5.0 μM atrazine, followed by a stabilization in FRI . Experiment A demonstrated a gradual fluorescence decrease in the range of 3.0 to 7.5 μM atrazine. In all the terbutryn experiments (A, B, and C), saturation first appeared at .05 μM (Figure 3-1c). In experiment C, there was a gradual increase in fluorescence with increasing terbutryn concentration, beyond the saturation point, whereas, in experiments A and B there were no further increases in fluorescence. For the pre-conditioned atrazine experiment A, fluorescence decreased from 0.5 to 2.0 μM followed by an dramatic increase in FRI to a maximal level at 5.0 μM , followed by a slight decrease at 7.5 μM atrazine (Figure 3-1d). In the pre-conditioned atrazine experiment B, fluorescence decreased from 0.5 to 1.0 μM followed by a gradual rise to a maximal fluorescence at 2.0 μM (Figure 3-1d). For pre-conditioned atrazine experiment C, saturation occurred at 1.0 μM followed by a gradual increase in fluorescence with increasing concentrations of atrazine (Figure 3-1d). In

simazine experiment A, saturation appeared at about 3.0 μM , followed by a gradual increase in FRI with increasing simazine concentrations (Figure 3-1c). In the other two simazine experiments B and C, there was indication that saturation first appears at 0.5 μM , followed by a pronounced increase in FRI with increasing concentration, with a second saturation phase occurring at 3.0 μM (Figure 3-1c). At this point, fluorescence gradually increased in experiment B, with increasing simazine concentration, whereas, in experiment C, fluorescence decreased gradually. Hence it appears that, a biphasic fluorescence response may have occurred in simazine experiments B and C, and in pre-conditioned atrazine experiment A.

3.4) DISCUSSION

The occurrence of a biphasic fluorescence response for simazine treated cells may be interpreted in two ways. The first is that, this demonstrates a different degree of binding at the " B site " for simazine compared to atrazine and terbutryn. It has been demonstrated that simazine is actively absorbed by several species of green algae and then metabolized to a form that is bound with protein, thus inactivating it. The assimilation of the carbon of the side chain of simazine into amino acids of this protein has been confirmed (Kruglov and Mikhailova 1974; Kruglov and Paromenskaya 1980). Also, Vance and Smith (1969) concluded that two species of green algae (Chlamydomonas pyrenoidosa and Scenedesmus quadricauda) were able to dechlorinate simazine to its 2-hydroxy analogue, thus inactivating it. Shimabukuro (1985), revealed that conjugation with glutathione is the major

detoxification reaction of s-triazines. If detoxification of simazine was occurring at low concentrations, then this might explain the appearance of an early saturation, followed by a second saturation phase at higher concentrations, where primary inhibition is the dominant effect. Certainly, this biphasic pattern was not evident in any of the atrazine and terbutryn experiments, so it may be interpreted that detoxification of these two triazines at low concentrations was not occurring to any noticeable extent throughout the duration of the experiment. One simazine experiment (A), was exceptional in that, the biphasic fluorescence response was not evident. In the simazine experiments, the biphasic fluorescence response was seen in cells acquired from continuous (steady state) cultures whereas, with the monophasic response cells were acquired from batch culture. Since photochemistry may vary with respect to the physiological state of the organism (Roy and Legendre, 1979) and fluorescence response is closely related to photosynthetic capacity (Samuelsson and Öquist, 1977), it is possible that the occurrence or non-occurrence of a biphasic fluorescence response is due to the physiological state of the alga. Samuelsson and Öquist (1977) determined that when photosynthesis was inhibited in green algae by DCMU, only a small fluorescence increase was obtained during the period of low photosynthesis compared to fluorescence increase when the photosynthetic rate was high. So, fluorescence increase induced by DCMU was high in exponentially growing cultures but, decreased when cultures approached the stationary phase of growth. Certainly, cultures in steady state would possess a high rate of photosynthesis as compared to a culture in late exponential phase. The higher K_{FRI} value (.2676) for the simazine experiment A, compared to the lower K_{FRI} values of .1776 and .0517 from simazine experiments B and C, supports the claim by Samuelsson and Öquist

(1977) that low induced fluorescence response is indicative of low photosynthetic capacity. Therefore, it appears that the cells obtained from batch culture had a lower photosynthetic capacity than those from continuous cultures, probably because they were in a later stage of growth. In some instances, fluorescence continued to increase, particularly at the higher concentrations of herbicide. Samuelsson and Öquist(1977) showed that fluorescence gradually increased in ageing cultures of Selenastrum capricornutum. They suspected that ageing might cause alterations of the photosynthetic apparatus, by retarding the reoxidation rate of Q. Hence, a cell culture at a later stage of growth might account for the occurrence of a continual increase in fluorescence response.

Another possible explanation for the biphasic fluorescence response is that electron flow may be inhibited at two sites in the PSII complex. It has been suggested that diuron (an inhibitor of electron transport) can bind directly to the P₆₈₃ complex, as well as the "B site" between Q and PQ of the photosynthetic electron transport chain (Moreland, 1980). If simazine were to behave in a similar manner by binding to the two sites, this might explain the two phase pattern, if electron transport is blocked at one site at low concentrations and at the other at high concentrations. Isolated chloroplasts from atrazine-tolerant lambsquarters (Chenopodium album) were shown to alter the triazine-induced fluorescence response, and that this was due to one or more modified PSII constituents, which decreased the binding affinity for triazines at their inhibition site (Souza Machado, et al., 1978) This supports the hypothesis that early fluorescence saturation seen with simazine is an expression of tolerance at these low concentrations, due to possible alterations in PS II constituents.

A somewhat, similar biphasic fluorescence response was observed in the pre-conditioned atrazine experiment A. In this case, the biphasic fluorescence response was not likely due to separate binding sites, but possibly to detoxification since, a similar response curve was not observed in the atrazine experiments. Apparently, pre-conditioning with atrazine altered the fluorescence response such that, early saturation occurs at low concentrations, and is perhaps indicative of a tolerance mechanism. A reduced fluorescence response was observed in atrazine-tolerant corn leaves, which reflected the detoxification of atrazine, leading to a reversal of photosystem II inhibition (Ducreuet and Gasquez,1978). In all instances, pre-conditioning with atrazine (at EC_{50} for growth rate= $2.46 \mu\text{M}$) took place in batch cultures (inoculated from continuous cultures), and not in continuous cultures. For this reason, there was no assurance that the physiological states of the algae were similar in the three batch cultures that were used. This might account for the different fluorescence responses seen in the three experiments. It would have been preferable to conduct triplicated experiments with cells pre-conditioned with atrazine from a common continuous culture.

A more typical fluorescence response was seen with the atrazine and terbutryn treated experiments. A similar response pattern was reported by Goldsborough and Robinson (1984), in which they reported a hyperbolic response of fluorescence to increasing concentrations of terbutryn in cultures of Nitzschia acicularis. Terbutryn was shown to be the greatest inhibitor of photosynthetic electron transport. It possessed the lowest K_{FRI} , indicative of a high herbicide binding capacity. It has been suggested that the methylthio-s-triazines are more affective in inhibiting the Hill reaction with isolated

chloroplasts, as compared to the chloro-s-triazines (Souza Machado et al., 1978; Radosevich et al., 1979). Simazine and atrazine were not different in their inhibitory action, by expressing similar values for K_{FRI} . Although, simazine possessed a biphasic response in two instances, the overall K_{FRI} was not different from that for atrazine. Fluorescence response reached a maximum at $2\mu\text{M}$ atrazine, with no further effect on fluorescence with greater concentrations (Figure 3-1a) . This is in good agreement with the reported value of $3.0\mu\text{M}$ atrazine, in cultures of Chlorella pyrenoidosa (Zweig, et al., 1963) . The molar concentration at which 50% of the fluorescence intensity increase above equilibrium intensity is achieved, has been termed F_{50} (i.e. K_{FRI}) and the corresponding pF_{50} value is the negative logarithm of F_{50} (Zweig, et al., 1963). The determined averaged pF_{50} values of 6.97, 6.76, and 6.78 for atrazine, pre-conditioned atrazine, and simazine, respectively, are in good agreement with the reported values of 6.52 for atrazine and 6.10 for simazine for fluorescence responses seen in cultures of Chlorella pyrenoidosa (Zweig, et al., 1963). The lowest K_{FRI} values, seen in terbutryn experiments again suggest that it has the greatest binding efficiency of the three triazines tested.

In conclusion, chlorophyll fluorescence response proved to be a useful bioassay in assessing the differential primary effects of the three triazines in question. It is suspected that the physiological state of the organism was directly related to the fluorescence response pattern. The degree of herbicide binding and possible detoxification was perhaps also linked to the physiological status of the algae. For this reason, it is desirable to use continuous cultures that are in steady state or preferably to conduct

induced chlorophyll fluorescence on synchronous cultures in different cell cycle stages.

CHAPTER 4 TRIAZINE-INDUCED INHIBITION OF CARBON ASSIMILATION IN CHLAMYDOMONAS GEITLERI

4.1) INTRODUCTION

The primary effect of triazine herbicides is inhibition of photosynthetic electron flow on the reducing side of photosystem II (Moreland,1980). This action alters the production of NADPH, and ATP required for subsequent carbon fixation, as well as other metabolic reactions (Moreland,1980). Herbicides that are strong inhibitors of photosynthetic electron transport must inhibit the NADPH-dependent step from P-glycerate to triose-P in the Calvin cycle (Diaz et al.,1980), and ultimately inhibit the formation of glucose.

Carbon dioxide fixation has been used by several researchers, as a measure of triazine-induced inhibition of photosynthesis in unialgal cultures (Hawxby et al.,1977; Plumley and Davis, 1980; Stratton, 1984; Larsen et al., 1986), phytoplankton communities (DeNoyelles et al.,1982; Larsen et al., 1986) and in periphyton communities (Goldsborough and Robinson, 1983; Herman et al., 1986; Hamilton et al., 1987). Larsen et al.(1986) demonstrated that the EC₅₀ values for ¹⁴C uptake of eight algal species ranged from 37 to 308 µg/L atrazine and that in a multispecies system the EC₅₀ ranged from 103 to 159 µg/L for ¹⁴C uptake. In addition, Plumley and Davis (1980) stated that a 10⁻⁵ M concentration of atrazine reduced the rate of photosynthesis in unialgal diatom cultures. Also, Stratton (1984), concluded the EC₅₀ for carbon fixation, for several species of green

and blue-green algae ranged from 0.1 to 0.50 $\mu\text{g/L}$ atrazine. Reported EC_{50} values for phytoplankton communities were in the range of 95 to 131 $\mu\text{g/L}$ atrazine (Herman *et al.* 1986; Larsen *et al.* 1986) and in periphyton communities, the EC_{50} was between 100 and 1000 $\mu\text{g/L}$ for simazine, and less than 10 $\mu\text{g/L}$ for terbutryn (Goldsborough and Robinson, 1983). DeNoyelles *et al.* (1982) were able to demonstrate that $^{14}\text{CO}_2$ uptake by phytoplankton communities in ponds declined in the presence of atrazine, but later returned to control values. Simazine was shown to induce a slight stimulation in CO_2 fixation, in periphyton at 0.50 μM (Goldsborough and Robinson, 1987). Also, DeNoyelles *et al.* did indicate that phytoplankton treated with 0.45-1.35 mg/L simazine, were more resistant to a subsequent 0.10 mg/L atrazine treatment.

It was the intent of this study to characterize the inhibition of photosynthesis in a green alga by three triazine herbicides (atrazine, simazine, and terbutryn) by determining effects on rates of inorganic carbon assimilation. Pre-conditioning of cells with atrazine, as a means of inducing increased tolerance, was also investigated.

4.2) MATERIALS AND METHODS

The test organism used was *Chlamydomonas geitleri*. It was grown in batch and continuous culture, in a manner similar to that described in Chapter 2.

For each experiment 20 mL of exponentially growing batch culture or continuous culture (100,000 cells/mL) was dispensed into each of 30 mL

glass culture tubes. The treatments consisted of 8 herbicide concentrations and 2 sets of controls, with each treatment being triplicated. One set of controls was treated with 100 μ L methanol, while the other was untreated. Herbicide-treated samples contained 100 μ L of herbicide stock in methanol (separate stocks for each concentration). The range of herbicide concentrations were from 0.10 to 10.0 μ M for atrazine and simazine, and from .001 to .01 μ M terbutryn.

At any one time, two treatments were inoculated with either methanol or herbicide stock, and 1.0 mL of standardized $^{14}\text{C-HCO}_3$ (2 μ Ci/mL). All samples were incubated at 23 $^\circ$ C under 250 $\mu\text{E m}^{-2} \text{sec}^{-1}$ supplied by cool white fluorescence VHO lights (Sylvania) , for 1 hour. A time gap of 10 minutes was established between each set of two treatments to allow for filtration. Following incubation, samples were vacuum-filtered onto .45 μm membrane filters and washed with deionized water. Filters were acidified for 1 minute by fuming over concentrated HCl (12N) to remove residual $^{14}\text{C-HCO}_3$. Filters were placed into glass scintillation vials containing 5 mL of Scinti-Verse (Fisher Scientific) scintillation cocktail, and allowed to stand for at least 48 hours. Specific radioactivity (dpm) of samples was determined with a Beckman LS 3801 Scintillation Counter. The rate of inorganic carbon uptake for each sample was determined as follows:

$$\text{Rate of uptake} = \text{dpm}(\text{sample}) / \text{dpm}(\text{added}) \times \text{DIC} / 50 \times 1.05$$

The value of 1.05 is a discrimination factor for the preferential uptake of ^{12}C .

DIC is the dissolved inorganic carbon content of the medium and was derived from alkalinity (APHA, 1971). The rate of inorganic carbon uptake

was finally expressed as pgC/cell/hr for each treatment. Cell counts were conducted with a Model B Coulter Counter. Each experiment for each triazine was conducted 3 times.

For assessment of induced tolerance, 3 experiments were conducted as described above, with the exception that cells were pre-exposed in batch culture to 2.46 μ M atrazine for a 4 day period of exponential growth. The value of 2.46 μ M is an approximate EC₅₀ determined from growth rates of the organism exposed to atrazine. The pre-exposed culture was centrifuged in at 4000 rpm for 15 minutes. The cells were washed with sterilized medium, and re-centrifuged before being resuspended in a volume of medium that was half the original volume.

Values for inorganic carbon uptake were expressed as a percentage of the methanol controls, from which EC₅₀ values were calculated for each triazine. Percent inhibition was calculated by:

$$100 \times (\text{methanol control} - \text{herbicide treatment}) / \text{methanol control}$$

4.3) RESULTS

A single factor ANOVA applied to all experimental groups (Table 4-1), indicated that there were significant differences (at $\alpha=.05$) between triplicated experiments for all experimental groups, and that significant differences (at $\alpha=.05$) existed between identical treatments in triplicated simazine experiments. For this reason, individual experiments were analysed

TABLE 4-1: Summary of ANOVA results testing for differences in inhibition of inorganic carbon fixation in C. geitleri expressed as a percent of methanol controls, between experiments, for different treatment levels, and for identical treatment levels in triplicated experiments using: exponentially growing cultures exposed to atrazine, simazine, and terbutryn; and with cultures pre-conditioned with 2.46 μ M atrazine, followed by supplementary exposure to atrazine. Analyses were performed at $\alpha=.05$ level of significance.

<u>Variable</u>	<u>P-value</u>			
	<u>Atrazine</u>	<u>Simazine</u>	<u>Terbutryn</u>	<u>Pre-conditioned Atrazine</u>
Experiment	.0066*	.0282*	.0005*	.0003*
Different Treatment	.0001*	.0001*	.0001*	.0001*
Identical Treatment	.6323	.0001*	.1290	.2986

*Significantly different at $\alpha=.05$

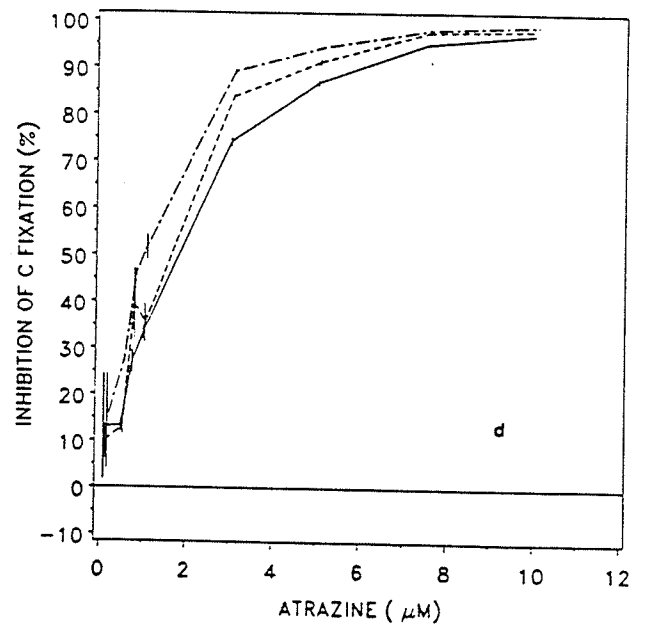
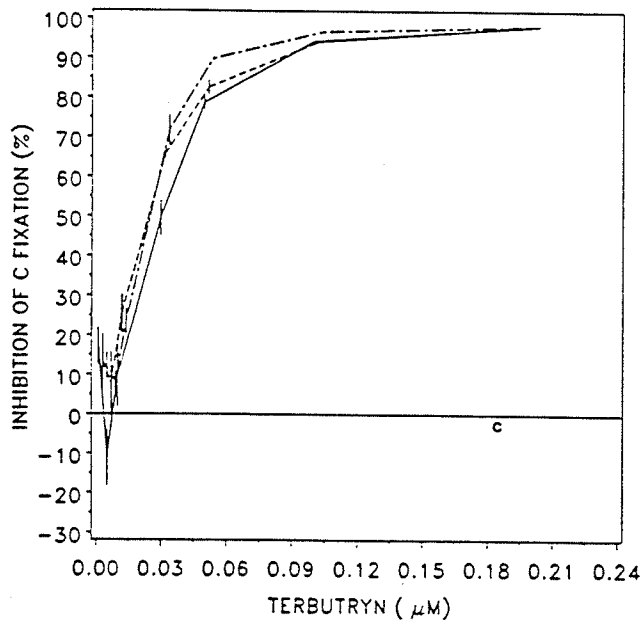
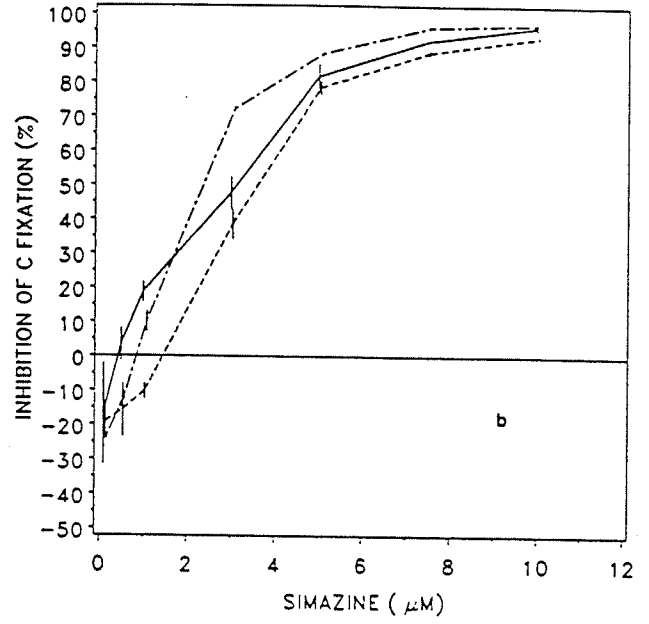
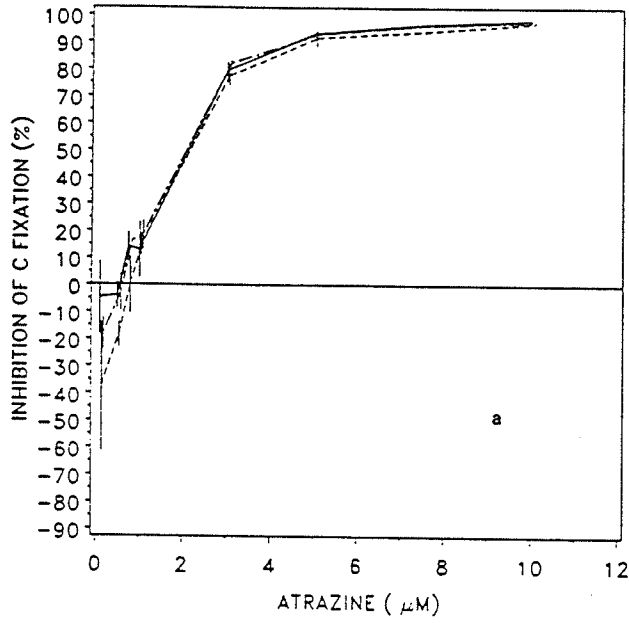
separately. Inhibition values as a function of herbicide concentration, are presented in Figure 4-1.

Results of the atrazine experiments clearly illustrated stimulation of carbon fixation at atrazine concentrations in the range of 0.10 to 0.50 μM in experiments A and C (Figure 4-1a). Stimulation occurred in atrazine experiment B, from 0.10 to 0.75 μM followed by increased inhibition with increasing atrazine concentration. In all three experiments inhibition increased dramatically from 1.0 to 3.0 μM atrazine, and eventually plateaued from 5.0 to 10.0 μM atrazine. The greatest stimulation with atrazine occurred at the 0.10 μM treatment levels in all experiments, a,b, and c, with respective mean values of -4.8, -37.7, and -18.2%.

Simazine experiments A and C, (Figure 4-1b) expressed stimulation of carbon fixation at concentrations of 0.10 and 0.50 μM , whereas, in experiment B, stimulation was evident at 0.10 to 1.0 μM simazine. From this point, in all three experiments, inhibition increased dramatically with increasing simazine concentrations up to 5.0 μM , followed by a gradual increase in inhibition. Stimulation was greatest at the lowest simazine concentration of 0.10 μM with mean values (n=3) of -16.7, -19.2, and -23.5% for experiments A, B, and C, respectively.

Terbutryn caused little stimulation in carbon fixation rate (Figure 4-1c). Only in experiment A, was stimulation expressed with a mean value of 11.1% occurring at the lowest terbutryn concentration of .005 μM . Inhibition increased markedly with increasing terbutryn concentrations to 0.10 μM , following which there is a slight increase in inhibition.

FIGURE 4-1: Inhibition of inorganic carbon fixation in C. geitleri expressed as a percent of methanol controls, as a function of herbicide concentration in replicated experiments A (—), B (-----), and C (---), in cell cultures treated with atrazine (a), simazine (b), terbutryn (c), and with cell cultures pre-conditioned with 2.46 μ M atrazine, and subsequently treated with atrazine (d). Error bars are standard errors of replicates (n=3). Negative values represent stimulation of carbon fixation.



There was no indication that stimulation of carbon fixation took place in experiments where cells were first pre-conditioned with 2.46 μM atrazine, then subsequently exposed to atrazine concentrations (Figure 4-1d). Aside from the absence of stimulation, the response pattern was similar to that of non-conditioned cells, in that inhibition increased with increasing concentration, except that saturation appeared at a greater concentration of 7.5 μM atrazine.

The greatest variation in replicate treatments ($n=3$), occurred at the lowest treatment levels, in all experimental groups, with coefficient of variation values that were greater than 100%.

Negative inhibition values were made positive (by the addition of 100), so that a linear response curve following a Woolf transformation (Figure 4-2), could be constructed for each experiment. Values of EC_{50} listed in Table 4-2, were derived from equations determined from linear regression analysis. The linear correlation was good for all experiments ($r^2=.9853-.9992$). A Scheffe multiple comparison of means was applied to the mean values of EC_{50} for each experimental group (Table 4-3). The comparison clearly indicated that the EC_{50} for terbutryn was significantly different from all other experimental groups. EC_{50} 's for atrazine and simazine were not significantly different . Similarly, atrazine and supplementary atrazine exposure to cells pre-conditioned with atrazine, showed no significant difference (at $\alpha=.05$) in EC_{50}

Terbutryn clearly demonstrated the greatest inhibition of carbon fixation (EC_{50} of 0.0141 μM) and is 102 and 127 times more toxic than atrazine and simazine, respectively. Cells pre-conditioned with atrazine and

FIGURE 4-2: Regression plots of coded values of carbon fixation inhibition as a function of herbicide concentration, following a Woolf transformation, in C. geitleri using, atrazine (a), simazine (b), terbutryn (c), atrazine (cells pre-conditioned with 2.46 μ M atrazine) (d). Replicated experiments were combined as a single regression plot. Data from experiments A, B, and C are combined (See Appendix D).

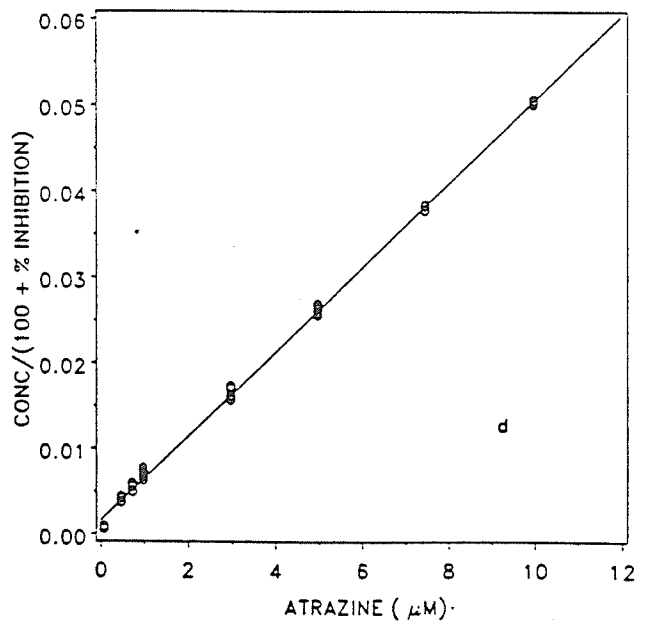
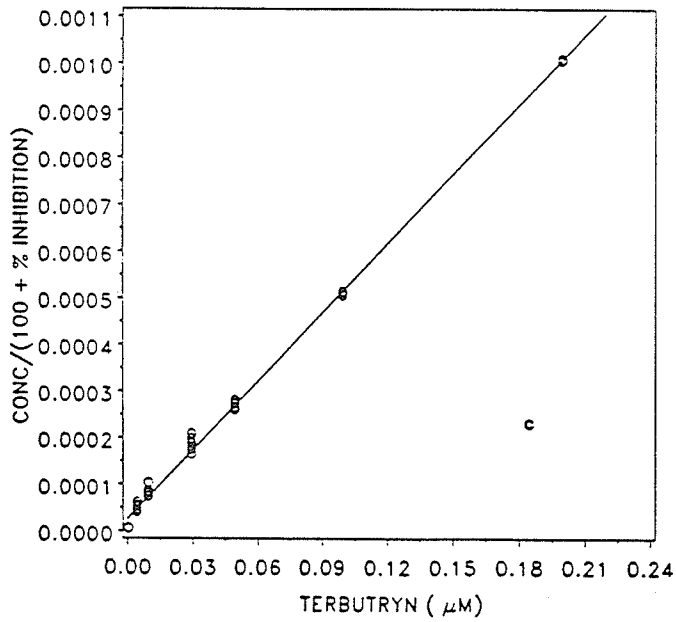
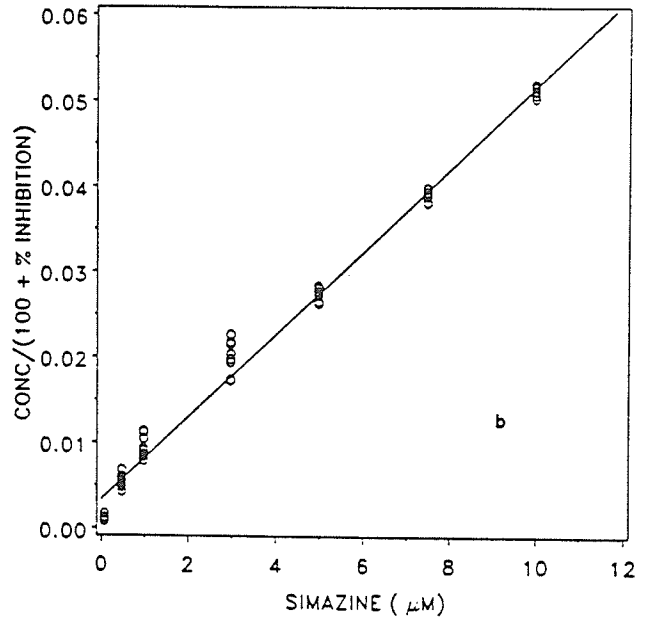
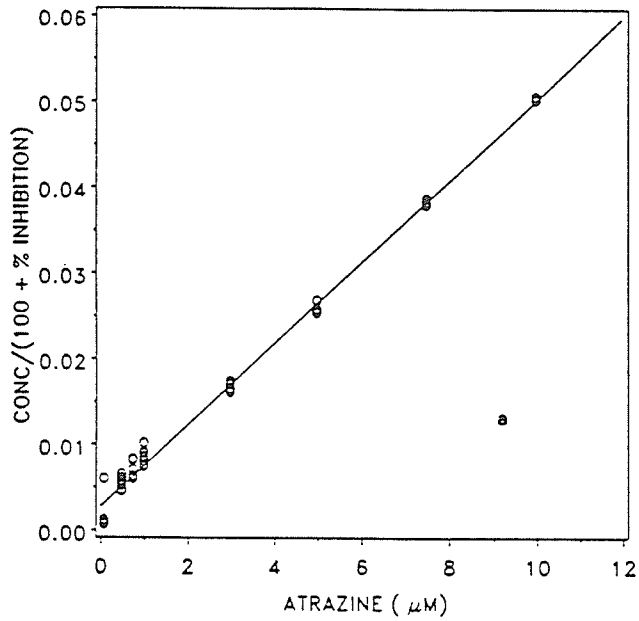


TABLE 4-2: Values of EC₅₀ derived from linear regression analysis for triplicated herbicide-induced inhibition of carbon fixation experiments (A,B,C) using cultures of C. geitleri exposed to atrazine, simazine, terbutryn, and with cell cultures pre-conditioned with 2.46 μ M atrazine, and subsequently exposed to atrazine.

<u>Triazine</u>	<u>Experiment</u>	<u>EC₅₀</u>
Atrazine	A	1.3391
	B	1.7166
	C	1.2682
Simazine	A	1.6346
	B	2.2573
	C	1.4656
Terbutryn	A	0.0182
	B	0.0131
	C	0.0112
Pre-Conditioned Atrazine	A	1.0865
	B	0.9238
	C	0.6919

TABLE 4-3: Summary of Scheffe multiple comparison of means of EC₅₀ inhibition of carbon fixation caused by atrazine, simazine, terbutryn, and pre-conditioned atrazine. Means with the same letter are not significantly different from each other. Analysis was performed at $\alpha=.05$ level of significance.

<u>Experiment</u>	<u>Means</u>	<u>Letter</u>
Atrazine	1.4413	A
Simazine	1.7858	A
Terbutryn	0.0141	B
Pre-condition atrazine	0.9007	A

subsequently treated with atrazine expressed a 38% reduction in EC₅₀ over cells treated with atrazine, indicating that previous exposure to atrazine increased sensitivity. This difference was not, however, significant (at $\alpha=.05$).

Variation in EC₅₀ was greatest for terbutryn and smallest for atrazine with coefficient of variation values equalling 25.4 and 16.7% respectively.

Table 4-4 is a summary of Scheffe multiple comparison of means testing for differences in inhibition between controls with methanol and controls without methanol. In most cases, controls without methanol were not significantly different from controls with methanol.

4.4) DISCUSSION

At low concentrations of herbicide, especially with atrazine and simazine, there was a strong stimulation of carbon fixation. Such stimulation has been reported elsewhere. Goldsborough and Robinson (1987) noticed a slight stimulation of periphyton photosynthesis at 0.5 and 0.05 μ M simazine. It has also been demonstrated that hydroxyatrazine, a degradation product of atrazine quoted as being non-toxic at 4.6x10⁻⁴ M induced a stimulation in photosynthetic activity of approximately 10% in cultures of Anabaena variabilis (Stratton, 1984). Veber et al. (1981) concluded that 90% of the atrazine in the medium was absorbed by Chlorella vulgaris in less than 1

TABLE 4-4 Summary of Scheffe comparison of inhibition means of CO₂ fixation between controls without methanol and controls with methanol. Inhibition values are combined means (N=9) of controls without methanol, from triplicated experiments using atrazine, simazine, terbutryn, and supplementary atrazine treatments.

<u>Experiment</u>	<u>Inhibition mean of CO₂ fixation (%)</u>
Atrazine	-9.121
Simazine	-8.012
Terbutryn	-1.975
Pre-conditioned Atrazine	17.971 *

* Means are significantly different (at $\alpha=.05$) from controls with methanol.

hour. If similar absorption occurred in C. geitleri then perhaps detoxification occurred throughout the duration of the experiments (1 hr), reported here, and hence the production of hydroxy analogues of atrazine and simazine might explain the stimulation of carbon fixation at low concentrations.

Ries et al. (1967) concluded that simazine at subtoxic levels (0.1 μ M), increased respiration rate in rye plants (Secale cereale) by more than 10%. Coupled with this was a lower rate of carbohydrate accumulation, suggesting a greater energy requirement in simazine-treated plants. With this decrease in carbohydrate came an increase in activity of nitrate reductase, which stimulated protein synthesis. This was further supported by evidence of stimulation of amino acid content by atrazine, coupled with a decrease in leaf weight in Chenopodium album L., which was photosynthetically sensitive to atrazine inhibition (Marriage et al. 1981). It has been postulated that the degradation of carbohydrate reserves in Chlamydomonas segnis resulted in formation of PEP (phosphoenolpyruvate), which would enhance the fixation of CO₂ by PEP carboxylase and that this was enhanced at later stages of development in 5%-CO₂ adapted cells (Tan and Badour, 1982). Hence, it would appear that a possible mechanism for stimulation of carbon assimilation, is one whereby the demand for increased energy for nitrate reduction, that is stimulated by atrazine and simazine is centered around PEP carboxylation.

Results presented here clearly demonstrate that of the three triazines tested terbutryn was the strongest inhibitor of CO₂ fixation. Goldsborough and Robinson (1983), concluded that simazine at 5.0 and 24.8 μ M inhibited ¹⁴C uptake by 95% in periphyton communities. They also showed that terbutryn at .04 μ M and higher inhibited ¹⁴C uptake by > 90% in similar

communities. Our results with a single species indicated that 95% inhibition with simazine occurred between 7.5 and 10 μM and 90% inhibition with terbutryn occurred between 0.05 and 0.10 μM . Laboratory results with the single species of *C. geitleri*, indicated that slightly higher concentrations of simazine and terbutryn were required to attain the same level of toxicity observed in periphyton communities. Comparison of the two types of assays is interesting in itself, since in one case ^{14}C uptake is measured after prolonged triazine exposure (weeks), and in the other, after only 1 hour exposure. Despite these differences, the concentrations of simazine and terbutryn required for close to 100% inhibition in single species experiments, were quite comparable with those values for periphyton communities. In other studies utilizing periphyton and pond communities however, the EC_{50} for CO_2 fixation of 0.44 and 0.46 μM atrazine (Herman *et al.*, 1986, Larsen *et al.*, 1986) were somewhat lower than our determined EC_{50} of 1.44 μM atrazine.

The fact that simazine and atrazine were shown to inhibit CO_2 fixation the least was expected since it is known that the methylthio-s-triazines (like terbutryn) are more toxic than the chloro-s-triazines (like atrazine and simazine) (Radosevich *et al.*, 1979).

In experiments where cells were treated with atrazine following a pre-conditioning treatment with atrazine, the EC_{50} value decreased in comparison to the direct atrazine treatment, although not significantly. There was, however, an absence of stimulation of CO_2 assimilation in the pre-conditioned cells, perhaps indicating that pre-conditioning also increased sensitivity of cells to low atrazine levels. However, maximum inhibition (>90%) did occur at a higher concentration in pre-conditioned cultures (7.5

μM) as compared to atrazine treated cultures ($5.0 \mu\text{M}$). Hence, any increased tolerance caused by the pre-treatment was slight, and certainly, not significant. There appears to be a contradiction between the absence of CO_2 uptake stimulation and a slightly higher saturation concentration for fixation in pre-treated cell cultures receiving supplementary atrazine exposure.

Single-species algal assays with Chlamydomonas reinhardi and Chlorella vulgaris revealed EC_{50} values for atrazine of $0.22 \mu\text{M}$ and $1.51 \mu\text{M}$ respectively (Larsen et al., 1986). The latter value is in good agreement with our determined value of $1.44 \mu\text{M}$. Stratton (1984) found the EC_{50} for atrazine ranged from 0.46 to $2.32 \mu\text{M}$ for CO_2 uptake for several species of blue-green and green algae (Anabaena, Chlorella, and Scenedesmus), and is also comparable with our EC_{50} values of 1.44 and $1.79 \mu\text{M}$ for atrazine and simazine respectively. In unialgal cultures of Nitzschia sigma and Thalassiosira fluviatilis, CO_2 fixation was significantly reduced after a 7 day exposure to $10 \mu\text{M}$ atrazine, while at $1 \mu\text{M}$ exposure, results were variable (Plumley and Davis, 1980). Our results with atrazine indicate that inhibition was near 100% at $5.0 \mu\text{M}$ thus indicating a lesser degree of tolerance by C. geitleri to atrazine.

This reported bioassay, utilizing CO_2 uptake, provided some insight into the inhibitory action of the three triazines tested. The reported stimulation of carbon fixation at low concentrations of atrazine and simazine, which was not clearly evident with terbutryn, and was not observed with supplementary atrazine treatment, has clear implications in aquatic environments in which, these herbicides are present in trace amounts. Certainly, there is no clear explanation for these occurrences. Clearly, cells pre-conditioned for 72 hours with $2.46 \mu\text{M}$ atrazine, reduced the stimulatory

mechanism, probably due to the accumulation of atrazine within the chloroplast membrane thus, indirectly preventing some biochemical pathway that might be responsible for the enhanced carbon assimilation (i.e. PEP carboxylation). Also, it can be concluded that pre-treatment with atrazine reduced tolerance rather than increased it.

CHAPTER 5

SUMMARY

Overall, the strongest inhibitor of growth, chlorophyll *a* accumulation, and carbon fixation was terbutryn, followed by atrazine with simazine being the least toxic. Inhibition of growth was 108 and 207 times greater with terbutryn than with atrazine and simazine respectively. CO₂ fixation was inhibited by terbutryn by as much as 127 times the inhibition by both atrazine and simazine. Also primary inhibition as measured by fluorescence response was 32 and 50 times greater with terbutryn than with atrazine and simazine respectively. Atrazine was 2 to 4 times more toxic than simazine at inhibiting growth .

Although EC₅₀ values for inhibition of CO₂ fixation were not significantly different between atrazine and simazine, they were significantly different for growth rate and final yield, suggesting that the subsequent effect of the two herbicides on the growth of the organism is substantially different. Likewise, the degree of the induced fluorescence response is the same for both herbicides. Therefore, one can conclude that with short term exposure (1hr) to atrazine and simazine, their action on the binding site and subsequent inhibition of carbon assimilation was the same. Long term exposure (6-8 days) on the other hand, resulted in simazine being the least toxic. This may be attributed to enhanced chlorophyll synthesis, that acted as a tolerance mechanism . Also it was possible that detoxification took place in batch cultures that might have accounted for the reduced toxicity of simazine compared to atrazine and terbutryn .

Pre-conditioning with atrazine did not enhance tolerance by increasing growth and CO₂ fixation, or by reducing the chlorophyll fluorescence response, upon subsequent treatment with atrazine.

The photoheterotrophic potential induced by atrazine was insufficient to counteract herbicide toxicity, in terms of its contribution to growth. This was perhaps due to an inadequate supply of either glucose or acetate.

The EC₅₀ values determined here for a single species are of significance in that they may also provide us with an indication of the immediate responses of multispecies systems to these chemicals.

Clearly from this study the three triazines were shown to be not completely specific in that they expressed some secondary effects. These effects included stimulation in growth, chlorophyll synthesis, and carbon fixation. This information may be valuable in the choice of triazines in the control of specific groups of organisms.

One area of concern dealt with the physiological consistency of cells in different experiments. For this reason either continuous or synchronous cultures should be used to assure that identical physiological states are maintained. This would also permit information on the toxicity of triazines at different stages of growth.

It has been established that different degrees of tolerance were expressed for each of the three herbicides. Also it is suggested in the case of simazine exposure, that tolerance was achieved by enhanced chlorophyll synthesis. Adaptation to a sublethal level of atrazine was not apparent. Although pre-adaptation with simazine and terbutryn was not tested for,

certainly this addition should come under investigation given that the three triazines had different effects on the experimental organism.

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APPENDIX A

Phosphorus Limitation of Final Yield

Summary of results from a batch culture experiment for phosphorus limitation of final yield in C. geitleri. The asterix (*) indicates the concentration of K_2HPO_4 used in all subsequent experiments. Values for growth rate and final yield are means of 3 replicates.

<u>Concentration</u> <u>of K_2HPO_4 (μM)</u>	<u>Growth rate</u> (G, generation time, hrs.)	<u>Final Yield</u> (cells/mL)
5	10.83	122995
10 *	9.77	194443
20	8.91	350157
25	9.01	376079
30	9.90	403726
35	9.62	428544
40	9.87	485951

APPENDIX B

Derived equations for determination of fluorometric chlorophyll a

Equations used for chlorophyll a determination at different fluorometer sensitivities, derived from the calibration of standard chlorophyll extracts (lettuce leaves), where, ' x ' is the chlorophyll a concentration as $\mu\text{g Chl. a/L}$ and ' y ' is the fluorometer scale reading .

<u>Fluorometer sensitivity</u>	<u>Equation</u>
1	$x = (y - 4.9741) / 0.0155$ (1)
3	$x = (y - 6.2503) / 0.0588$ (2)
10	$x = (y - 2.9716) / 0.2283$ (3)
30	$x = (y + 0.8340) / 0.8407$ (4)

APPENDIX C

Relationship equating spectrophotometrically and fluorometrically determined chlorophyll a

Equation (5) was derived using linear regression analysis, of the relationship of fluorometric chlorophyll a as a function of spectrophotometric chlorophyll a (ug/mL). Identical samples were used for each of the two determinations.

$$x = (y - 0.0103) / 0.8193 \quad (5)$$

where ' x ' is the spectrophotometrically determined chlorophyll a and ' y ' is fluorometrically determined chlorophyll a .

APPENDIX D

Comparison of regression functions for carbon fixation experiments

A comparison of regression functions for each experiment, in each herbicide group, indicated that they were not significantly different from each other (at $\alpha=.05$), thus the transformed data was combined to produce a single regression curve for each herbicide group. A summation of this comparison is outlined in the following table.

TABLE D.1 Summary of analysis utilizing the F value to test whether regression functions are identical. Analysis performed at $\alpha=.05$ level of significance.

<u>Experiment</u>	<u>F* value</u>	<u>Critical F value</u>	<u>Conclusion</u>
Atrazine	0.6031	3.10	Equal regressions
Simazine	1.1816	3.29	Equal regressions
Terbutryn	0.7550	3.29	Equal regressions
Supplementary			
Atrazine	1.6675	3.10	Equal regressions

The test statistic is given by:

$$F^* = \frac{SSE(R) - SSE(F)}{(n_1+n_2+n_3 - 3) - (n_1+n_2+n_3 - 6)} \div \frac{SSE(F)}{(n_1+n_2+n_3 - 6)}$$

where $SSE(R)$, is the error sum of squares for combined data, and $SSE(F)$, is the sum of individual error sum of squares, and where $n = 7$ or 8 .