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

by

Derek Louis François

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AN INVESTIGATION OF TOLERANCE TO THREE TRIAZINE HERBICIDES IN *Chlamydomonas geitleri* Ettl.

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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 CO_2 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 ^{14}C-glucose.

Terbutryn was the greatest inhibitor of growth and CO_2 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 CO_2 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 CO_2 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, CO_2 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 CO_2 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 ametyrn, 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 Amesz,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 ferrodoxin, 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 µg/L, coincided with the time of application (Muir et al., (1978). Levels of simazine even reached as high as 250 µg/L in flowing canal water, following ditchwater application (2.25-7.43 kg/ha), but rapidly declined to less than 5 µg/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 proceeded 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 $^{14}CO_2$ uptake occurred at 100 and 200 µg/L atrazine, within 2 weeks of application. $^{14}CO_2$ 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 500µg/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 µg/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.