The effects of atrazine on the competitive interactions of three green algae

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Thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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THE EFFECTS OF ATRAZINE ON THE COMPETITIVE INTERACTIONS OF THREE GREEN ALGAE

by

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Abstract

The toxic effects of the herbicide Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) on the three common fresh water phytoplankton species *Scenedesmus quadricauda*, *Chlamydomonas musicola*, and *Chlorella vulgaris* were examined. Initially the EC50 values for atrazine were determined for each species using standard batch culture methods. The EC50 values were then recalculated from growth data taken from unialgal continuous culture experiments, and a comparison was made between the results of these two bioassay protocols. The species were then grown in pairs, in continuous cultures, in competition for phosphate. From cell density data the competition coefficients $\alpha_{1,2}$ and $\alpha_{2,1}$ were determined. The competitive cultures were exposed to concentrations of atrazine ranging from 0 to $1.0X10^{-6}$ mol/L. From these experiments it was possible to describe the pattern of changes in the competition coefficients with changes in levels of atrazine exposure for each of the test species pairs. Finally series of model equations were developed from the empirical data and a series of computer simulations were used to predict the outcomes of competition at different initial population densities and differing atrazine exposures. In all species pairs tested the alpha value dose responses showed a biphasic pattern, with a threshold which coincided with the thresholds of the physiological responses seen in the unialgal experiments. These alpha value dose responses led to competitive outcomes and species compositions that were not predicted from the single species toxicological tests. This multispecies competitive bioassay, holds the promise of predicting community level toxicological responses.

Acknowledgments

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Chapter 1

General Introduction

In 1981, while a graduate student at the Natural Resources Institute (NRI) at the University of Manitoba, I had the privilege to attend a workshop organized by G. E. Beanlands of the Institute for Resource and Environmental Studies, Dalhousie University. The workshop, held in Brandon, Manitoba was one of several held across the country which brought together members of the Federal Environmental Assesment and Review Office (FEARO), the university community, and industry in an attempt to increase the scientific credibility of evironmental impact assessments (EIA) in Canada. The resulting report (Beanlands and Duinker, 1983) provided the basic scientific framework that has been adopted for many environmental assessments conducted in Canada. One of the major recommendations of this report was that there be an attempt to develop ecologically based protocols for the determination of the environmental impacts of toxic substances. It was felt that more ecological data were critical if sound environmental managment decisions were to be made. It is towards that end that I undertake this thesis. Inspired by Dr. Beanlands and his vision, I hope that this study may in some small way contribute to the understanding of the ecological effects of toxic substances in our aquatic environment.

A. Introduction to ecotoxicology

It has been over 30 years since Rachel Carson (1962) published her book "Silent Spring" which described the hazards of chemical pollution and the use of persistent pesticides on the environment. This book and similar publications such as Rudd (1964) and Moore (1966), have served to focus the attention of the public and scientific community on the risks that we and the rest of nature face due to exposure to these chemicals. During the last two decades there have been countless meetings of experts, large numbers of conferences and scientific congresses, and a myriad of articles in both the scientific and popular press that have helped to make the public aware of problem situations, some of which have been in existence for years. In spite of this flood of information, it appears that governing politicians of the industrialized nations have yet to make a concerted effort towards concrete decisions that would alter the route that the civilized world has taken. Evidence of this, includes the reluctance of the authorities in developed countries to enforce the legal restrictions on large companies that are often major sources of industrial pollution. Agricultural chemicals are a vast and constant source of biosphere contamination. Ma and Selim (1996) estimate that in the United States, 62% of the agricultural land is treated with pesticides, of which 69% are herbicides, 19% insecticides and 12% fungicides. Pimental and Levitan (1986) estimate that only 0.1% of these agricultural pesticides actually reach the target species, the rest (over 99%) being distributed into the ecosystem. Giesy and Graney (1987) have estimated that there are about 63,000 chemicals in use worldwide, and that over 8.5

million chemicals have been documented in the American Chemical Society's Chemical Abstract Service as of April 1988. They also estimate that the world's chemical industry is marketing up to 200-1000 new synthethic chemicals each year. In recent years the global scale effects of acid rain, ozone depletion, and global warming have made their way into the popular psyche. It is hoped that this awareness will again focus attention on pressing environmental management problems.

The term ecotoxicology was first used by Truhaut (1969), and was intended as a natural extention of the science of toxicology, the effects of toxins on individual organisms, to the ecological effects of pollution. Toxicology is both a descriptive and an explanatory science with respect to the way it seeks to define the mechanisms governing the actions of poisons on individual organisms. Toxicology attempts to describe the effects of a toxicant on a living organism at the biochemical and physiological levels. Death is often described as the end point of the toxic interaction, and it is often the dose or quantity of a toxicant, and the exposure time needed to cause mortality, that are of interest to the researcher. Much of the early toxicological research focused on the toxic effects of chemicals on our own species, or the physiologically similar Norway rat. The effect of pollution on our food species was the next major area of interest. Such studies could be viewed as a part of the field of ecotoxicology, ie. as the effects of chemicals in the human environment.

Several terms have arisen to define an "environmental poison". The word poison is almost never used except in relation to humans. Moriarty (1983) defines a pollutant as a substance that occurs in the environment at least in part as a result of human activities,

and which has a deleterious effect on living organisms. This term has also been applied to physical alterations to the environment (such as the release of heat or noise). It is often useful to distinguish between a pollutant and a contaminant. A substance released by human activity is a contaminant, unless it is suspected of having some biological effect.

B. Goals and directions of ecotoxicology

The effects of a sudden pulse release of pollutants, or the continued long-term exposure to low concentrations of a pollutant, are usually observed at too late a stage to undertake any mitigating action. The need to predict the short-term and long-term effects of these pollutants on ecosystems is one of the reasons for the development of the science of ecotoxicology. This is truly an integrated multidisciplinary effort, drawing tools from sciences such as chemistry, physics, toxicology, physiology, ecology and mathematics. de Kuijt (1988) summarizes some of the questions that ecotoxicologists attempt to answer:

1. What chemicals are we dealing with?
2. What happens to them in nature?
3. How can we measure their effect on organisms?
4. What are the relevant measurements for a species or a community in its usual environment?
5. Do model systems tell us anything that we cannot discover more easily in other ways?
6. How should we monitor the effects of pollutants?

7. How can we predict what will happen with a chemical once it is released into the environment and what effects does it have on the environment?

To investigate these questions, we trace three main steps in a chain of causality. (1) The first consideration is the source of the contaminants. There are generally two types of sources. A point source is when one localized source for the contaminant such as a particular pulpmill or smoke stack can be identified. A diffuse or non-point source defines a more wide spread source, such as agricultural or urban runoff. (2) The second step in the process is to determine the behaviour and fate of chemicals. Once a pollutant has been released into the environment, it will be subject to physical, chemical and biological processes. These processes will influence its movement through, or accumulation in, various compartments of the ecosystem. Such processes could also contribute to the degradation or alteration of these pollutants into other forms. (3) The last step is to determine the effects of the pollutants on living organisms. While the effects of pollutants usually become evident at the community or ecosystem level, all of the effects start with the individual organism. This accounts for the development of single-species toxicity tests (or bioassays) in the mid 1940's. In these tests, representative organisms from the major groups within an ecosystem are used to gain some understanding of the effects of the pollutant on that group of organisms (Cairns and Pratt 1989). For example, *Daphnia sp.* are often used to test toxicant effects on zooplankton (Evans and McNaught 1988), and *Scenedesmus sp.* are often used the illustrate how phytoplankton may respond (Sicko-Goad and Stoermer 1988). Lake Trout, *Salvelinus namaycush* (Walbaum) have been used as indicator species for tests on the effects of

pollutants in cold water, oligotrophic communities, and the walleye *Stizostedion vitreum* (Mitchill) have been used as an indicator species for warmer, nutrient-rich, mesotrophic environments (Robertson 1988). Since toxicity tests came into general use in the early 1950's, they have almost all been based on single species.

C. Bioassays - Principles, scientific basis, and problems

In the broadest sense, a bioassay evaluates the effects of a substance (toxic or otherwise) on a living organism by exposing that organism to the substance and measuring some response. Bioassays are often used to determine the level of exposure that yields no response (or no observable adverse effects). This level is then deemed to be a safe exposure. Such tests have also been used to help identify susceptible species, populations, or other components of an ecosystem and therefore identify biological resources that might be at risk. One of the most commonly used bioassay is the *Selanastrum capricornutum* Printz Algal Assay Bottle Test (Miller and others 1978). The majority of bioassays are carried out under the assumption that the test organisms are representitive of the larger number of species that comprise the natural community.

In order to understand the current state of development of biological methods for the estimation of the effects of pollutants in the environment (bioassays), it is important to understand some of the past history and some of the problems, scientific and otherwise, that must be overcome. It may be surprising to learn that non-biological methods have long been considered superior to biological methods for evaluating the potential hazards

that chemicals pose to the environment. Many of the oldest and most longstanding journals in the fields of pollution control, water resources and environmental management are housed in engineering libraries. During the 1940's and 1950's, the pollution control field was dominated by chemists and sanitary engineers (Cairns and Pratt 1989). It was not until recently that ecologists were able to convince regulatory personnel that biological evidence should be used in conjunction with the chemical and physical determinations, that until then were the primary basis for regulatory decisions.

The single-species bioassay has come to be considered the standard method for biological testing of toxicants. This was no doubt due to the origins of aquatic ecotoxicology in mammalian toxicology, with its emphasis on the toxic effects of substances on humans. Even today, one rarely encounters a multispecies toxicity test being used by industry or a regulatory agency (Cairns 1988), despite the recognized importance of interactions in complex multispecies communities (Odum 1953). At each level of biological organization (from subcellular, through cellular, tissue, whole organism, population, community to ecosystem), new interactions and system properties are added that cannot be effectively studied at the lower levels of organization. Reluctance on the part of the environmental managers to move to multispecies tests is due in part to the problems of extrapolating from one biological level of organization to another. Another problem is the inability to predict toxic effects on complex naturals systems from the results of single-species tests carried out under laboratory conditions.

The National Research Council (1981), in the exectutive summary of it's report on methods of ecotoxicology, list some of the problems of single species toxicology tests:

(1) Current laboratory tests examine only the responses of individuals, which are then averaged to give a mean response for the test species.

(2) With given constraints of limited finances and numbers of personnel, it is not possible to identify the most sensitive species or group of species.

(3) The data are too limited in scope for extrapolations to be made from them to responses of other (even closely related) species.

(4) Indirect effects resulting from population or species interactions cannot be observed.

(5) Conditions within which single-species tests are performed lack the realism of natural habitats.

As if all of these problems were not enough, there are some questions as to the scientific bases of these tests. Bioassays are generally considered to be scientific tests, since biologists, ecologists, or chemists usually carries out these tests. As Cairns (1988) points out, the scientific basis for using bioassays will depend on how accurately the test results can be validated in the natural ecosystems. The scientific method has, at its core, the statement of a null hypothesis and an experiment to test this hypothesis. In toxicology, a null hypothesis usually takes the form of a negative statment, such as "The pesticide has no effect of the death rate of the species in question". If the null hypothesis is not supported by the results of the experiment, we gain more confidence that the toxin does effect the organisms or system in question. A good experiment must possess sufficient and rigorous controls. Scientists must also be able to compare their evidence and hypotheses to those generated by others. Cairns and Pratt (1989) suggest that, because aquatic toxicology was an outgrowth of mammalian toxicology, the basic hypothesis in aquatic toxicology is often only implicit. The development of a scientific basis for ecotoxicology has been held back, because the goals of aquatic ecotoxicology and mammalian toxicology do not share (or should not share) a common key hypothesis. In

mammalian toxicology one attempts to extrapolate toxicological results from surrogate test organisms (e.g., rats, mice, *Rhesus* monkeys, etc.) to humans. As more information on these test organisms becomes available, the accuracy of that extrapolation improves. An explicit statment of the hypothesis underlying ecotoxicological bioassays is very difficult to locate in the literature.

There is an implicit hypothesis which governs the practices of many government departments charged with the responsibility of environmental protection. This is illustrated by the present standard practice of the American Environmental Protection Agency (EPA), and the standard methods described by the American Public Health Organization (APHA)(1976). As Miller and others (1978) specifically states: "The AA:BT [algal assay bottle test] can be used to define the potential stimulatory and/or inhibitory properties of new product formulations introduced into receiving waters". Implicit in this statement is that the response of *Selenastrum capricornutum* to any toxicant will be representitive of the responses of all the primary producers in any aquatic ecosystem. APHA (1976) describes the standard methods for bioassays using an alga, a mollusk, a fish, and several tentative bioassay methods including three zooplankton, a crustacean, an aquatic insect, a marine annelid, and a coral.

American Public Health Association (APHA 1976) defines safe concentration (SC) as:

> The maximum concentration of a toxicant that has no observable harmful effects after long-term exposure over one or more generations. When the test species is the important local species that is most sensitive to the material or waste under consideration, the SC so determined serves as the basis for a water quality standard for that material or waste in that area. Most important species are those having economic (food), recreational, forage (important in the food chain), or ecological importance.

APHA, (1976) standard methods goes on to the define the maximum allowable toxicant concentration (MATC) as:

> The concentration of toxic waste that may be present in the receiving water without causing significant harm to its productivity and all its various uses. The MATC is determined by a long-term bioassay of a partial life cycle with sensitive life stages or a full life cycle of the test organism in which a range of concentrations of the toxicant under test that do not demonstrate significant harm to the test organism is determined.

APHA (1976) informs researchers that these terms are adequate for the expression of nearly all results of bioassays in aquatic environments, and that these terms must be used as standard symbols for expression of bioassay results. However, most ecologists would agree that single-species toxicity tests are totally inadequate to fulfill the objectives of environmental protection implied in these definitions (Cairns and Pratt 1989)..

Definitions such as "Safe concentration", and "Maximum allowable toxicant concentration", imply that single species tests are intended to be used to protect an entire ecosystem. For this goal to be realized, some evidence must be presented that extrapolations made from single-species tests to higher levels of ecological organization are reliable. Cairns and Pratt (1989) lament the fact that the scientific basis for applying information generated from bioassays has not increased in the past few years. Macek (1982) sums up the problem thus: "There has been an incredible increase in data but virtually no increase in knowledge". It should be pointed out that with respect to the development of a scientific basis for prediction, ecologists are not much further ahead. Ecology has traditionally been largely a descriptive science, and most of what we know of ecology is of an empirical nature (Emlen, 1977). It is true that until a considerable amount

of descriptive work had been done it will be impossible to build any sort of theoretical framework. Ecology has only recently developed some of this theoretical framework that can now be used to structure and direct the investigation of the complex interactions that typify ecosystems. It is time that ecotoxocologists began to incorporate some of the developing ecological theory into the development of methods of environmental management.

Several authors and scientific organizations have reached this same conclusion. The United States National Research Council (1981), in its review of the methods of ecotoxicology, stated that single-species tests would be of greatest value if used in combination with tests that can provide data on population interactions and ecosystem processes. The executive summary states that "Research and development should be directed toward designing and validating test systems and procedures that will detect changes in ecosystem and population attributes." This committee recommended a multi-level approach to data collection. They suggest that before a decision on the potential hazard of a chemical can be made, four classes of information must be gathered.

> 1. *Characterization of test substance*: information on chemical and physical properties, estimates of fate within and among ecosystems, and estimates of dose and exposure time for biotic compontents of an ecosystem.
> 2. *Physiological responses of species*: data on individuals of representative species indicating morphological, biochemical, genetic, and pathological changes related to the presence of the chemical.
> 3. *Multi-speies responses*: information on the changes in interactions among organisms, including changes in population or system structure and changes in patterns of interactions among species (e.g., predation, **competition** (bold mine) and migratory behavior patterns).
> 4. *Ecosystem responses*: data on changes in functional processes that affect the resistance and resilience of the ecosystem.

D. Algal Bioassay Methods

Batch culture systems

As Rhee (1989) points out, pollutants that enter an aquatic system affect that system at the population and community level. It is important that an effective bioassay yield information at both these levels, and that the information be predictive of potential impacts of toxins on the structure of the community or ecosystem as a whole. Whether the assay is single-species or mixed-species, the type of culture system used is very important. As this thesis will show, the ecological relevance of the bioassay results depends as much on the culture system as on the experimental design.

The culture systems used in algal assays can be divided into two types, open systems and closed systems. The most common culture system is the batch culture, due to its simplicity and low cost. This is a closed system in which there is no input or output of materials. The algal population cell density increases constantly until the exhaustion of some limiting factor, while other nutrient components of the culture medium decrease over time. Any products produced by the cells during growth also increase in concentration in the culture medium. Batch culture systems are highly dynamic. The populations show a typical pattern of growth that has been described by many authors (Monod 1949, Fogg and Thake 1987, Bolier and Donze 1989). Monod (1949) distinguished a succession of six phases in the growth of bacteria in batch culture. These phases are characterized by variations in growth rate, and are summerized in Table 1.

Table 1. Description and interpretation of the different phases of batch culture growth curves, as characterized by Monod (1949).

	phase	growth	interpretation
1	lag	zero	physiological adaptation of the innoculum to changing conditions
2	acceleration	increasing	trivial
3	exponential	constant	population growth changes the environment of the cells
4	retardation	decreasing	effects of changing conditions appear
5	stationary	zero	one or more nutrients (or light) are exhausted down to the threshold level of the cells
6	decline	negative	the duration of stationary phase and the rate of decline are strongly dependent o the kind of organisms

In batch cultures, cell properties such as size, internal nutrient composition, and metabolic function vary considerably during the above growth phases. This can often make interpretation of the results difficult (Kubitschek 1970). During the exponential growth phase, cell properties tend to be constant. However, this phase usually only lasts for a short period of time. Bolier and Donze (1989) have shown that if one wishes to estimate growth rates of the exponential phase of batch cultures, daily sampling appeared to be insufficient to allow a reasonably accurate estimate. It was also found that the accuracy of growth rate determination was highest in artificial, defined media as compared to cells grown in natural surface water media. This would make suspect the use of batch culture as a bioassay technique for the determination of toxic substances in natural waters. If, however, the toxic substance is added at known concentrations to artificial, defined

growth media, better results should be obtained. A significant advantage of batch culture systems is their operational simplicity. The culture vessels most often consist of an Erlenmeyer flask with a 20% sample volume to flask volume ratio, in order to prevent carbon dioxide limitation. This volume ratio is only critical if the flasks are shaken by hand once a day during the culturing run. If the flasks are cultured on a rotating shaker table a sample volume to flask volume of 50% is permitted (Miller and others 1978). Cells of the algal test species are inoculated into the culture medium, and the cells are allowed to grow for up to 96 hours. Samples are taken at regular intervals and cell densities estimated. A variety of methods have been used to directly estimate cell densities, from direct microscopic counts with a hemocytometer (Ibrahim 1990) or Sedgwick-Rafter counting cell (APHA 1976) to electronic particle/cell counting techniques. Other indirect estimators of biomass have also been used in batch culture studies. Optical density has been a popular method of estimating algal biomass (Klapwijk and others; 1989, Lukavský 1992), as are the estimation of chlorophyll *a* (Kratky and Waren 1971; Maule and Wright 1984), and dry weight determination (Clotaire and Gschwind 1994). The duration of these bioassay tests is most often 96 hours (APHA 1976; Miller and others 1978). There appears to be no significance to this 96 hour duration apart from the fact that assays can be started on a Monday and terminated on a Friday. A time of 96 hours should allow the cultures of most species time to achieve stationary phase under normal culture conditions, but cultures may not reach this phase under stressed conditions.

Continuous culture methods

Continuous culture systems have been widely used to culture microbes for industrial and research purposes (Kubitschek 1970; Tempest 1970; Veldkamp 1976, 1977; Rhee 1980; Munawar and others 1989). In recent years these culture techniques have found their way into the bioassay methods of ecotoxicology (Rhee 1980, 1989). The early development of a continuous culture system can be traced back to the work of Novik and Szilard (1950*a*,*b*) who developed the first "chemostat", and by Monod (1950) who called his apparatus a "bactogen".

Batch and continuous culture systems differ in that in a continuous culture system, nutrients are supplied to the cell culture at a constant rate, and in order to maintain a constant volume, an equal volume of cell culture is removed. This allows the cell population to reach a "steady state" (ie. growth and cell division where the growth rate and the total number of cells per milliliter of culture remains constant). Fogg and Thake (1987) make the distinction between "turbidostat" and "chemostat" continuous culture systems. In the turbidostat system, fresh medium is delivered only when the cell density of the culture reaches some predetermined point, as measured by the extinction of light passing through the culture. At this point, fresh medium is added to the culture and an equal volume of culture is removed. The diluted culture increases in cell density until the process is repeated. In a chemostat, the medium is delivered at a constant rate, which ultimately determines growth rate and cell density. All of the experiments in this thesis used a chemostat continuous culture system.

In many chemostat continuous culture systems, the nutrient medium is delivered to the culture at a constant rate by a peristaltic pump or solenoid gate system (Kubitschek 1970). The rate of media flow can be adjusted, and is often set at approximately 20% of culture volume per day. Air is pumped into the culture vessel through a sterile filter. This bubbling air has three effects: it supplies CO_2 and O_2 to the culture, aids in circulation and agitation of the cultures, and pressurizes the head space of the culture vessel so as to provide the force to "remove" an amount of media (and cells) equal to the volume of inflowing media. The culture may be aseptically sampled by opening the clamp on a sample port. The magnetic stirrer and aeration help to prevent the cells from collecting in the bottom of the culture vessel. A truly continuous culture will have the medium delivered at a constant volume per unit time. However it has been this researcher's experience that delivery systems such as peristaltic pumps or solenoid gates are inherently unreliable. It is difficult to adjust such systems to deliver equal amounts of medium to several cultures simultaneously. This is what is needed if competition experiments are to be truly replicated. In order to deliver exactly the same amounts of medium to several cultures growing at once, a "semi-continuous" approach can be taken. In a semi-continuous system the fresh medium is delivered to the culture all at once, by simply opening a valve in the medium delivery line. Fresh medium flows into the culture vessel, and spent culture flows out into a collecting vessel. Once the required medium has entered the culture, the valve is closed, and the culture is allowed to grow for 24 hours, when the procedure is repeated. Our lab has used both methods and it has been

shown that there are significant advantages in the consistent medium delivery of a semi-continuous system.

The rate of flow of medium into a continuous culture system is known as the "dilution rate". When the number of cells in the culture vessel remains constant over time, the dilution rate is said to equal the rate of cell division in the culture, since the cells are being removed by the outflow of medium are being replaced by an equal number through cell division in the culture (Kubitschek 1970).

The principal advantage of continuous culture is that the rate of dilution controls the rate of microbial growth via the concentration of the growth-limiting nutrient in the medium. As long as the dilution rate is lower than the maximum growth rate attainable by the algal species, the cell density will increase to a point at which the cell division rate ("birth rate") exactly balances the cell washout rate ("death rate"). This steady-state cell density is also characterized by a constancy of all metabolic and growth parameters. On the other hand, if the dilution rate exceeds the maximum cell division rate, then cells are removed faster than they are produced and total washout of the entire cell population eventually occurs. Figure 1.1 illustrates a typical setup of such semi-continuous culture equipment.

The growth rate that is achieved under steady state conditions within a continuous culture is determined by the dilution rate. The only potential complication with a continuous culture system might occur if one wished to investigate the effects of very high levels of a toxin on the growth of an algae. If growth is so severely affected by the toxic

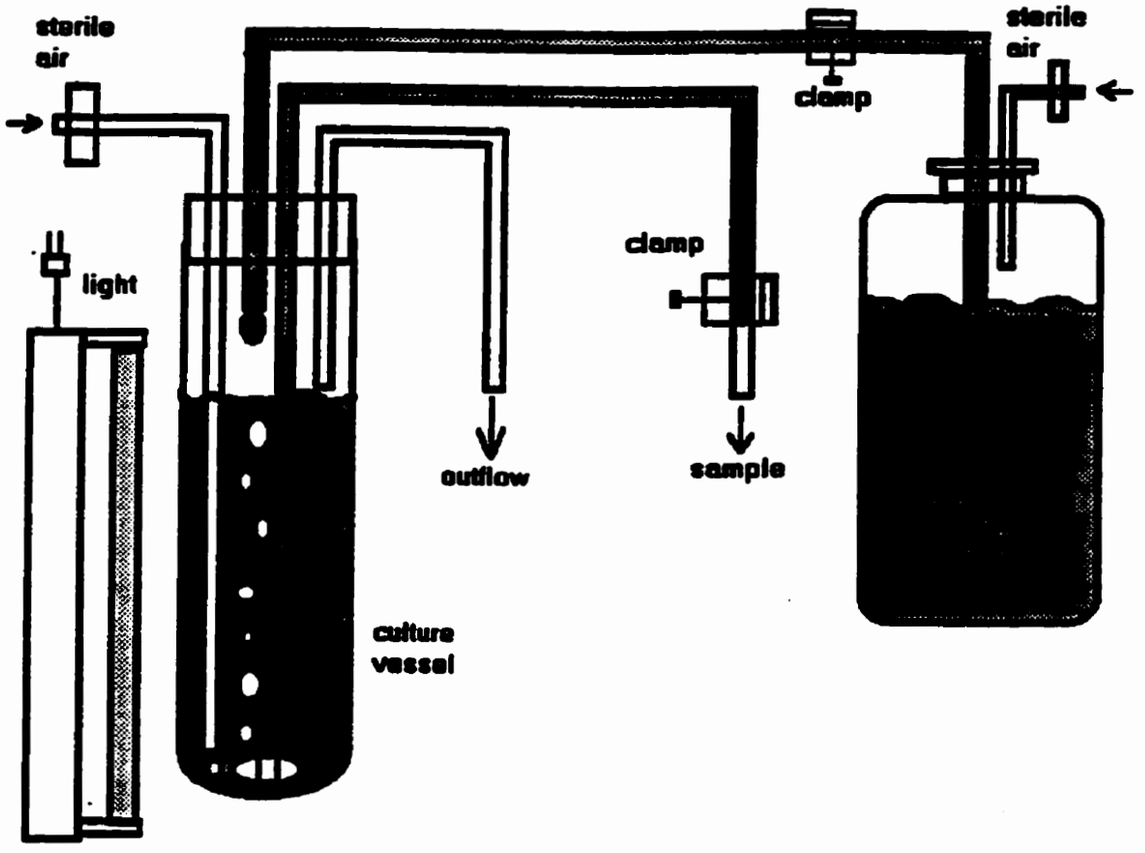


Figure 1.1 Diagramatic representation of a semi-continuous culture system.

effects of the herbicide that the growth rate declines below the dilution rate, the culture would wash out. If one wished to use a continuous culture system to test the effects of an extremely high concentration of toxin, a two-stage chemostat could be used (Rhee 1989). In such a system there are two media reservoirs each supplying a separate culture vessel (stage). The first stage operates as a single-stage chemostat. However, as can be seen in figure 1.2, the outflow from the first stage flows into the second stage culture vessel which also receives fresh medium from its own reservoir at a constant rate. The dilution rate of the second stage is the sum of the outflow from the first stage and the inflow from the second stage fresh medium reservoir. If a toxicant were added to the second stage in the second stage medium which caused significant reduction in growth rate so that the second stage would normally wash out, a supply of fresh healthy cells could be provided from the outflow of the first stage. This constant supply of cells could maintain a constant cell density in stage two even at extremely low division rates. Many researchers have used variations on this two stage type of system to investigate important ecological interactions such as the regeneration of nutrients in aquatic ecosystems (Gude 1985). Hendzel (1986) describes the use of a two chamber chemostat where the two growth chambers are separated by a porous membrane, and each supplied with its own medium. This apparatus allowed the study of competition between two species growing in a shared medium, while kept physically seperated. This system facilitated the enumeration of each species during the growth experiments.

It was Monod (1942) that first expressed the dependence of microbial growth rate on the concentration of limiting nutrient. Later the relationship was interpreted in terms of

Michaelis-Menton kinetics. The model of a single enzyme-mediated reaction as the one rate-limiting step may not be completly accurate. Waltman and others (1980) point out that some of the observed deviations from the Monod relationship in microorganisms may be due to the possibility that the limiting factor is an energy source or an essential nutrient such as a vitamin or mineral. However Waltman and others (1980) do feel that the competition theory developed from the microbial growth equations of Monod remain the most general theory available, and will serve as the most practical tool for probing the mechanics of microbial competition.

E. Atrazine

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-trazine) is a member of the group of heterocyclic nitrogen herbicides commonly known as the *triazines*. These compounds are six member rings containing three nitrogens in a heterocyclic azine ring. Figure 1.3 shows the molecular structure of atrazine. This selective herbicide has been used worldwide since 1952 to control annual weeds in corn and sugarcane (Ma and Selim 1996). The wetable powder formulation of atrazine sprayed as an aqueous suspension has also been recommended as a post-emergence herbicide to control target graminoid and broadleaf weeds in crops of sorghum (Horowitz 1964). The U.S. Environmental Protection Agency estimated that between 32,000 and 34,000 metric tonnes of atrazine were used in U.S. agricultural crop production in 1993, making it now the most heavily used of all pesticides in North America (Aspelin 1994).

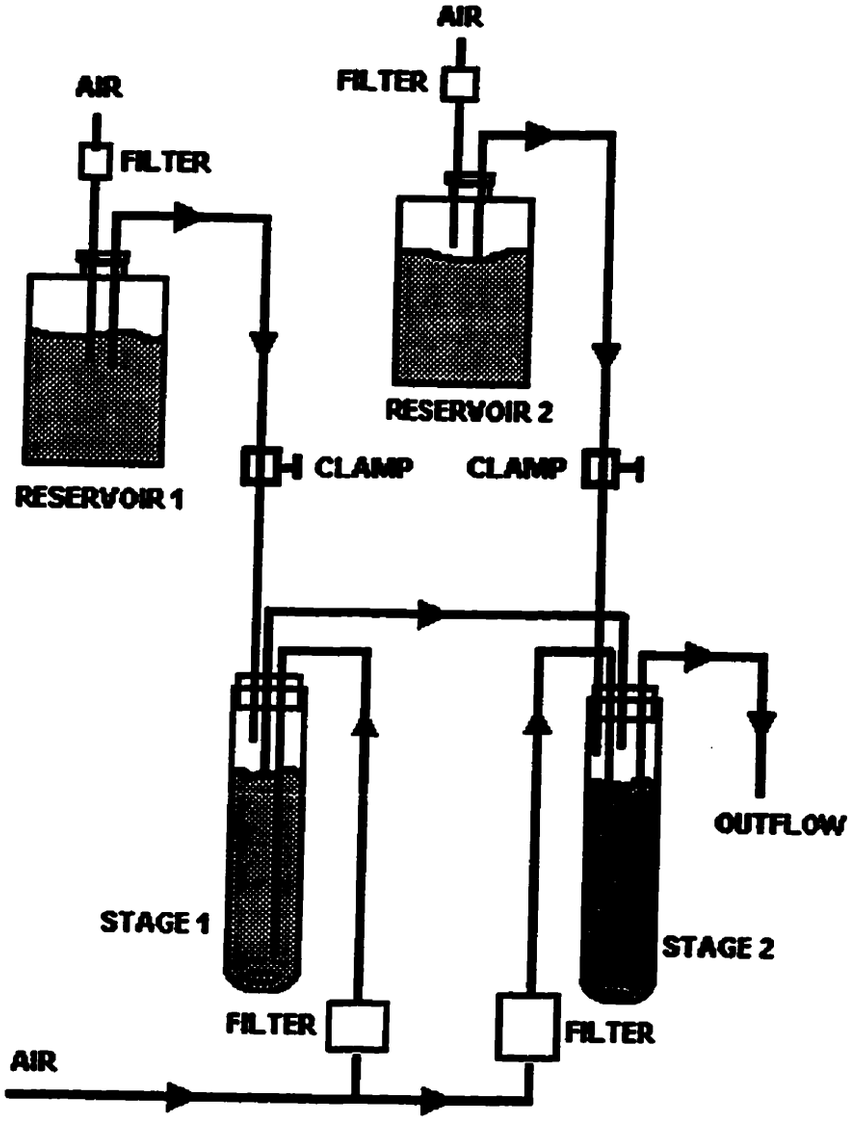


Figure 1.2 Schematic diagram of a two-stage chemostat. (After Rhee 1989).

Atrazine acts as an inhibitor of photosynthesis, by blocking electron transfer between Q_A and Q_B, the primary and secondary plastoquinone electron acceptors of photosystem II, respectively. This herbicide appears to bind to the 32-34 kilodalton, D1 polypeptide on the acceptor side of the photosystem II core complexe at the Q_B site. Purcell and others (1990) discussed evidence for a second site of triazine action located on the donor side of the photosystem II. By studying PSII particles as well as intact thylakoids isolated from atrazine resistant biotypes of *Chenopodium album* L., their data were discussed in terms of an atrazine binding site on the luminal side of the thylakoid membrane, which presented the same resistance characteristics as the site on the acceptor side of photosystem II.

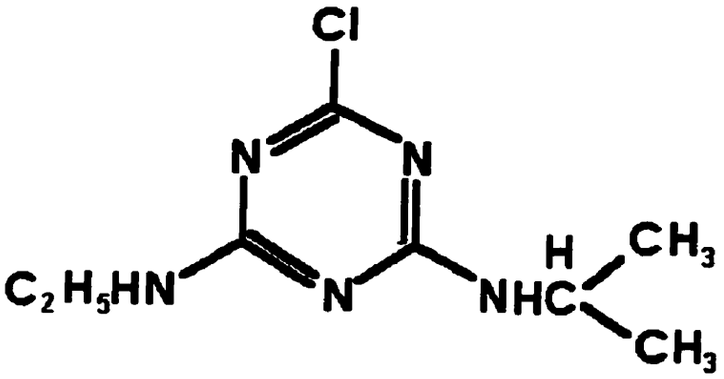


Figure 1.3 Molecular structure of Atrazine.

F. Autecology of test species.

All of the species chosen for these experiments have been classified by Lee (1992) in the same division, Chlorophyta, and the same class, Chlorophyceae. Members of the division Chlorophyta, commonly called green algae, have chlorophylls *a*, and *b*, and form starch within the chloroplast, usually in association with a pyrenoid. Members of this division are primarily freshwater, with only about 10% being marine. The freshwater species have a cosmopolitan distribution, with few species endemic to a certain area.

The cell walls generally have cellulose as the main structural polysaccharide. Chloroplast pigments are similar to those of higher plants, Chlorophylls *a* and *b* are present, and the main carotenoid is lutein. The carotenoid β-carotene can accumulate outside of the chloroplast under conditions of nitrogen deficiency. Chloroplasts are surrounded only by the double-membrane chloroplast envelope. The thylakoids are grouped into bands of three to five thylakoids without grana. The starch formed in the chloroplast, is similar to that of higher plants and is composed of amylose and amylopectin. The photosynthetic pathways are similar to those of higher plants, many of these pathways first being worked out in green algae such as *Chlorella*.

Characteristics which distinguish members of the class Chlorophyceae are the presence of a theca outside the cells (secondarily lost in some), and a collapsing telophase spindle that brings the daughter cells close together, followed by cell division by a phycolast. The Chlorophyceae whose sexual reproduction is known produce a dormant zygote, with meiosis usually occurring when the zygote germinates.

Chlamydomonas musicola Schmidle, is a member of the order Volocales, family Chlamydomonadaceae. Cells in this family are unicellular, flagellated and motile. All of the genera are uni-nucleate, and the chloroplast is usually cup-shaped. *Chlamydomonas musicola* Schmidle, is a unicellular, biflagellate organism with a cupshaped, basal chloroplats containing a central pyrenoid (see figure 1.4). The cells are uninucleate, with two contractile vacuoles at the base of the flagella. This species had no distinct eyespot. Although no reproduction was observed directly, asexual reproduction in this genus typically begins with the cells coming to rest and retracting or discarding its flagella. The protoplast then divides into 2 to 16 daughter protoplasts within the parent cell wall. These protoplasts develop walls and flagella and are released on gelatinization of the parent wall. Most species of *Chlamydomonas* exhibit isogamous sexual reproduction. Morphologically similar gametes of opposite mating strains fuse at their anterior ends to form the primary zygote. The nuclei fuse within 24 hours and a resistant secondary zygote wall is laid down. Meiosis occurs in the zygote to produce 4 to 8 zoospores. The zoospores are released from the zygote wall to mature into vegetative cells.

The other two species used in these studies are in the order Chlorococcales. This order is characterized by unicellular or nonfilamentous colonial algae. If colonial, then daughter colonies are formed as coenobia. All vegetative cells are nonmotile. Asexual reproduction occurs by zoospores or aplanospores that are commonly autospores. These autospores are probably no more than daughter cells of the parent thallus. Sexual reproduction can be isogamous, anisogamous, or oogamous.

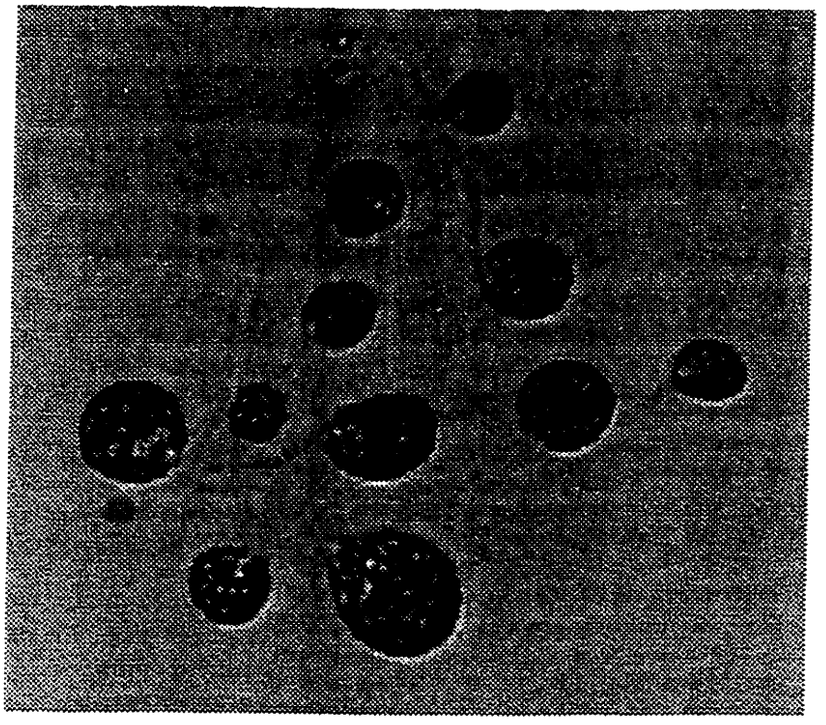


Figure 1.4 *Chlamydomonas musicola* Schmidle.

Chlorococcalean algae, such as *Chlorella vulgaris* Beyerinck, producing daughter cells of the same shape as the parent cell (autospores) are placed in the family Oocystaceae. *Chlorella vulgaris* cells are spherical with a cup-shaped chloroplast (Figure 1.5). The only method of reproduction in *Chlorella* is by daughter cells that resemble the parent cell.

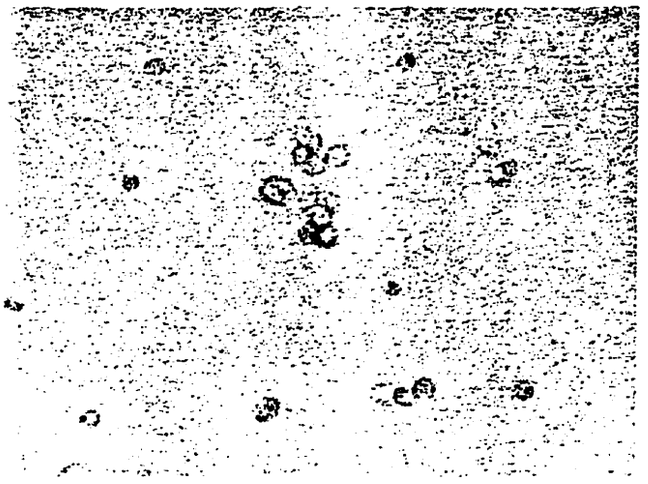


Figure 1.5 *Chlorella vulgaris* Beyerinck.

In many taxonomic schemes, algae reproducing by autospores that become apposed to one another to form a colony of a definite number of cells (coenobium) are placed in a separate family, the Scenedesmaceae. It has been shown that it is possible to vary the morphology of *Scenedesmus quadricauda* (Turp.) de Brébisson, from a unicell to a colony by changing the medium (Trainor and others 1976). This has lead Lee (1992) to suggest that perhaps *Scenedesmus* should be included in the family Oocystaceae. Scenedesmus is a common alga (Figure 1.6), often forming a dominant part of the

phytoplankton community. Cells in the colony occur in multiples of 2, with four or eight cells being the most common. The species differ mostly in the number ant type of spines on the cells and the texture of the wall. The uninucleate cells have a single laminate chloroplast.

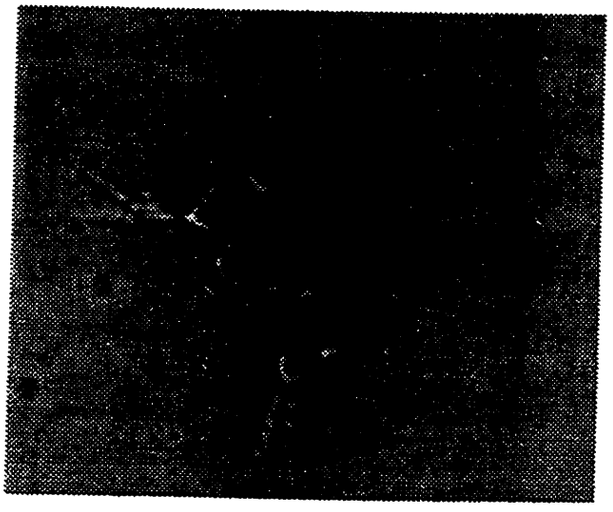


Figure 1.6 *Scenedesmus quadricauda* (Turp.) de Brébisson

G. Objective

The objective of this thesis was to provide data on the changes in the competitive interactions between selected phytoplankton species caused by their exposure to the herbicide Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine)(CAS No. 1912-

24-9). This thesis has added to the body of ecotoxicological knowledge in three areas: (i) It has compared the results of a standard single species algal bioassay to those produced by the competition bioassay. (ii) It has provided some basic information on the competitive interactions between three ubiquitous algal species, *Scenedesmus quadricauda* (Turp.) de Brébisson, *Chlorella vulgaris* Beyerinck, and *Chlamydomonas musicola* Schmidle, under phosphate limitation. (iii) It has described how different concentrations of the herbicide Atrazine altered the competitive interactions of these species.

Chapter 2

The effect of Atrazine on the growth responses of three green algae.

A. Abstract

The EC50 values for the herbicide Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) were determined for the three phytoplankton species *Scenedesmus quadricauda*, *Chlorella vulgaris*, and *Chlamydomonas musicola*. A standard batch culture protocol was used to culture these algae, and the EC50 values were estimated using weighted probit analysis of two growth parameters, doubling time (G) in hours, and 96 hour cell density values. The 96 hour cell density EC50 values showed considerably less variation than the EC50 values estimated from the doubling time data. In an experiment run in 1993, the EC50 values for *Scenedesmus quadricauda*, *Chlorella vulgaris*, and *Chlamydomonas musicola* were estimated at $9.93X10^{-8}$, $1.60X10^{-7}$, and $1.31X10^{-7}$ mol/L respectively. These estimates were found not to be statistically different from the EC50 estimates of experiments run in 1991 (*Scenedesmus quadricauda* $2.05X10^{-7}$ mol/L, *Chlorella vulgaris* $1.80X10^{-7}$ mol/L and *Chlamydomonas musicola* $3.9X10^{-7}$ mol/L).

B. Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is an s-triazine, one of the better known photosynthetic inhibitors. It blocks electron transport between Q_A and Q_B, the primary and secondary plastoquinone electron acceptors of photosystem II respectively (Purcell and others 1990). This herbicide has been used worldwide since 1952 to control annual weeds such as henbit (*Lamium amplexicaule L.*), annual bluegrass (*Poa annua L.*), and annual sowthistle (*Sonchus oleraceus L.*) in corn and sugarcane fields (Frank 1966; Ma and Selim 1996). The wettable powder formulation of atrazine sprayed as an aqueous suspension has also been recommended as a post-emergence herbicide to control target graminid and broad-leaf weeds in fields of sorghum (Horowitz 1964). Jurgensen and Hoagland (1990) reported that atrazine was the second most heavily used pesticide in the United States. The U.S. Environmental Protection Agency (USEPA) estimated that between 32,000 and 34,000 metric tonnes of atrazine were used in U.S agricultural crop production in 1993, making it now the most heavily used of all pesticides (Aspelin 1994).

Atrazine can be transported into water bodies adjacent to agricultural fields in runoff via clay particles, organic matter, and in solution, particularly when application is followed by a heavy rain (Jurgensen and Hoagland 1990). As could be expected with such heavy use, numerous reports of ground water and river contamination have been reported (Isensee and others 1988; Pionke and others 1988; Periera and Rostad 1990). Southwick and others (1992) detected atrazine concentrations as high as 403 µg/L ($1.9X10^{-6}$

mol/L/L) in 1.0 meter deep tile-drain water samples 7 days post-application in Sharkey clay soil. Aquatic toxicologists have detected atrazine in natural waters in concentrations within the range of 0.1 - 30 μg/L ($4.65X10^{-9}$ to $1.4X10^{-7}$ mol/L/L) (Muir and others 1978). Detection of atrazine in surface waters has prompted many studies into its possible effects on photosynthetic phytoplankton which are the primary producers of these aquatic ecosystems.

As deNoyelles and others (1982) correctly point out, due to the complex network of interspecific interactions, the response of an aquatic community to a toxic chemical stress is the sum total of the combined responses of its members. Understanding the physiological responses of individual members of a community to the toxicant is the first step in understanding the response of the community as a whole to such a toxic assault. In this chapter I investigate the growth responses of three common green algae to various environmentally relevant concentrations of Atrazine. Calow (1989) suggests that there is far too little information about the relative sensitivities of phytoplankton species to such toxins. I will attempt to follow Calow's recommendation that an experimental program be developed to rigorously compare sensitivities between species. In support of the overall thesis, I will provide the example of the standard method of assessing and predicting the effects of a toxic chemical on the aquatic environment, the single species batch culture bioassay. This study will also compare the use of growth rate data with the maximum standing crop assessment recommended by Miller and others (1978). Lastly, these experiments will also serve to determine if the responses of the test species that were used

for the two species competitive herbicide bioassay, which is discussed in Chapter Five, changed over the four year duration of those experiments.

C. Materials and Methods

The three test species used in this study, *Scenedesmus quadricauda*, *Chlorella vulgaris*, and *Chlamydomonas musicola*. were isolated from water samples collected on May 28, 1989, from channels of the Delta Marsh, on the southern end of Lake Manitoba, Canada (99°19'W, 50°7'N). Isolation procedures are described in Appendix 2. The unialgal isolates were maintained as stock cultures in 150 mL of the phosphate limited WC' algal growth medium modified from Guillard and Lorenzen (1972) by Healy and Hendzel (1979). The complete WC' medium composition is given in Appendix 1. These algal stock cultures were stored in 250 mL Erlenmeyer flasks sealed with a cheesecloth covered cotton bung, on a rotary shaker table, at 19 °C, under continuous illumination with General Electric Cool White fluorescent lamps at 70 $\mu Em^{-2}s^{-1}$. The stock cultures were subcultured once a week by aseptically transferring about 30 mL of the old culture into 150 mL of fresh medium. A stock solution of $1.0X10^{-4}$ mol/L atrazine was created by dissolving 0.0215 g of technical grade atrazine (>97.7% active ingredient) into 10 mL of absolute methanol. This methanol solution was then suspended in 90 mL of double distilled water. This stock atrazine solution was stored in the dark at 5 °C.

In order to determine if the dose response of each test species to atrazine remained constant over the 4 year duration of the continuous culture competition experiments, two separate dose response experiments were carried out for each test species. The first set of

dose response experiments were conducted between April and June of 1991, and the second in June and July of 1993. The dose response experiments were modified from Miller and others (1978) as follows. Six 250 mL Erlenmeyer flasks were filled with 150 mL of WC' medium, then a sufficient volume of $1.0X10^{-4}$ mol/L/L Atrazine was added to each flask to create the following Atrazine concentrations: 0 mol/L/L, $1.0X10^{-8}$ mol/L/L, $5.0X10^{-8}$ mol/L/L, $1.0X10^{-7}$ mol/L/L, $5.0X10^{-7}$ mol/L/L, $8.2X10^{-7}$ mol/L/L, and $1.0X10^{-6}$ mol/L/L. These flasks were stoppered with a cheesecloth-covered cotton bung and autoclaved at 121 °C for 30 minutes. Four replicate cultures of each of the above concentrations were prepared for each dose response experiment.

Each test species was subcultured as above and allowed to reach the log phase of growth. Ten milliliters of this log phase culture were then used to aseptically inoculate the four replicate flasks of each of the six herbicide concentrations. The 24 experimental flasks were randomly placed on a rotary shaker table (60 RPM) at 19 °C, under continuous illumination with General Electric Cool White fluorescent lamps at 70 $\mu Em^{-2}s^{-1}$. Each flask was removed from the shaker table and aseptically sampled daily for 4 days (96 hours). After each sampling the flasks were randomly replaced on the rotary shaker table. Cell density estimates (cells per milliliter) were made using a Reichert-Jung, Bright-line Improved Neubauer Hemocytometer. From the resulting growth curves (cells per milliliter vs. time) three parameters were calculated, μ' the relative growth rate, G the doubling time (in days), using the following equations from Fogg and Thake (1987). The maximum yield of the culture (Y_{max}) was determined from the day four cell density. The

"mean" μ', G, and Y_{max} values represent the mean values of the four replicates of each herbicide concentration.

$$\mu' = \frac{\log_{10} N_1 - \log_{10} N_0}{t_1 - t_0} \qquad 1$$

$$G = \frac{\log_{10} 2}{\mu'} \qquad 2$$

Where N_1 = cell per milliliter at time t_1 (day1) and N_0=cells per milliliter at time t_0 (day0).

In addition the parameter "r", the exponential growth rate, was calculated using the following formula from deKruijk (1988).

$$r = \frac{\ln\left(\frac{N_0}{N_1}\right)}{t_0 - t_1} \qquad 3$$

This r value was then used to determine the % inhibition using the following formula from deKruijk (1988),

$$\textbf{\% inhibition} = (1 - r_1/r_2) \times 100 \qquad 4$$

Where r_1 equals the exponential growth rate in the presence of herbicide and r_2 equals the exponential growth rate of the herbicide-free control. The % inhibition values were transformed to probit values according to the table of Y=probit(p) for p=0.1 to 99.9 percent found in Hubert (1984). The Y=probit(p) values were plotted against the $\log_{10}$ of the herbicide concentration and a straight line fitted through these point using linear regression analysis. Y=probit(p) = a + bX was then used to estimate the median effective dose (EC50) for the parameter being measured, by setting Y=probit(p) = 5. EC50 can be

defined simply as the concentration of atrazine which produced a 50% level of the parameter under consideration. As Hubert (1984) points out, there is a real statistical problem of how to fit the best line through the herbicide, probit data. The use of ordinary regression techniques requires the assumption that the variability of the observations (the values of Y) is the same for each value of X. For probit plots, however, it can be argued that, in general, this is not the case. To avoid this problem each probit value was adjusted using the weighting coefficients taken from Hubert (1984), and these adjusted values used to calculate the 95% confidence intervals.

The mean maximum cells per milliliter on day 4 of the experiment was used as an estimator of the "Maximum Standing Crop" parameter as described in Miller and others (1978). These day-four cell density values were used to calculate a second set of EC50 values, using the same method as for the "r" values. The EC50 estimates resulting from the "r" values and maximum cell numbers were then compared.

The algal growth responses observed during the 1991 experiments were compared to those from the 1993 experiments using an approximate randomization test (Manly 1991). Two different culture parameters, growth rate expressed as Doubling time (G) in hours, and day 4 cells per milliliter were compared in order to determine whether they showed the same response patterns. The QBasic® program used to perform the approximate randomization test is given in Appendix 3.

D. Results and Discussion.

The growth responses, expressed as the mean % inhibition of four replicate cultures vs. $\log_{10}$ of atrazine concentration, of the three test species *Scenedesmus quadricauda, Chlorella vulgaris*, and *Chlamydomonas musicola* in 1991 and 1993 are compared in figures 2.1, 2.2 and 2.3. The % inhibition response for these graphs were calculated from the day 4 (96 hour) cell density data. This growth parameter was selected because it yielded a smaller variation between the replicate samples as compared to doubling time (G hours) (Tables 2.1 and 2.1). In all experiments, the % inhibition of the algae increased as the herbicide levels were increased,. *Chlamydomonas musicola* and *Chlorella vulgaris* show a similar response of only slight inhibition in growth at herbicide concentrations below $1.0X10^{-7}$ mol/L/L, and then dramatic decrease in growth at atrazine levels in excess of this herbicide level. Since there was no significant difference in the growth of these organisms at the three lowest herbicide levels tested, it is likely that these species are all capable of mitigating the effects of the herbicide when it is present in the environment at relatively low concentrations ($<10^{-7}$). It should be remembered however, that this apparent response was observed in isolation. The algae were responding at the organismal level. These responses show the results of physiological stresses on the species due the presence of the herbicide under phosphate limitation. All other nutrients were provided in excess, and there were no interspecific interactions involved. It should also remembered that the nutrient status of the cultures is unknown due to the batch culture technique. It is assumed that the cultures have exhausted the phosphate supply, but there is no assurance of this.

The EC50 values and the upper and lower 95% confidence intervals that were calculated from the growth rates (r) from the 1991 and 1993 experiments are summarized in Table 2.1. The corresponding EC50 values calculated from the day four cell density values are given in Table 2.2. Miller (1974) states that such unialgal batch culture bioassays can be used to define the effects of toxicants on the productivity of aquatic ecosystems. The basic principle of such tests is the development of an estimator of toxicity, based on the inhibition of the growth of the algae in the presence of the toxicant compared to growth responses in its absence. The EC50, or the median effective concentration that yields a growth response that is only 50% of the toxicant free control value, is often used as a way to compare the relative toxicity of different toxicants on one species, as well as to compare the relative sensitivities of several species to a single toxic substance.

A comparison of Tables 2.1 and 2.2 would tend to support the assertion of Miller and others (1978) that EC50 values should not be calculated from growth rate data. Table 2.1 shows an almost two order of magnitude range in the 95% confidence intervals on the EC50 estimates. Table 2.2, however, shows a very narrow range in the 95% confidence intervals for the EC50 estimates made from the day four cell density data. The estimates of EC50 taken from the day four cell density data tended to be lower then those estimates taken from the growth rate data. However, in both cases, the EC50 estimates were similar, and comparisons of EC50 values between species and between years showed similar patterns.

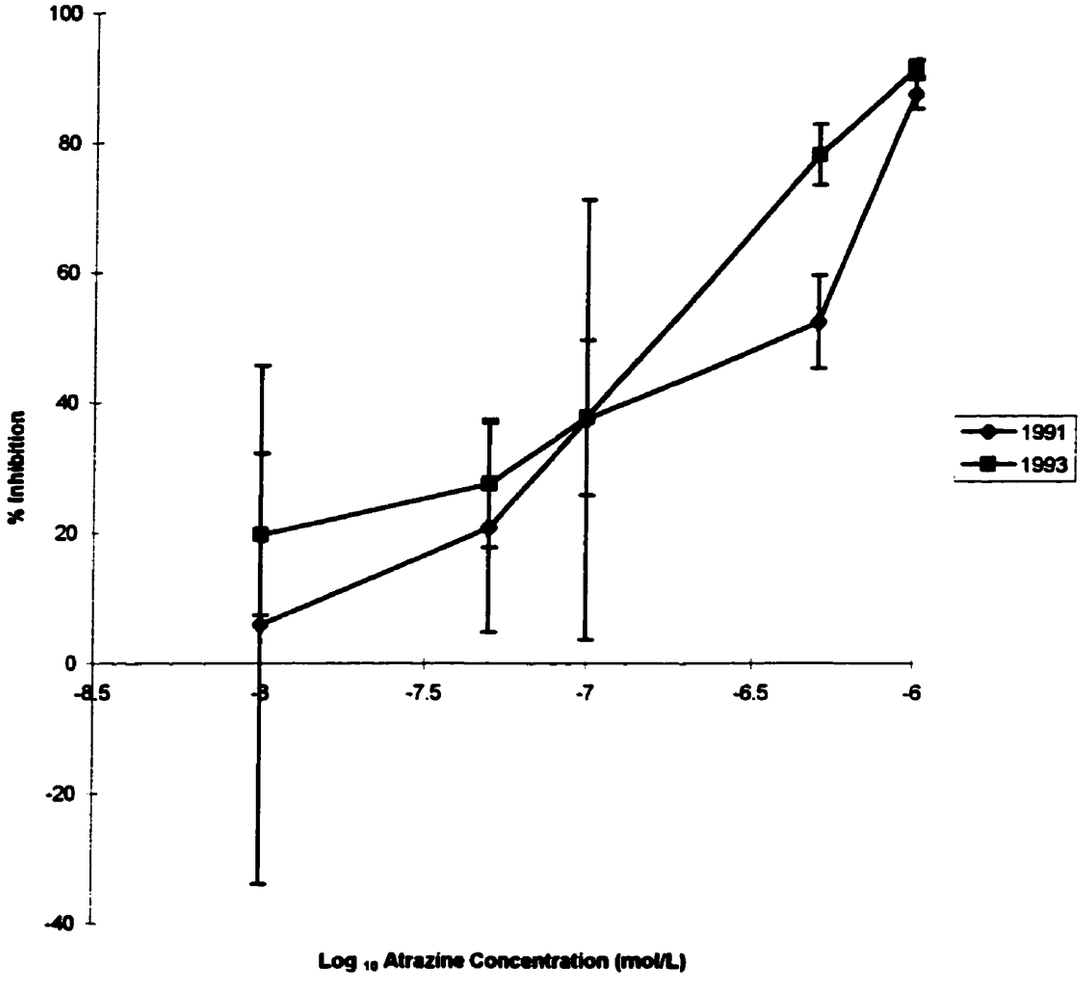


Figure 2.1 Batch culture dose responses of *Scenedesmus quadricauda* to Atrazine. The % inhibition was calculated from 96 hour cell density data. The error bars represent the standard deviation of the four replicate cultures.

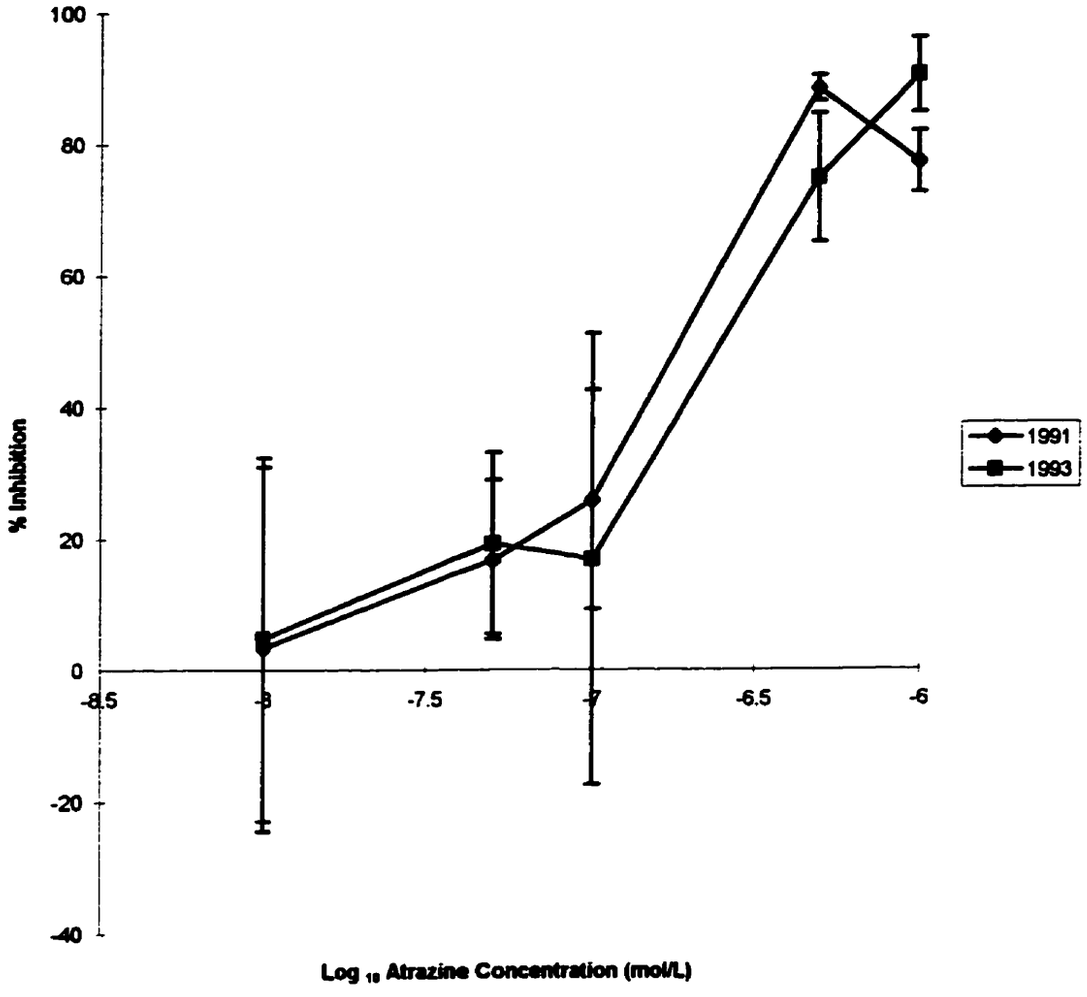


Figure 2.2 Batch culture dose responses of *Chlorella vulgaris* to Atrazine. The % inhibition was calculated from 96 hour cell density data. The error bars represent the standard deviation of the four replicate cultures.

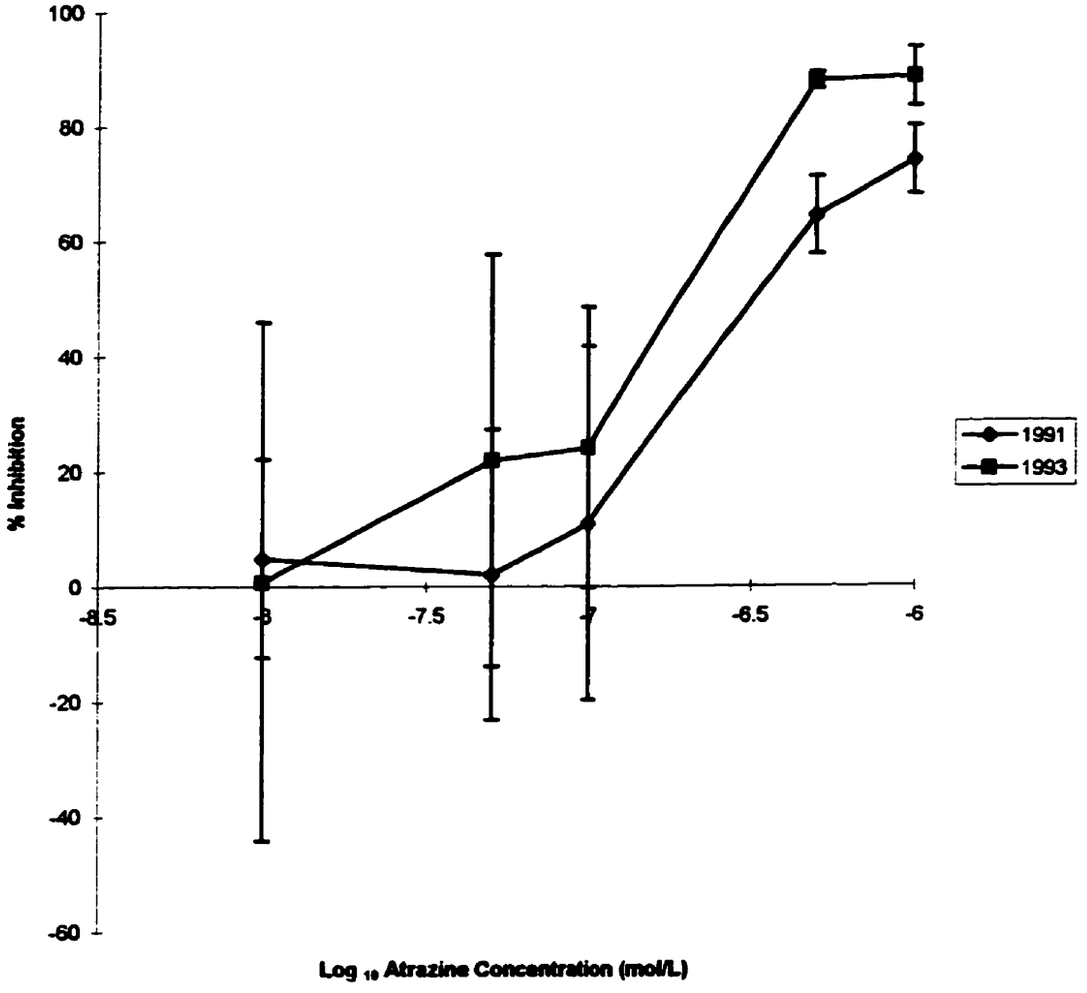


Figure 2.3 Batch culture dose responses of *Chlamydomonas musicola* to Atrazine. The % inhibition was calculated from 96 hour cell density data. The error bars represent the standard deviation of the four replicate cultures.

Table 2.1 EC50 values from the growth rate "r" analysis for the three phytoplankton species *Scenedesmus quadricauda*, *Chlorella vulgaris*, and *Chlamydomonas mucicola*.

Species	Year	EC50 (mol/L/L)	95% upper (mol/L/L)	95% lower (mol/L/L)
Chlorella vulgaris	1991	$3.99X10^{-7}$	$5.97X10^{-6}$	$2.68X10^{-8}$
Chlorella vulgaris	1993	$1.39X10^{-7}$	$9.49X10^{-7}$	$2.05X10^{-8}$
Chlamydomonas mucicola	1991	$4.38X10^{-7}$	$1.10X10^{-5}$	$1.62X10^{-8}$
Chlamydomonas mucicola	1993	$2.18X10^{-7}$	$5.27X10^{-6}$	$9.12X10^{-9}$
Scenedesmus quadricauda	1991	$3.37X10^{-7}$	$2.19X10^{-6}$	$5.20X10^{-8}$
Scenedesmus quadricauda	1993	$2.67X10^{-7}$	$1.39X10^{-6}$	$5.16X10^{-8}$

Table 2.2 EC50 values from the day four cell density analysis for the three phytoplankton species *Scenedesmus quadricauda*, *Chlorella vulgaris*, and *Chlamydomonas mucicola*.

Species	Year	EC50 (mol/L/L)	95% upper (mol/L/L)	95% lower (mol/L/L)
Chlorella vulgaris	1991	$1.80X10^{-7}$	$1.80X10^{-7}$	$1.80X10^{-7}$
Chlorella vulgaris	1993	$1.60X10^{-7}$	$1.60X10^{-7}$	$1.59X10^{-7}$
Chlamydomonas mucicola	1991	$3.90X10^{-7}$	$3.91X10^{-7}$	$3.90X10^{-7}$
Chlamydomonas mucicola	1993	$1.31X10^{-7}$	$1.31X10^{-7}$	$1.30X10^{-7}$
Scenedesmus quadricauda	1991	$2.05X10^{-7}$	$2.07X10^{-7}$	$2.06X10^{-7}$
Scenedesmus quadricauda	1993	$9.93X10^{-8}$	$9.95X10^{-8}$	$9.90X10^{-8}$

In the literature there is no one culture parameter that is consistently used to estimate the toxicity of a man-made chemical or "xenobiotic". Some of the parameters used to estimate the toxicity of atrazine include uptake rates for carbon, phosphate and ammonium (Brown and Lean 1995). Other physiological responses such as chlorophyll accumulation and degree of chlorophyll fluorescence have been used to illustrate toxic effects (Roy and Legendre 1979; Francois and Robinson 1990). The parameter most often used is growth rate, which can be expressed in several forms: doubling time (hours); doublings per day; specific growth rates (Hersh and Crumpton 1987, Mayasich and others 1987, Okay and Gaines 1996), or simply as changes in absorbance (Maule and Wright 1984). As we have seen from Tables 2.1 and 2.2, one should be very careful when making comparisons between such EC50 estimates throughout the literature since even with a consistent culture method, the accuracy of the estimate will vary greatly with the parameter used. Brown and Lean (1995), using carbon fixation rates, estimated the EC50 for atrazine in natural lake water samples to be $4.6X10^{-7}$ mol/L/L. Hersh and Crumpton (1987) did not calculate an EC50 as such, however they did report a 55% reduction in the growth of the *Chlorella sp.* they designated as /osa-2 at $1.0X10^{-7}$ mol/L/L and no growth at $1.0X10^{-6}$ mol/L/L atrazine. They also found an 8% increase in the growth of *Chlamydomonas reinhardtii* at $1.0X10^{-7}$ mol/L/L atrazine over the no herbicide control, and no growth at $1.0X10^{-6}$ mol/L/L. Francois and Robinson (1990) carried out an analysis of the radiant energy utilization of *Chlamydomonas geitleri*. They used chlorophyll Fluorescence Response Index (FRI) as an indirect measure of atrazine toxicity. From this analysis the estimated EC50 from triplicate experiments were found to be, $2.8X10^{-6}$,

$2.4X10^{-6}$, and $1.43X10^{-6}$ mol/L/L. Maule and Wright (1984) used a 25 X 5 mL compartment "Repli-dish" system and a turbidometrically measuring 96 hour cell densities to estimate an EC50 of $2.8X10^{-7}$ mol/L/L. atrazine for *Chlorella pyrenoidosa*. Kirby and Sheahan (1994) used 250 mL batch cultures to obtain an estimated the EC50 value for atrazine of $9.7X10^{-8}$ mol/L/L for *Scenedesmus subspicatus*. This value corresponds very closely to the EC50 of $9.9X10^{-8}$ mol/L/L found in this study for *S. quadricauda*.

The question of whether the algae would change their responses to the atrazine over the extended duration of the continuous culture experiments was addressed by performing two separate experiments, one in 1991 and the other in 1993. There are two possible ways in which responses may have changed over time. The species may have developed a resistance to the herbicide, and therefore would have shown a decrease in doubling time and an increase in day four cells/mL. Alternately the species may have developed greater susceptibility to the herbicide. This would be manifested in increased doubling times and decreased day four cells/mL. values. Two culture responses were measured, growth rate, calculated as doubling time (G) in hours, and the cell density (cells per milliliter) on day four of the experiment. For each herbicide concentration, the response of the test species in 1991 was compared to the response in 1993, under similar culture conditions. Comparisons were made using an Approximate Randomization Test, to test the null hypothesis that there was no difference between the responses of the algae to the herbicide in 1991 and 1993. The results of these comparisons are given in Table 2.3. For resistance there must have been a significant decrease in the doubling time and also a significant increase in the day four cells per milliliter values.

Table 2.3. Comparison of 1991 and 1993 herbicide dose responses for *Scenedesmus quadricauda*, *Chlorella vulgaris*, and *Chlamydomonas musicola*. A positive sign + indicates a significant increase in the response in 1993 compared to 1991, while a negative sign - indicates a significant decrease in that response in 1993 compared to 1991. NS indicates no significant change in that response.

Species	Atrazine Concentration (mol/L/L)	Response Doubling Time (G) hours	Response Day Four Cell Density (cells/mL)
Scenedesmus quadricauda	0	NS	NS
	$1.0X10^{-8}$	-	-
	$5.0X10^{-8}$	-	NS
	$1.0X10^{-7}$	NS	NS
	$5.0X10^{-7}$	NS	NS
	$1.0X10^{-6}$	NS	-
Chlamydomonas mucicola	0	NS	NS
	$1.0X10^{-8}$	NS	NS
	$5.0X10^{-8}$	NS	NS
	$1.0X10^{-7}$	-	NS
	$5.0X10^{-7}$	NS	-
	$1.0X10^{-6}$	NS	-
Chlorella vulgaris	0	NS	-
	$1.0X10^{-8}$	NS	-
	$5.0X10^{-8}$	NS	-
	$1.0X10^{-7}$	+	NS
	$5.0X10^{-7}$	NS	+
	$1.0X10^{-6}$	NS	-

Several interesting observations arise from Table 2.3. At an atrazine concentration of $1.0X10^{-8}$ mol/L/L, *Scenedesmus quadricauda* appears to decrease its doubling time, that is grow faster, indicating an increase in resistance. However it also decreased the day four cells/mL. level, which indicates a greater susceptibility. It is difficult to rationalize the apparently contradictory responses. One explanation could be that no effort was made to ensure that the inoculum culture cells/mL levels used in 1991 were the same as those used to inoculate the cultures in 1993. If the cells per milliliter of the inoculum was lower in 1993, the cultures could very well have had a doubling time decrease, but simply not have

had sufficient time to reach the same day four cell densities as were seen in 1991. This is likely the explanation for the other responses which show no significant difference in doubling times, but do show a decrease in day four cell densities. For *Chlorella vulgaris* at $1.0X10^{-7}$ mol/L/L atrazine, an inoculum of a slightly higher cell density in 1993 could explain how an increase in doubling time could occur and yet the cultures were still able to attain the same day four cell density as in 1991. At a doubling time of 9.38 hours in a WC' growth medium with no herbicide, *Chlorella vulgaris* would have gone through approximately 2000 generations in the batch stock cultures in the time between these two test experiments. Under these same conditions both, *Scenedesmus quadracuada* and *Chlamydomonas mucicola* would have under gone through 1000 generations. It is conceivable that in that number of generations genetic drift could have resulted in the development of atrazine resistance. I feel confident from the results of these experiments that this was not the case for any of these test species.

An examination of either Table 2.1 or 2.2, show that my EC50 estimates are either equivalent or below those described in the studies of Francois and Robinson (1990) and Maule and Wright (1984). It is also evident from my results that there is a distinct difference in the susceptibility of each of our test species to atrazine. These test species were isolated from the same water sample and from the examination of other water samples taken from southern Manitoba lakes, it appears that they often occur together in natural waters. Such variations in their sensitivities to atrazine could produce dramatic changes in community structure during prolonged exposure. This assertion has no direct supporting evidence from these unialgal, batch culture experiments, but as with many

things in ecology, there is a solid logic in such a statement. Unfortunately, many policy and decision makers either cannot see such logic, or because of other social and economic demands, must have more evidence. These types of unialgal batch culture bioassays are important first steps in understanding the organismal level responses to the presence of herbicides. However, questions on a larger ecological scale require a more complex type of test. In the next chapter of this thesis I will begin to develop a bioassay system that will attempt to answer community level questions.

Chapter 3

The effect of Atrazine on the growth responses of three green algae grown in semicontinuous culture.

A. Abstract

An unialgal semicontinuous culture method was used to estimate the EC50 values for three species of green algae, *Scenedesmus quadricauda*, *Chlamydomonas musicola*, and *Chlorella vulgaris*, to the s-triazine herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine). Each species was exposed to several individual concentrations of atrazine and allowed to come to steady state. The EC50 estimates made using a probit analysis of the maximum steady state cell densities achieved during day 5 through day 10 of the experiment, were found to be: *Scenedesmus quadricauda* ($5.59X10^{-7}$ mol/L), *Chlamydomonas musicola* ($1.51X10^{-7}$ mol), and *Chlorella vulgaris* ($5.68X10^{-7}$ mol/L). Each of these EC50 estimates exceeds those values estimated for the same species using a batch culture technique (ch.2). This may indicate that due to confounding effects, EC50 estimates made using batch culture methods could overestimate the toxicity of a compound. The steady state system also produced an interesting stimulatory effect in *Scenedesmus quadricauda* at $5.0X10^{-8}$ mol/L atrazine and in *Chlorella vulgaris* at $1.0X10^{-8}$, $5.0X10^{-8}$ and $1.0X10^{-7}$ mol/L.

B. Introduction

The exposure of aquatic ecosystems to toxic xenobiotic compounds has been steadily increasing over the last decade. In response to this exposure, environmental authorities have requested toxicity tests which test the inhibition of growth and photosynthesis of microalgae as part of effluent control schemes (Kusk and Nyholm 1991).

Although the culture technique of choice in most algal bioassay protocols is the batch culture, the culture environment to which the cells are exposed in these systems is constantly changing. As the algae grow, they consume nutrients from the medium, produce oxygen and other metabolic wastes. Furthermore, cell density increases the cells begin to shade each other, decreasing the amount of light each cell receives. Several authors have documented problems with batch culture methods that could interfere with their use in studying the effects of xenobiotics on population level responses. Nielsen (1978) discussed the problems that can arise when batch culture techniques are used to evaluate the effects of toxicants on growth rate. Nielsen suggests that the maximum growth rate found during the logarithmic growth phase may not represent the real growth rate in toxicological bioassays where the toxicant is present in the medium when the algae are inoculated. Nielsen (1991) recommended that the inoculum used must undergo logarithmic growth from the very beginning of the experiment. The inoculum culture must be in logarithmic phase, and also be growing under exactly the same conditions as the experimental culture. Stratton and

Giles (1990) have noted that there is a lack of uniformity between results obtained from batch culture assays performed in different laboratories. They suggest that this may be due to the lack of standardization in the bioassay conditions, particularly the choice of total test volume and the number of test subjects (cell density). These two factors determine the amount of toxicant to which each cell is exposed at any given toxicant concentration. This in turn could have a profound influence on estimated EC50 values. Trainor (1984) suggests continuous culture methods could yield more useful data than these using batch culture techniques, since the growth conditions would more closely mimic nature.

In recent years, more and more researchers have carried out experiments using the continuous culture technique. Continuous cultures differ from batch cultures in that, as the algae grow, fresh medium is added while an equal volume of culture (medium plus organisms) is removed (Fogg and Thake 1987). A "chemostat' is a continuous culture system in which the concentration of one nutrient in the inflowing medium is limiting to growth. Kubitschek (1970) describes some of the advantages of continuous culture systems over batch culture techniques. These include:

1. Rates of growth and division are more easily controlled and maintained for long periods of time.
2. Cell concentrations can be set and maintained independently of growth rate.
3. Cells can be grown for longer periods in a constant chemical environment.
4. Very low levels of critical nutrients, growth factors, mutagens, or toxic agents can be maintained during the growth of the culture.
5. Cell size and biochemical composition are more easily selected and maintained with a given strain, because these cell characteristics depend upon the rate of growth.

Many of these advantages have to do with the degree of control the technique gives to the researcher. Fogg and Thake (1987) point out that one of the most important advantages of the continuous culture system is that the growth rate of an organism in a chemostat is determined solely by the medium dilution rate, and that the cell concentration is determined by the concentration of the limiting nutrient in the inflow medium. By including a toxic substance in the fresh medium, the continuous culture system can be used to determine the effects on cell density of different concentrations of toxicant at a constant dilution rate.

The simplest form of the continuous culture system involves the manual rather than automatic addition of fresh medium, and the manual removal of an equal volume of 'old' culture. This type of system is more accurately termed a semicontinuous culture. They are relatively cheap and easy to set up and maintain, and have been used in a wide range of research with phytoplankton and photosynthetic bacteria (Fogg and Thake 1987).

An important activity of scientific research in the field of ecotoxicology is to investigate how one bioassay technique relates to others in terms of sensitivity and reliability of the responses, through intercalibration exercises (Calow 1989). This chapter will investigate the use of an unialgal continuous culture system to examine the growth responses of three phytoplankton species (*Scenedesmus quadricauda*, *Chlorella vulgaris*, and *Chlamydomonas* musicola) to the herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine). This study will also serve to set baseline responses of these test algae to the differing atrazine concentrations in unialgal

cultures. The next phase in this thesis will be to examine the competitive responses of the algal species to differing atrazine concentrations. I have used a semicontinuous culture system to determine the steady state cell densities of the three test algae exposed to different concentrations of the herbicide. These growth responses will be used to estimate EC50 values for the herbicide. These values will then be compared to those estimated from the batch culture experiments in the previous chapter.

C. Materials and Methods

The three phytoplankton species tested in this experiment were *Scenedesmus quadricauda*, *Chlorella vulgaris*, and *Chlamydomonas musicola*. These species were isolated by the author from water samples collected on May 28, 1989, from Delta Marsh, on the southern end of Lake Manitoba, Canada (99°19'W, 50°7'N). Stock algal cultures were maintained in WC' medium (Healy and Hendzel 1979), as described in the last chapter. Stock cultures were subcultured three days prior to the start of a continuous culture experiment to allow inoculum batch cultures to reach log phase. Each unialgal semicontinuous culture experiment began with six, 500 mL culture flasks. Four of the flasks contained atrazine at the concentration being tested, and two flasks served as the herbicide free controls. The flasks were incubated under a constant illumination of 70 to 75 $\mu Em^{-2}\ s^{-1}$ from four General Electric Cool White fluorescent lamps. Temperature was kept constant at 19C°. Each flask was aerated with humidified filtered air, to prevent CO_2 limitation and to keep the algal culture constantly suspended. Fresh medium equivalent to 20% of the culture volume was

delivered in one dose at the same time each day for the duration of the experiment. Sterile fresh medium was stored in 10 L carboys, which were connected to the inflow ports of the culture flasks. Figure 1.1 illustrates the components of the semicontinuous culture apparatus. For each test species, the unialgal semicontinuous culture herbicide-free control data were derived from a run consisting of six replicate cultures. Data for the various herbicide levels were derived from the unialgal cultures, which were run as controls for the two-species competition experiments (see chapter 4). These competition experiments consisted of six cultures, four containing the two competing species, and two other cultures each containing one member of the competing pair. As a result, the herbicide dose response data for this unialgal continuous culture study were derived from four cultures for each algal species. Even though these cultures were grown under the same conditions, they could not be considered true replicates since they were performed at different times.

Each algal species was tested for its responses to the following concentrations of atrazine; $1.0X10^{-8}$ mol/L; $5.0X10^{-8}$ mol/L; $1.0X10^{-7}$ mol/L; $5.0X10^{-7}$ mol/L; $8.2X10^{-7}$ mol/L; and $1.0X10^{-6}$ mol/L. The required volume of $1.0X10^{-4}$ mol/L stock atrazine solution was added to experimental fresh medium carboys in order to establish the above herbicide concentrations. Ten milliliters of the stock algae were inoculated into each semicontinuous culture vessel through the sterile inoculation port on day zero of the experiment. Each experimental run lasted an average of 10 days.

Each day of the experiment a 5 mL sample was taken from each culture and preserved with Lugol's iodine for cell density determination. Cell density estimates

(cells per milliliter) were made using a Reichert-Jung, Bright-line Improved Neubauer Hemocytometer. The specific growth constant "r"produced by a 20% dilution rate for each test species as determined each day from the dilution rate and the daily cell density estimates using the following formula from deKruijk (1988).

$$r = \frac{\ln\left(\frac{N_0}{N_1}\right)}{t_0 - t_1}$$

where r equalls the specific growth constant, N_0 is the cell density in cells/mL. at time t_0, and N_1 is the cell density in cells/mL. at time t_1. This specific growth rate will serve as the unialgal growth rate (20% dilution rate) estimate for each species under each herbicide concentration.

For each algal species, the maximum steady state cell density (MSD) produced under each herbicide concentration was determined from the average cell densities on days 5 through 10. Using these MSD values, EC50 estimates were made from the % inhibition vs. herbicide concentration results from weighted PROBIT analysis as described in Chapter 2 and Hubert (1984). The % inhibition estimates were determined using the following formula from deKruijk (1988),

$$\textbf{\% inhibition} = (1\text{-}C_1/C_2) \times 100$$

where C_1 equals the MSD in the presence of herbicide and C_2 equals the MSD of the herbicide free control.

D. Results and Discussion

In these semicontinuous culture experiments, the algal species were inoculated into the culture vessels and the 20% dilution rate was begun the next day. The algae were exposed to the herbicide from the beginning of the experiment. This system would best be described as a chronic exposure system, since the herbicide level is maintained at a constant level for the duration of the experiment. The initial inoculum produced a cell density in the culture of approximately $1.0X10^4$ to $5.0X10^4$ cells/mL on day zero. This initial cell density is so low that, for the first four to five days of the experiment, the cells experienced no phosphate limitation, and therefore the algal population grows as if in log phase of a batch culture. By day five the cell density had increased to a point where the phosphate limitation slowed their rate of growth until it was equal to the dilution rate. At this point the culture was said to be in "steady state". Kubitschek (1970) describes a culture as being in a steady state of growth and division when the average value of every individual cell property remains constant. As a result, one can assume that in a culture in steady state, the average cell values of RNA, DNA, protein, mass volume, generation time and all other structural and functional characteristics of the cell remain constant. As a result of this definition we can determine that a culture has achieved steady state when the cell density, at a given dilution rate, remains constant from day to day, or when growth rate "r" is equal to the dilution rate.

Each of the species-herbicide concentration experiments was analyzed to determine that the cultures were indeed in steady state from day 5 to the end of the run. This analysis consisted of comparing the mean dilution rates (day 5 to end) and the calculated mean growth rate "r" using an approximate randomization method. The mean dilution rate and mean growth rates were found to be different in only two of the 21 species-herbicide concentration experiments. These were the herbicide free controls for *Chlamydomonas musicola* and *Chlorella vulgaris*. In order to be certain that the responses we were observing were those of a population in steady state, data were only analyzed from days five through to the end of the experiment.

It is important to realize that such a steady state condition may not be any more ecologically realistic than the log phase of a batch culture. However such a steady state condition, which can be maintained for extended periods of time, does give the experimenter more control over the culture parameters than does the batch culture method. This semicontinuous culture method is ideally suited for investigating the effects of long term chronic exposures.

In such semicontinuous cultures, if the growth rate of the culture were to drop below that of the dilution rate, due to the toxicity of some xenobiotic incorporated into the medium, more cells would be removed each day than would be produced by cell division. In this case the cell density would decline over time and eventually the culture would "washout". It was interesting to note that under no atrazine concentration tested in these experiments did any of the cultures show any trends

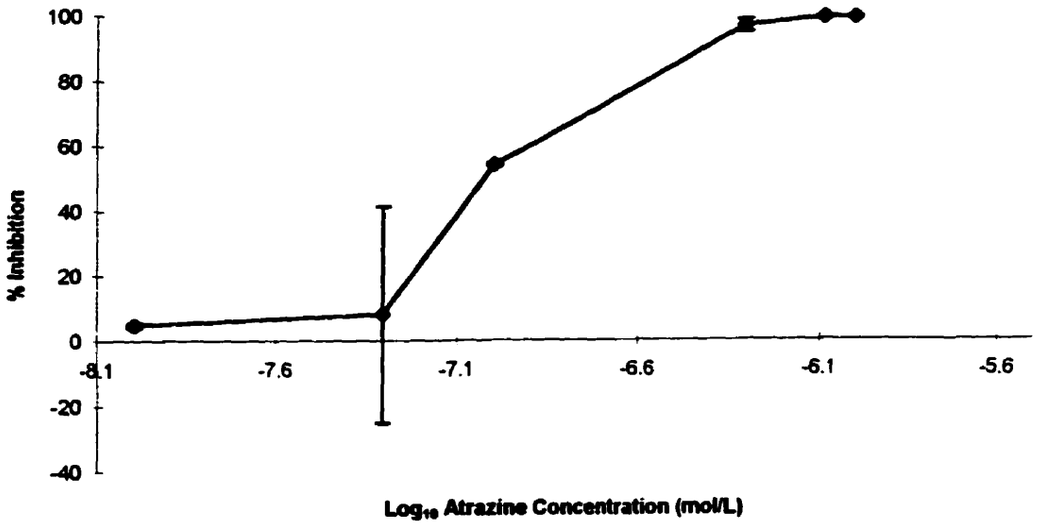


Figure 3.1 The growth response of *Chlamydomonas musicola* to different concentrations of atrazine, expressed as % inhibition. Error bars represent one standard deviation about the mean.

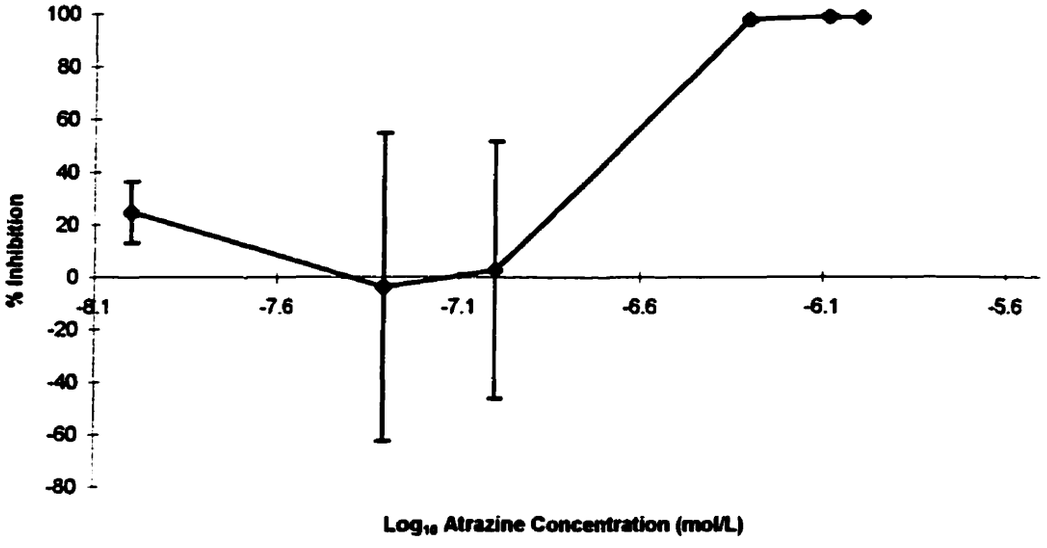


Figure 3.2 The growth response of *Scenedesmus quadricauda* to different concentrations of atrazine, expressed as % inhibition. Error bars represent one standard deviation about the mean.

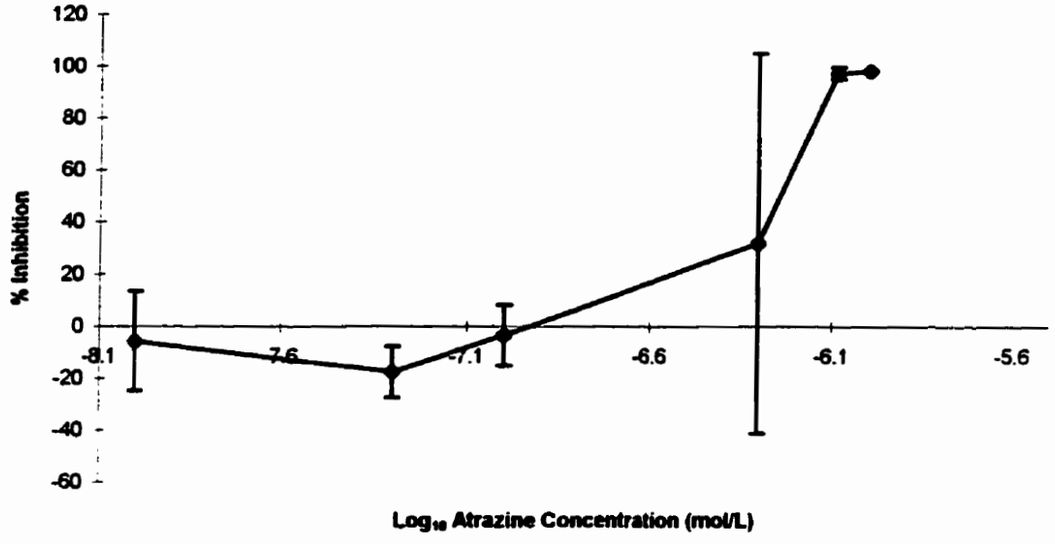


Figure 3.3 The growth response of *Chlorella vulgaris* to different concentrations of atrazine, expressed as % inhibition. Error bars represent one standard deviation about the mean.

towards washing out. Even at the highest concentration of $1.0X10^{-6}$ mol/L the cultures reached a new stable steady state growth rate.

As one would expect, there was a distinct decline in the steady state cell density with increasing herbicide concentration. This trend can be seen in Figures 3.1, 3.2, and 3.3. In order to determine at which herbicide concentration the growth responses of test algae changed significantly from the response seen in the no herbicide control culture, the MSD values for each herbicide concentration were compared to those of the herbicide free controls. This comparison was made using the approximate randomization method described in Chapter 2. The results of this analysis are presented in Table 3.1. As before, the null hypothesis in this test was that there was no difference in the means of the two samples (herbicide free control vs. herbicide response), and that the p value represents the probability of obtaining a difference between the two means that is greater than the observed difference between the two means by random chance alone. In order to avoid being characterized as one of "that group of people whose aim in life is to be wrong 5% of the time" (Manly 1991), I regarded the p values generated by this approximate randomization test as measures of the strength of evidence against the null hypothesis rather than showing whether the data are significant or not at an arbitrarily predetermined level. There appears to be a similar response pattern to the different concentrations of atrazine in these semicontinuous culture experiments as there was in the batch culture experiments. The lowest concentrations produce no detectable effect, however after a certain threshold level is reached the MSD significantly decreases.

Table 3.1 Results of the approximate randomization test of significance of the maximum steady state cell density (MSD) responses (herbicide free control vs. atrazine concentration), for *Scenedesmus quadricauda*, *Chlamydomonas musicola*, and *Chlorella vulgaris*. NS indicates no significant change in the response, while S indicates there was a significant change in the response when compared to the zero herbicide control.

Species	Atrazine concentration (mol/L)	P values	Significance
Chlamydomonas musicola	$1.0X10^{-8}$	0.928	NS
	$5.0X10^{-8}$	0.7188	NS
	$1.0X10^{-7}$	0.671	NS
	$5.0X10^{-7}$	0.0053	S
	$8.2X10^{-7}$	0.053	S
	$1.0X10^{-6}$	0.0133	S
Scenedesmus quadricauda	$1.0X10^{-8}$	0.1403	NS
	$5.0X10^{-8}$	0.9017	NS
	$1.0X10^{-7}$	0.9900	NS
	$5.0X10^{-7}$	0.048	S
	$8.2X10^{-7}$	0.007	S
	$1.0X10^{-6}$	0.0468	S
Chlorella vulgaris	$1.0X10^{-8}$	0.8068	NS
	$5.0X10^{-8}$	0.342	NS
	$1.0X10^{-7}$	0.8555	NS
	$5.0X10^{-7}$	0.4288	NS
	$8.2X10^{-7}$	0.0078	S
	$1.0X10^{-6}$	0.0496	S

Although the results shown in Table 3.1 indicate there was no significant difference in the measured response of these algae to the very low concentrations of atrazine, the % inhibition analysis depicted Figures 3.1-3.3 does indicate an interesting response. In the *Scenedesmus quadricauda* $5.0X10^{-8}$ mol/L culture and in the *Chlorella vulgaris* $1.0X10^{-8}$, $5.0X10^{-8}$, and $1.0X10^{-7}$ mol/L cultures there was an increase in the MSD levels attained by these cultures as compared to the herbicide-free control. Even though the observed increase in MSD was not statistically significant,

this could indicate that the herbicide, at these very low levels had a stimulatory effect on these two species. This researcher has observed a similar response to very low herbicide concentrations on numerous occasions. Although it was beyond the scope of this study it would be very interesting to repeat these unialgal semicontinuous culture experiments with sufficient replication in order to more accurately determine the extent of this response. If this is a significant response, it could have profound implications of the population dynamics of natural algal assemblages. A compound which produces a selective stimulatory effect has just as much potential to alter the community structure as a selectively toxic substance.

The growth responses of these unialgal semicontinuous cultures to different atrazine concentrations were also used to estimate the EC50 values of atrazine for these three algal species (Table 3.2).

Table 3.2. Atrazine EC50 estimates and 95% confidence intervals for three green algae, determined by a weighted PROBIT analysis of maximum steady state cell density (MSD) data derived from semicontinuous culture dose response experiments.

Species	Atrazine EC50 (mol/L)	Lower 95% confidence interval	Upper 95% confidence interval
Chlamydomonas musicola	$1.51X10^{-7}$	$1.51X10^{-7}$	$1.51X10^{-7}$
Scenedesmus quadricauda	$5.95X10^{-7}$	$5.95X10^{-7}$	$5.95X10^{-7}$
Chlorella vulgaris	$5.68X10^{-7}$	$5.68X10^{-7}$	$5.68X10^{-7}$

If one compares these EC50 values to those estimated from the 1993 day four (96 hour) cell density batch culture experiments (Chapter 2) one sees that for each species the semicontinuous culture EC50 estimates are all higher than those estimated from the batch culture method. This would indicate that atrazine is less toxic to each

species in semicontinuous culture than to the same species in batch culture. I would submit that the semicontinuous culture EC50 estimates are the more accurate estimation of the toxicity of atrazine to these phytoplankton species. One reason for this assertion is what I term "per capita exposure rate". When one compares the 1993, day four cell density from the batch culture experiments with the mean maximum steady state cell density values from the semicontinuous experiments, in only 4 out of 21 experiments did the 1993 day four batch culture cell density values exceed the MSD values for that species-herbicide concentration experiment. Therefore, in most cases the semicontinuous culture method produced a higher cell density. In a culture of a higher cell density, each cell is exposed to less herbicide than would be the case of a lower cell density at the same herbicide concentration. That is, in the semicontinuous culture system, there is a lower per capita exposure rate. Batch cultures, because of the rapid depletion of resources and the build up of waste products, could yield EC50 estimates that overestimate the toxicity of the herbicide due to an increased per capita exposure rate, and to a generally weakened physiological state of the cells in stationary phase.

From these results, I would recommend that estimates of toxicity of xenobiotics on phytoplankton species would best be made from experiments employing a semicontinuous culture or continuous culture methods.

Chapter 4

The effects of Atrazine on the competitive interactions of three green algae.

A. Abstract

In this study, the effects of the herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) on the competitive interactions of *Chlamydomonas musicola*, *Chlorella vulgaris* and *Scenedesmus quadricauda* were examined. The test species were grown in pairs, in semicontinuous culture under phosphate limitation, and exposed to various levels of the herbicide. The changes in the competition coefficients were compared to the competitive abilities under no herbicide stress. In all cases the paired species systems showed a threshold response. For atrazine exposures between $1.0X10^{-8}$ and $5.0X10^{-7}$ mol/L, changes in the coefficients of competition ($\alpha_{1,2}$ and $\alpha_{2,1}$) were much the same, within each pair of species tested. However at atrazine exposures above $5.0X10^{-7}$ moles, response patterns shifted to a distinctly different equilibrium. Each species tested showed a different pattern of response depending on the other species in the pairing. It was shown that not only are there species-specific responses to xenobiotic toxicity, but system specific responses as well.

B. Introduction

The ability to predict the environmental fate of chemicals based on their physical properties is relatively well developed and widely practiced. The ability to predict the ecological impacts of these chemicals is much less well developed. Much effort has been expended in the development of mathematical models to predict such ecological impacts. These models are usually comprised of two parts, an ecological component and a toxicological component. The ecological components of these models are arranged into organismal, population and community levels of organization. At each of these levels there are different types of problems and questions which need to be answered, in order to set limits of exposure and regulations for the use of these chemicals. Many of the testing protocols are directed at the organismal level of organization, and yield information of the physiological effects of the toxin in question. Much less frequently, tests are designed to investigate community level effects. This should not be a surprising situation, given that there is precious little understanding of the underlying ecological theory on how processes such as competition and predation influence the structure of natural communities. The problems involved in ecological effects assessment are substantial. Community responses to xenobiotic exposure include direct lethal effects on any of the very large number of biotic species present, and any indirect, nonlethal effects brought about by alteration of characteristics of the interacting biotic species. As Lassiter (1986) points out, the ideal model for the analysis of ecological effects would possess the capability to predict responses at all these levels. We are obviously quite far from this ideal.

This study I hope to add to our understanding of how xenobiotics may affect community level processes by examining how the herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) affects the interspecific competition patterns between three species of phytoplankton, *Chlamydomonas musicola*, *Scenedesmus quadricauda*, and *Chlorella vulgaris*.

The principal approach to determining the effects of a toxic compound on an organism has been the bioassay. It is important to understand the limitations of the various bioassay methods that have been developed in the past few years. Robinson (1989) stressed the importance of precise and accurate standardized bioassment methods in providing input for making decisions that would minimize the impact of contaminant loadings to our freshwater resources. Calow (1989) describes the two principal functions of such environmental bioassays. They can be used predictively, to forecast the impact of a substance on an ecosystem prior to its release, or as a monitor of actual effects in nature. When used to predict the impact of a toxin on the environment, a perturbation is applied to the system and a response noted that should be interpreted relative to its environmental implications. When such bioassays are used as monitors, characteristics of the ecosystem are used to estimate the level of perturbation compared to an undisturbed reference site. Monitoring alone assumes that an addition will occur, and that if the resulting perturbation is deemed unacceptable some remedial action would then be taken. The problem with this approach is that the "damage is done" and as is the case with many compounds, once they are into a system they continue to affect that system for many years. With respect to the aquatic ecosystem, Stephan (1986) proposed that the goal of applied aquatic toxicology

should be to make a useful prediction concerning whether or not the addition of a toxic agent to a particular aquatic ecosystem will cause any unacceptable effect on that ecosystem.

In the early 1970's, a group of researchers from government, industry and academia joined forces to ascertain if algal growth in water samples could be used to gain an understanding of the chemistry of a body of water and therefore its carrying capacity. The basic idea was to determine if a series of assays using photosynthetic microorganisms could produce more information concerning the pollutants which were then known to be serious and troublesome additions to our lakes and streams. From these discussions and studies, it was determined that the single species algal bioassay (often referred to as the provisional algal assay procedure (PAAP)) was to become the standard test protocol, and *Selenastrum capricornutum* was to become the national (U.S.) test organism (Trainor 1984). There was a growing group of researchers who felt that continuous culture systems could yield more ecologically relevant data on the impacts of toxic substances on phytoplankton eg. Orcutt and others 1983, Trainor 1984, Rhee 1989, Burnett and Liss 1990 , Marsot and others 1995. Single-species continuous cultures had been in use, and had yielded extensive information on microorganism physiology. Much of our understanding of the effects of nutrient deficiencies, temperature, and light on the cell composition, physiological processes, and growth of phytoplankton has come from the use of this very powerful culture technique. Veldkamp (1976, 1977) provides a good review of these studies and techniques.

At the other end of the bioassay spectrum, Round (1991) and Sgro and Johansen (1995) have reviewed many papers written on protocols for using the structure, function and composition of natural associations of algae for monitoring water quality. The use of the taxonomic composition of natural assemblages as assessments of water quality has proved difficult. Algal information does not allow for easy unambiguous interpretation. Algae respond to a wide variety of environmental factors, including nutrient levels, substrate type, geomorphology, grazing, current velocity, photoperiod. Toxic contaminants all work in concert to influence community structure. Recognizing that laboratory single-species tests and field experiments on natural phytoplankton communities both have their drawbacks, many researchers sought a compromise system. The problem is that algal communities are too important to be ignored and yet too complex to be understood within the complete aquatic ecosystem. Addition of water samples containing natural assemblages to a laboratory culture system allows for greater control over the physical parameters and often a greater number of replicates, but this usually results in the loss of some portion of the natural assemblage. Taub (1984) suggests that 'transplanted" portions of natural systems may still show many of the same ecological properties as the natural community. An alternate approach is to isolate certain species from the natural community, culture them in separate unialgal cultures, and then combine selected species and study the behavior of the resultant system. Taub (1984) describes these simplified systems as "synthetic microcosms".

In this study I have taken a similar approach to synthetic microcosms. The goal was to develop a non-site specific synthetic microcosm protocol that could be used to

evaluate the effects of the herbicide atrazine on the competitive interactions of various two-species communities. This approach has the advantage that the same assemblage could be synthesized at a later date.

Walsh and Merrill (1984) review some of the potential benefits of the use of continuous culture systems in the testing of toxic substances. They point out that continuous culture methods can be used to identify sublethal effects of toxicants in long-term tests. Waltman and others (1980) provides a good review of the use of continuous cultures in experimental investigations of microbial competition. Competition has long been recognized as an important influence on the composition of natural communities, and much work has been done to understand how various environmental variables affect the abilities of algal species to compete for limiting resources. However, there has been very little work done on the direct effects of xenobiotics on competitive interactions of phytoplankton. In one study, Grochwski and Trainor (1987) demonstrated how a field phenomenon such as competitive exclusion may be better understood once the factors affecting the system have been determined by laboratory analysis using both batch and semicontinuous cultures. In that study they examined effects of a complex sewage effluent and changes in pH (due to different substrates) on the interspecific competition between *Ankistrodesmus falcatus* and *Chlorella regularis*. The authors simply noted the relative abundance's of the competing species on different substrates; no attempt was made to quantify coefficients of competition.

In a second study, Fisher and others (1974) investigated the effects of PCB's on interspecific competition between the diatom *Thalassiosira pseudonana* (formerly

Cyclotella nana) and *Dunaliella tertiolecta.* In this study, they carried out competitive runs in both batch and semicontinuous cultures, in order to evaluate the usefulness of each technique for the study of competitive interactions. They also used a complex multispecies natural assemblage grown in semicontinuous culture and exposed to PCB's to evaluate the ability of *T. pseudonana* to compete against a larger complex assemblage.

In this study I will attempt to ascertain all of the parameters necessary to construct a model of the effect of the herbicide atrazine on the pairwise interspecific competitive interactions between the three green algal species *Chlamydomonas musicola*, *Scenedesmus quadricauda*, and *Chlorella vulgaris*. Using the results of the atrazine single-species semicontinuous culture algal bioassay tests (chapter 4) as "no competition" controls, it should be possible to determine the effects of atrazine on competition. The development of this model requires three phases.

Phase 1. A method for estimating the competitive interactions of two algal species growing in continuous culture. This estimate must be derived from cell density data only.

Phase 2. Once the degree to which one species affects the growth of the other has been estimated, estimates will be made on how the presence of the herbicide atrazine changes the pattern of interspecific competition between the test species.

Phase 3. Once the relationship between ambient herbicide concentration and competitive ability has been established, the ambient herbicide

concentration will be included into the equations which describe the growth of the two algal species in a competitive situation.

Competition Theory

Before an effective experimental design could be developed, it was necessary to review the current theory on competition in order to determine which theory and model would be best used to generate a workable competitive bioassay. Practical considerations of time, cost and labor requirements are important if such a competitive bioassay is to become widely accepted.

The classic model of competition was developed independently by Lotka and Volterra in the 1920s. These simple mathematical models, based on the logistic growth equation, include three components to describe the rate of change of the population over time (dN/dt). For a two-species system the behavior of the two populations can be described by the following equations;

$$\frac{dN_1}{dt} = r_1 N_1 \left(1 - \frac{N_1}{K_1} - \frac{\alpha_{2,1} N_2}{K_1} \right)$$

$$\frac{dN_2}{dt} = r_2 N_2 \left(1 - \frac{N_2}{K_2} - \frac{\alpha_{1,2} N_1}{K_2} \right)$$

The first component of this model, rN, describes the unrestricted growth potential of the population in the absence of competition from other members of the same species. The second component -N/K embodies the damping effects of competition from other individuals of the same species (intraspecific) on the potential growth rate of the

population. This term includes the important ecological concept of carrying capacity (K). Most of the variation in a populations density is related to the availability of resources. Carrying capacity of the environment is the number of individuals of a species that can be sustained by the available resources. Because all organisms consume resources, consumer-resource interactions embodied by the concept of carrying capacity are often considered to be of central importance in ecology. The logistic equation can be modified to incorporate the effects of a second species on the carrying capacity of the first, that is interspecific competition. In its simplest form the term $(\alpha_{2,1}N_2)/K_1$ is the product of the population density of the competing species 2 (N_2) and a proportionality constant ($\alpha_{2,1}$) which relates the degree to which species 2 reduces the carrying capacity of species 1 (K_1) by either exploitation or interference competition. In a two-species system there would be a corresponding term ($\alpha_{1,2}$) which relates the degree to which species 1 reduces the carrying capacity of species 2 (K_2). It should be remembered that these α values indicate the sum total of the effects of one species on all of the resources used by the other species. This fact has made the measurement of the α values in nature very difficult, if not impossible. In the absence of any competition between the two species, $\alpha_{2,1} = \alpha_{1,2} = 0$ and each species would growth until it reached its' carrying capacity determined population density, as defined by the limiting resource. If the competition coefficients are positive values, then in the two species case there are 4 possible outcomes of the competition. Coexistence can occur with either of the species having a numerical advantage, or competitive exclusion can occur for one or the other species. Classical wisdom would suggest that a particular outcome depends only on the competition coefficients and the

carrying capacity of each species. Coexistence of the two competitors can occur if $\alpha_{2,1} < K_1/K_2$ and if $\alpha_{1,2} < K_2/K_1$. Competitive instability (the competitive exclusion of one of the species) occurs when both of these inequalities are reversed.

Many authors have described the Lotka-Volterra equations as "phenomenological". That is, the model gives us no insight into how the two species interact, only to what degree they influence each others growth (Tilman 1982, 1988, Hu and Zhang 1993). In an attempt to understand the mechanisms of competition, Tilman (1982) has developed a model that has been termed "Resource Competition Theory". This model includes variables for both consumers and resources explicitly, and therefore it incorporates some of the mechanisms by which species reduce each other's population growth rates. The principle of the theory is that one species reduces the carrying capacity of its competitors solely by reducing the concentration of the limiting resource to a point where the other species is unable to obtain sufficient resource to maintain an xenic rate of growth. This theory also holds that the outcome of competition for a single resource should be independent of the initial population densities of the competitors. Resource competition theory is a very powerful modeling tool which has provided much insight into species distribution and plant community structure with respect to resource gradients. However the resultant model requires the measurement of resource supply rates, and the rate of utilization of the limiting resources by all competing species.

For a bioassay based on changes in competition patterns to be practical, the underlying model should contain a minimum number of parameters that need to be measured in order to predict the outcome of the competition in the presence of the toxin.

The prediction of the outcome of the competition is the only important output of the model. While an understanding of the underlying mechanism should eventually be investigated, for the purposes of a predictive model they are not necessary. From this point of view, the simple phenomenological Lotka-Volterra competition model would be an adequate model to employ as the basis of this bioassay. The α values of this model have the advantage of being general enough to include all the possible causes of competitive displacement. As Tilman (1982) himself points out, the displacement of one population by another may not involve depression of a common resource. Allelopathy is one alternative mechanism. Under such interference competition, susceptibility to displacement is not solely determined by equilibrial resource requirements of a species. An organism with a lower equilibrial resource requirement may not be able to invade a habitat if the habitat contains toxic or growth-inhibiting compounds. Allelopathy may allow an organism which is an inferior competitor for a resource to maintain itself in a habitat when it otherwise could not.

Therefore the model of the effects of atrazine on the competitive interaction of these test algae will be based on the Lotka-Voltera equations. At some future date it is hoped that I will be able to investigate more thoroughly the underlying mechanisms of these interactions.

Classical competition theory gives us a way to measure the α values in a controlled system of two species competing for a single limiting resource. Ricklefs (1973) provides a graphical model of the conditions which would lead to the four possible outcomes of such a competition. Figure 4.1 illustrates the model. Each axis represents the population

density of a species. One could describe this phase plane as "species space". In the case of coexistence, there is an equilibrium point somewhere in this species space. The line defined by the intersection points K_1 and $K_1/\alpha_{2,1}$ represents the combinations of species one and two for which r_1 (growth rate of species 1) equals zero. The equation for this line is $N_1 = K_1 - \alpha_{2,1}N_2$. The $r_1 = 0$ line divides the graph into two regions. In the region closest to the origin $r_1 > 0$ and for any population density combination in this region species one would increase until the line was reached. The intercepts K_1 on the N_1 axis, and $K_1/\alpha_{2,1}$ on the N_2 axis have transparent biological meaning. K_1 is the number of individuals of species one that can be supported by the available limiting resource, (carrying capacity of species 1). If the population density of species 1 was larger than K_1, it would exceed its resource base and its population would decline. Below K_1 the species 1 is not fully utilizing its resource base, and can increase. The $K_1/\alpha_{2,1}$ represents the number of individuals of species 2 that would completely exploit the resources of species 1. When N_2 is greater than $K_1/\alpha_{2,1}$ the demands placed on the resources of species 1 by species 2 exceed the availability of the resource and prevent population growth of species 1. As this ecological overlap between the two species decreases, the competition coefficient $\alpha_{2,1}$ becomes smaller and the intercept of the line $r_1 = 0$ on the N_2 axis becomes higher as seen in figure 4.2b. One can see the effect of a set of α values which equal zero in figure 4.2e, and f. Figure 4.3 combines the two $r_1 = 0$, and $r_2 = 0$ lines on one graph. Point A on figure 4.4 represents an equilibrium point in a case where there is no competition between the two species. If one where testing the null hypothesis that there was no competition between two coexisting species, this point A on figure 4.4 might

represent the null hypothesis. Any deviation from this point would indicate the degree of competition.

In figure 4.4 point B represents a stable equilibrium point of two coexisting species. One can see as each α value becomes larger the $K_2/\alpha_{1,2}$ and the $K_1/\alpha_{2,1}$ intercepts move back toward the origin.

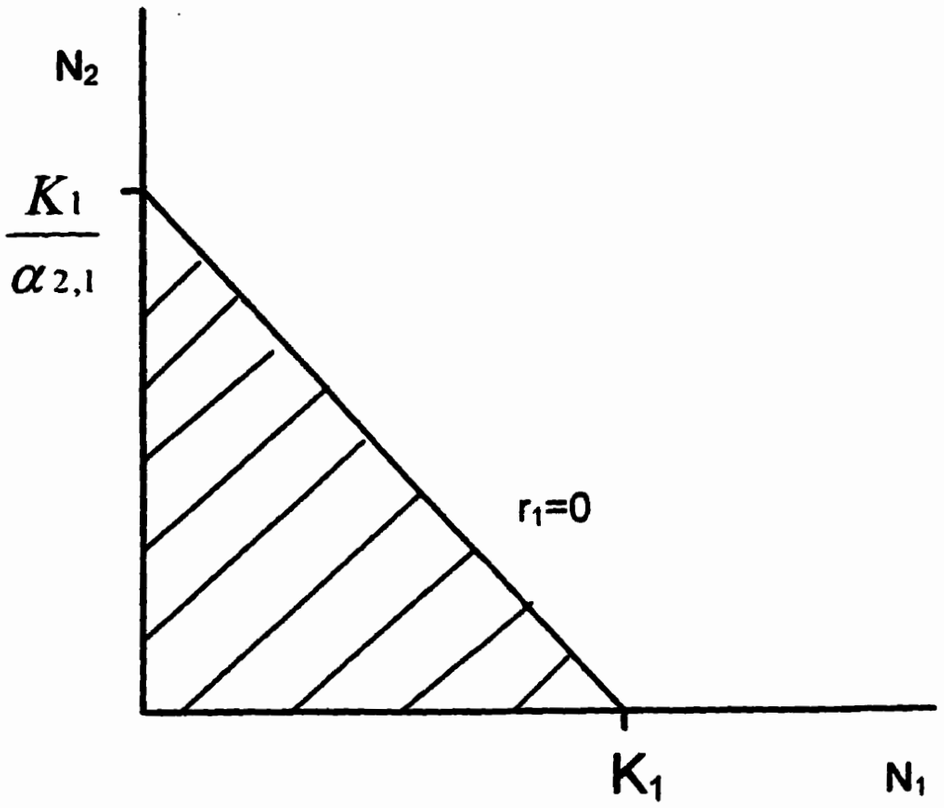


Figure 4.1 The zero growth isocline for two species competing for a single limiting resource. The line $r_1=0$ defines those combinations of populations of species 1 and 2, for which the growth rate is zero and the population is in equilibrium. N_1 represents the population density of species 1, N_2 represents the population density of species 2, K_1 represents the carrying capacity of species 1, $\alpha_{2,1}$ represents the competition coefficient (the effect of species 2, on species 1).

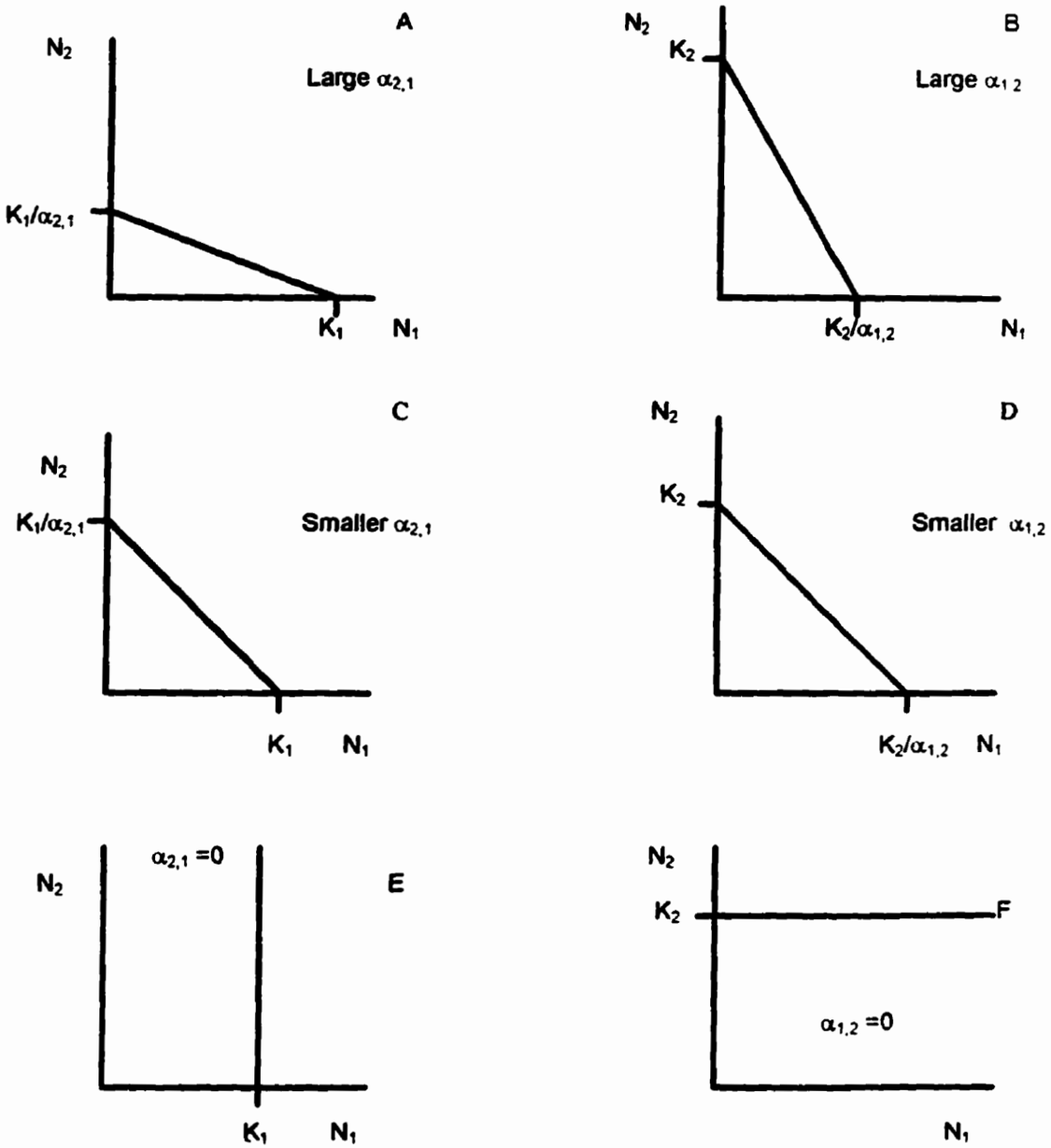


Figure 4.2. The effects of changing α values on the strength of interspecific competition. a) shows how a large $\alpha_{2,1}$ reduces the maximum population density of species 1 (N_1) at any population level of species 2. b) shows how a smaller $\alpha_{2,1}$ increases the maximum population level of species 1, at any population level of species 2. and c) shows the case when $\alpha_{2,1}$ =0 ie. on competition, species 1 reaches its carrying capacity K_1. d. e, and f show the corresponding situations for changes in $\alpha_{1,2}$.

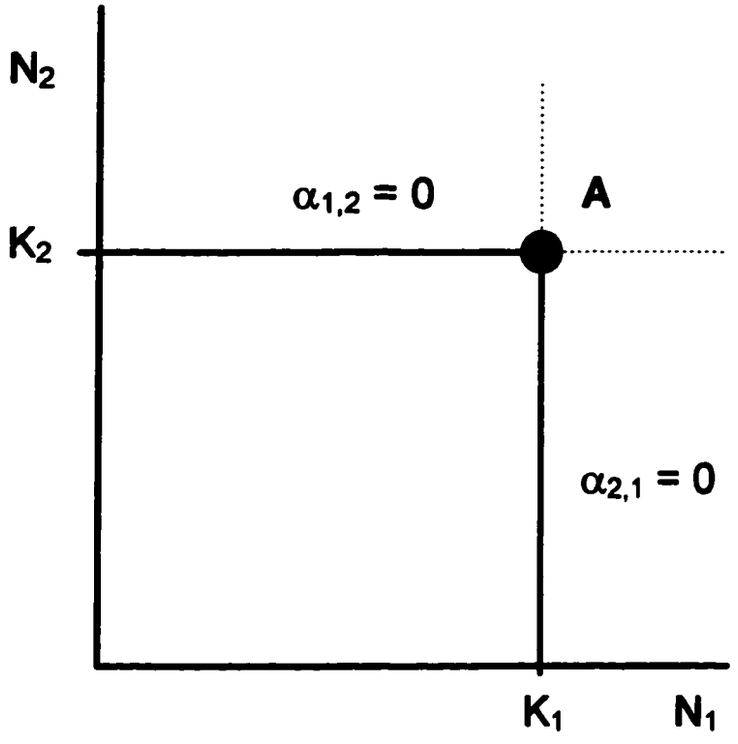


Figure 4.3. This is a combination of parts c and f in figure 5.8. Point A represents the theoretical, no competition equilibrium point, when $\alpha_{2,1}$ and $\alpha_{1,2}$ both equal zero.

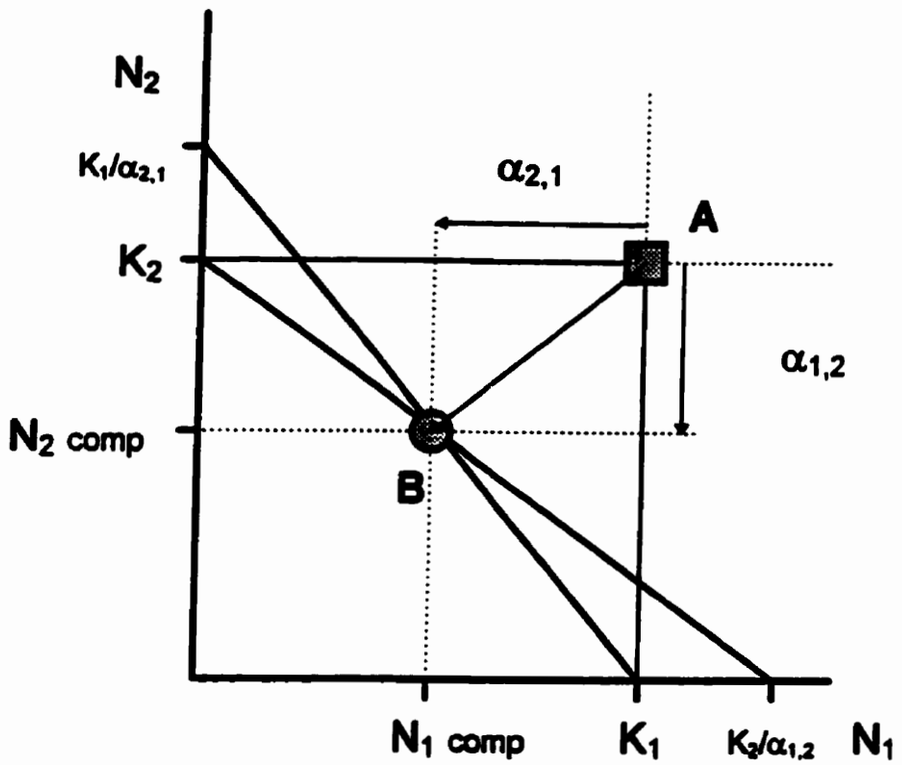


Figure 4.4. Estimation of $\alpha_{2,1}$ and $\alpha_{1,2}$ from the stable coexistence of two competing species. Point B represents the actual stable coexistence equilibrium, point A represents the theoretical no competition equilibrium population densities. N_{1comp} and N_{2comp} represent the population densities under competition, and $\alpha_{1,2}$ and $\alpha_{2,1}$ are the ratio the N_{1comp}/ K_{1uni} and N_{2comp} /K_{2uni} respectively.

One might speculate on how the combined effects of competition, and the metabolic stress caused by the presence of a herbicide, might affect the growth responses of two phytoplankters growing together in semicontinuous culture. A reasonable hypothesis might be that as herbicide concentration increases, the mean maximum steady state cell density of each species should decrease. It would be reasonable to expect the combined stresses of competition and herbicide toxicity should reduce the carrying capacity of the cultures below the level of reduction produced by either stress alone.

C. Materials and Methods

Culture Conditions

The test species used to create the synthetic microcosms, *Scenedesmus quadricauda*, *Chorella vulgaris*, and *Chlamydomonas musicola* were all isolated from water samples collected on May 28, 1989, from channels of the Delta Marsh, on the southern end of Lake Manitoba, Canada (99°19'W, 50°7'N). These algae were maintained in stock batch cultures of 150ml of modified WC' media (Healy and Hendzel 1979). These stock cultures were maintain in 250 ml Erlenmeyer flasks sealed with a cheesecloth covered cotton bung, on a shaker table at 19°C under a constant illumination of 70-75 $\mu Em^{-2} s^{-1}$. The cultures were subcultured into 150 ml of fresh medium every 5-7 days.

The competition experiments consisted of growing the test species in pairs, in 4 replicate semicontinuous cultures, at each of the following seven concentrations of

atrazine; 0 , $1.0X10^{-8}$, $5.0X10^{-8}$, $1.0X10^{-7}$, $5.0X10^{-7}$, $8.2X10^{-7}$ and $1.0X10^{-6}$ mol/L. This entire protocol was repeated and the results of these two sets of experiments were then pooled. The semicontinuous cultures were cultured in 500 ml of WC' medium, contained in 700 ml round-bottom culture flasks. These flasks received a constant illumination of 75 $\mu Em^{-1}\ s^{-1}$ provided by a series of 4 four foot long General Electric cool white fluorescent bulbs. Each culture was aerated with air passed through a water trap and a sterile filter. All culture equipment and media were autoclaved at 123°C for 40 minutes, and aseptically assembled in a laminar flow hood. The test algae were subcultured into fresh media and grown in batch culture for 4 days in order to produce an inoculum culture in log phase. Each semicontinuous culture was inoculated aseptically with stock culture through the inoculation port, using a sterile 10 ml syringe.

Each experiment consisted of six semicontinuous culture flasks arranged along the light tubes and positioned so that each culture received the same level of illumination. Four of the six culture flasks contained the two competing algae, and medium containing the particular atrazine concentration being tested. The other two cultures functioned as unialgal controls, containing one of the two algal species, and medium containing the atrazine concentration being tested. The six cultures were divided into two groups of three, on each side of the center of the fluorescent lights. Each group of three cultures consisted of two experimental cultures and one unialgal control. For each run, the control culture was randomly positioned, inside, outside or in the center with respect to the two experimental cultures.

The atrazine concentrations were produced by the addition of an appropriate volume of $1.0X10^{-4}$ mol/L stock atrazine, which was prepared from 99.8% research grade atrazine (Ciba Geigy). Six liters of the WC'-atrazine medium was mixed in 10L glass carboys and then autoclaved at 123°C for 90 minutes. Daily each semicontinuous culture was diluted by 20% with sterile medium delivered in one dose at the same time each day for the duration of the experiment. At the same time, a 5 ml sample of the culture was removed through the sterile sample port and preserved with Lugol's iodine and stored at room temperature for later examination. Estimates of cell density were made for each species in the sample, using a Reichert-Jung, Bright-line Improved Neubauer Hemocytometer. All subsequent culture parameters were calculated from these daily cells/ml data.

Estimation of α values.

The first step in this investigation was to make an estimate of the competition coefficients between each species. In order to estimate the competition coefficients, $\alpha_{2,1}$ and $\alpha_{1,2}$ one must grow the two species together in competition for one limiting resource. One of the drawbacks of the classical theory of competition is that it is very difficult to expand the theory into the complex interactions of the natural world, or to make such estimations of these α values for species not actually in competition. Waltman and others. (1980) list several of the problems with the biological assumptions of classical competition theory. These include the assumption of constant carrying capacity, ecological equivalence of all individuals within each population (no age dependent differences in birth

or death rates or in resource use, for example), no time lags, and constant, linear per capita effects on population growth rates within and between species. However, we can overcome many of the problems with these biological assumptions by creating a synthetic microcosm, using the semicontinuous culture method, controlling all of the environmental variables, and by using single celled photosynthetic phytoplankton as the test species. The use of the WC' medium (Healy and Hendzel 1979) establishes phosphate as the single limiting resource in our synthetic microcosms. Phosphate which is essential for phytoplankton growth is described by Tilman (1982) as an essential resource, that is no other resource can be substituted for it.

The α values can be calculated from the relative decrease in the population density of each coexisting species compared to the theoretical no competition point. In this study I estimated the theoretical, no competition equilibrium point, by growing each species separately in unialgal semicontinuous cultures.

These estimates of the theoretical, no competition equilibria, were then compared to the no herbicide competition semicontinuous culture steady state equilibria, in order to estimate the $\alpha_{2,1}$ and $\alpha_{1,2}$ values for that species pair. As the concentration of the herbicide increases there is a reduction in the mean maximum steady state cell density that can be achieved by that culture. There is therefore, a maximum carrying capacity associated with each herbicide concentration. What is of interest here is whether there is a change in the α values, with each different herbicide concentration, compared to the no herbicide α values for that species pair.

The competition experiments were run for 10 days, and the cultures achieved steady state on day 5. In none of the experiments did one or the other species disappear from the cultures by day 10. If it is assumed that the two species competed for phosphate, and reacted to the herbicide stress in the synthetic microcosms as they would in nature, then it is unlikely that a competitive exclusion that took longer than 10 days in our system would have any ecological meaning in nature. I therefore took the mean day 5-to-end, cell density of each competition experiment to be the coexistence equilibrium value.

The α values for each species pair, at each herbicide concentration were calculated as follows:

$$\alpha_{1,2} = \frac{N_{2comp}}{N_{2uni}}$$

Where, $\alpha_{1,2}$ represents the effect of species 1 on its competitor species 2, N_{2comp} represents the steady state cell density of species 2 in competition, and N_{2uni} represents the mean maximum steady state cell density of species 2 in unialgal culture. Note that this calculation of the alpha value will give an $\alpha = 1$ for the "no competition" case, rather than an $\alpha = 0$ for no competition as developed in the model in figure 4.4. The use of the above equation instead of $\alpha = (1 - N_{2comp} / N_{2uni})$ was chosen so the alpha values which indicate a "stimulation in growth" would be values greater than one, rather the negative values as would be the case in the model in figure 4.4.

"Delta alpha"

I was interested to see if there was a pattern in the way the alpha values changed with increasing atrazine concentrations. One of the key concepts of this approach is that changes in the competition coefficients ($\Delta\alpha$) of a species pair must be examined and interpreted together, since each had an influence on the value of the other.

Figure 4.5 summarizes the model of the $\Delta\alpha$ approach. The X and Y axes of the model represent the range of alpha values for each species in the two species system. The alpha values are themselves dimensionless ratios of the theoretical no competition equilibrium and the actual equilibrium observed in the no herbicide competition experiments. This model can be used to help answer two questions; 1) How does a specific herbicide concentration affect the final equilibrium levels of the competing species? and 2) By how much does the particular herbicide concentration affect the final equilibrium levels of the competing species? "How" implies a direction; in what direction does the equilibrium shift? The "How much" implies a length; to what extent does the equilibrium shift? The model described in figure 4.5 allows us to examine both these questions with respect to the results of our competition experiments.

To understand the population dynamics under herbicide stress, we can examine the general directions of the equilibrium's shift at various herbicide levels. From figure 4.5 we can see that for each species pair there will be an ordered pair of $\Delta\alpha$'s that define a vector with an origin of (0,0), into one of four quadrants. The (0,0) origin on the model represents the no herbicide alpha starting point. The original $\alpha_{2,1}$ and $\alpha_{1,2}$ no herbicide points for each species pair have been transformed to the origin to make it easier to

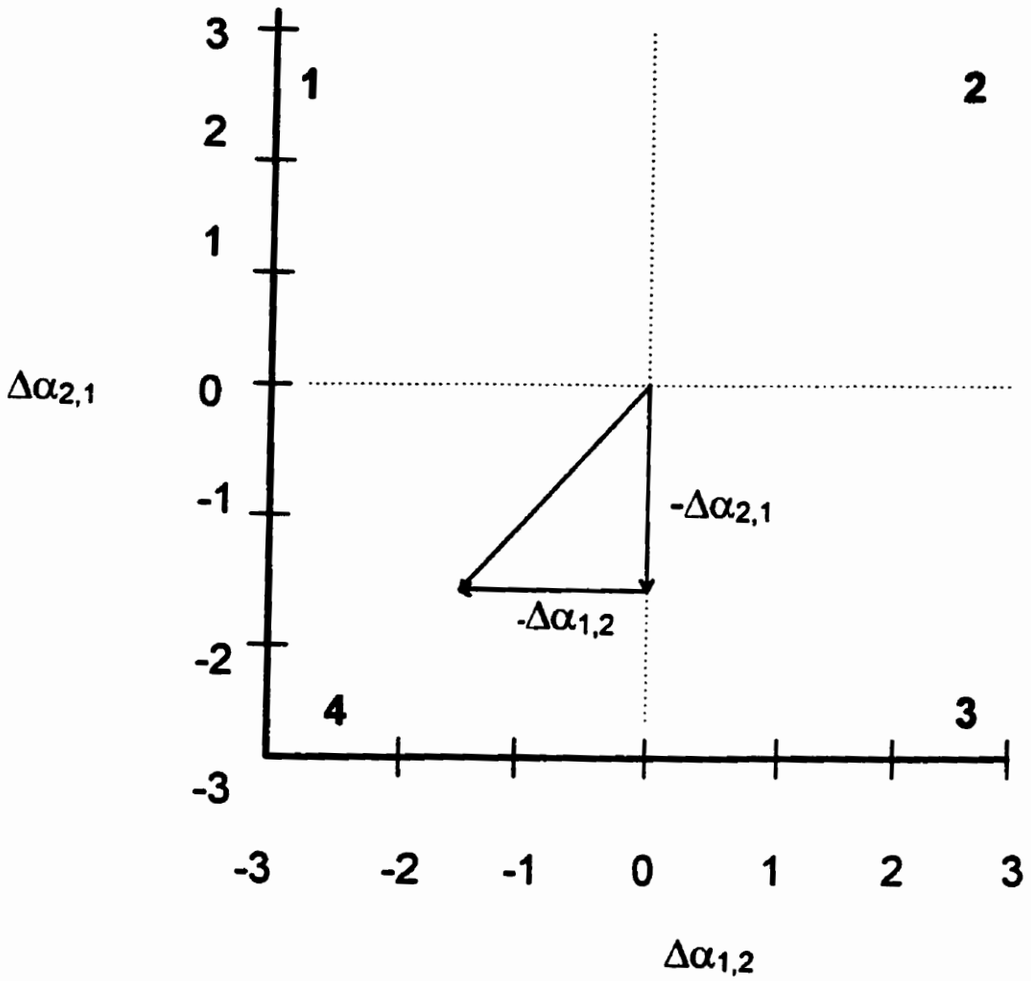


Figure 4.5 A model relating changes in the competition coefficients under different herbicide concentrations, for two species competing for one limiting resource.

compare the relative changes across species pairs. The direction of these vectors define the effects of that herbicide concentration on the new competitive equilibrium (the "how" question). Later we will examine the length of these vectors (the "how much" question). The competition experiments provided numbers which help us to see how the equilibrium changed with different herbicide levels, they were not designed to explain "Why" the populations changed in the way they did. However, in the descriptions of the exploratory model below, I allow myself the luxury of a little speculation on some of the possible mechanisms of these changes.

Four general classes of vectors can be defined, by the four quadrants that they occupy. Vectors into quadrant 1 have the form ($-\Delta\alpha_{1,2}$, $+\Delta\alpha_{2,1}$). Biologically this indicates that the ability of species 2 to reduce the carrying capacity of species 1 has increased, and the ability of species 1 to reduce the carrying capacity of species 2 has apparently decreased. Both responses could be the result of species 1 being more sensitive to the herbicide than species 2. This could be the case if the mode of action of the herbicide impacts the uptake ability of species 1, or otherwise utilize the limiting resource to a greater extent than it does on species 2. An additional explanation for this combination of $\Delta\alpha$s, is the possibility that species 1 is able to somehow absorb, detoxify or otherwise remove the herbicide from the medium thus reducing the exposure of species 2. This would tend to increase the carrying capacity of species 2 (K_2). One can see from figure 4.4, the effect of this would be to shift K_2 closer to N_2, (farther away from the origin), therefore increasing the competitive equilibrium cell density of species 2.

Quadrant 2 in figure 4.5 would contain vectors defined by ($+\Delta\alpha_{1,2}$, $+\Delta\alpha_{2,1}$). In this case the addition of the herbicide has improved the abilities of each species to reduce the carrying capacity of the other, as compared to their competitive abilities in the absence of the herbicide. We know that the addition of a physiological stress can, in some cases, cause a species to alter its' metabolism in order to minimize the effects of the stress. Anderson and Beardall (1991) describe how some CAM plants alter their mechanism of CO_2 assimilation under certain environmental conditions. There are examples in many organisms where the stimulation of such compensatory mechanisms produces a far greater reaction than is needed to overcome the stress. This can lead to a net stimulation of the affected metabolism or pathway. The stimulation of the production of vast quantities of antibodies by the human immune system by a relatively minor infection might be one such example. It is possible that a $\Delta\alpha$ response of ($+\Delta\alpha_{1,2}$, $+\Delta\alpha_{2,1}$) could have a similar explanation. Work in our lab has shown that there is an increase in the chlorophyll a per cell of *Scenedesmus quadricauda* and *Chlamydomonas reinhardtii* in response to increased atrazine stress. This response is likely related to the mode of action of this herbicide which binds to proteins in photosystem II blocking electron flow (Derkson 1996). The synthesis of more photosynthetic units (PSU's) and hence more chlorophyll, to replace the damaged units, could go a long way to reducing the effects of the herbicide. In such a scenario, over production of new PSUs in response to low levels of atrazine could lead to a stimulation of the growth and metabolism of the cells and perhaps an increase in their competitive abilities. Another possible explanation could involve the

production of allelopatric compounds by each species in responses to the stress which increases the impact of each species on the other.

Quadrant 3, in figure 4.5 produces an opposite vector set to quadrant 1. In this case the $\Delta\alpha$ responses would be ($+\Delta\alpha_{1,2}$, $-\Delta\alpha_{2,1}$). Here the effect of species 1 on species 2 increases and the effect of species 2 on species 1 decreases. Finally in quadrant 4, both $\Delta\alpha$s decrease, that is, both species become less capable of reducing the carrying capacity of the other. This response set is perhaps the easiest to explain. The relative competitive abilities of each species are reduced by the toxic effects of the herbicide.

The second question concerning the degree of the population equilibrium shift, can be explored by estimating the strength of the impact of a given concentration of herbicide, on the competitive abilities of the species. This can be represented by the length of these vectors in Figure 4.5. The length of these vectors can be calculated simply as a Euclidean distance, as given by the Pythagorean theorem using the following general formula;

Vector length $= \sqrt{(\alpha_{2,1})^2 + (\alpha_{1,2})^2}$. We now have a direction of the response, into one of four quadrants (or actually four different classes of response), and a distance (or strength) of that response.

If a response point ($\Delta\alpha_{2,1}$,$\Delta\alpha_{1,2}$) lies with in quadrant 2 or 4, it indicates that both of the species are either increasing there abilities to effect one another (Quadrant 2) or there has been a decrease in that ability in both (quadrant 4). One or the other species may still retain the advantage depending on the relative amounts of these changes. If one calculates the ratio of $\Delta\alpha_{2,1}/\Delta\alpha_{1,2}$ one can gain some idea as to which species might have gained the competitive advantage. A ratio of $\Delta\alpha_{2,1}/\Delta\alpha_{1,2} = 1$ would indicate the ordered

pair lies on a line, 45° from either axis that bisects the quadrant. This would indicate that each species was able to match the increase in competitive abilities of the other, so neither has gained an advantage. A ratio of $\Delta\alpha_{2,1}/\Delta\alpha_{1,2} < 1$ would place the response point below the 45° line and would indicate that species 1 has gained the competitive edge. Similarly, a ratio of $\Delta\alpha_{2,1}/\Delta\alpha_{1,2} > 1$ would place the response point above the 45° line giving species 2 the advantage. Figure 4.6 graphically illustrates the positions of all the possible $\Delta\alpha_{2,1}/\Delta\alpha_{1,2}$ ratios in our model from figure 4.5.

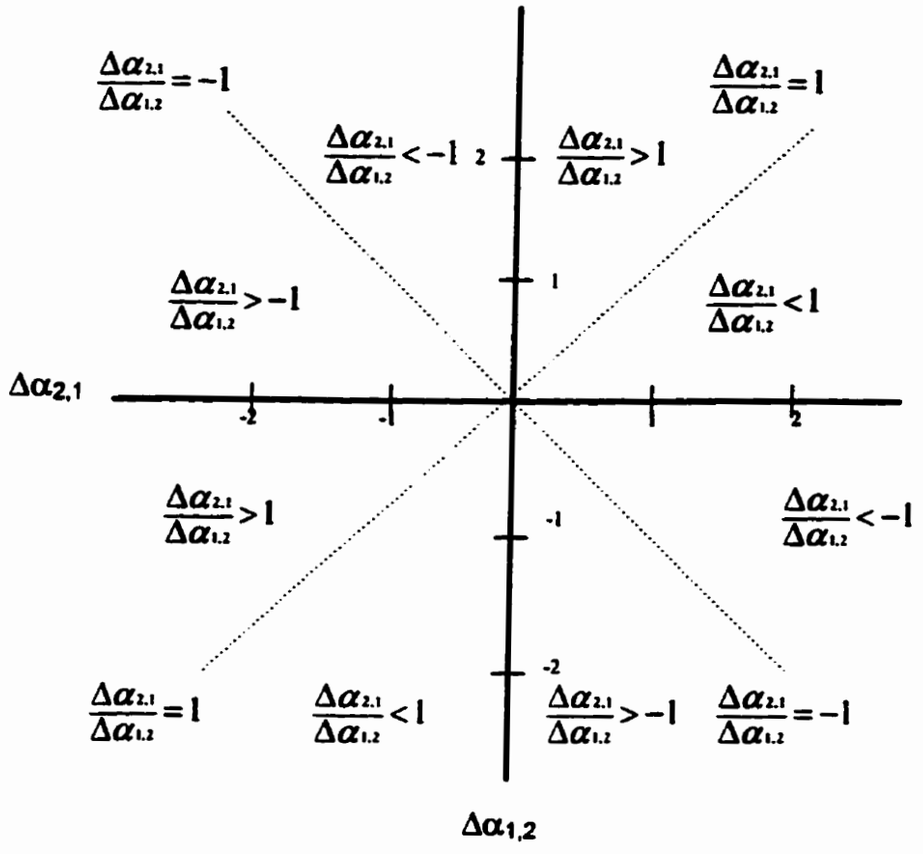


Figure 4.6 A graphical representation of the position of all possible $\Delta\alpha_{2,1}/\Delta\alpha_{1,2}$ ratio values.

D. Results and Discussion

In order to examine the patterns of atrazine dose responses in these test species, I will present the results of the estimation of the α values, and the analysis of the $\Delta\alpha$ patterns separately for each species pair.

Scenedesmus quadricauda* and *Chlamydomonas musicola

The mean maximum steady state cell density of each unialgal semicontinuous culture and the mean maximum steady state cell density of each species in competition were plotted against atrazine concentration (figure 4.7, 4.8).

Three interesting phenomena can be seen in some of these results. Although not statistically significant, in the *Scenedesmus quadricauda* unialgal cultures, the mean maximum steady state cell density seemed to increase at low herbicide concentrations compared to the controls. When *Scenedesmus quadricauda* was grown in competition with each of the other two test species, in both cases the addition of the competitive stress appeared to reduce this stimulatory effects at the low herbicide levels.

When *Scenedesmus quadricauda* and *Chlamydomonas musicola* were grown in competition with no atrazine added, the mean maximum steady state cell density of each species was higher in the competitive culture than they were in the unialgal culture, with *Scenedesmus quadricauda* having a significantly higher cell density. In the competitive culture, as the concentration of herbicide rose to $1.0X10^{-7}$ mol/L, the mean maximum

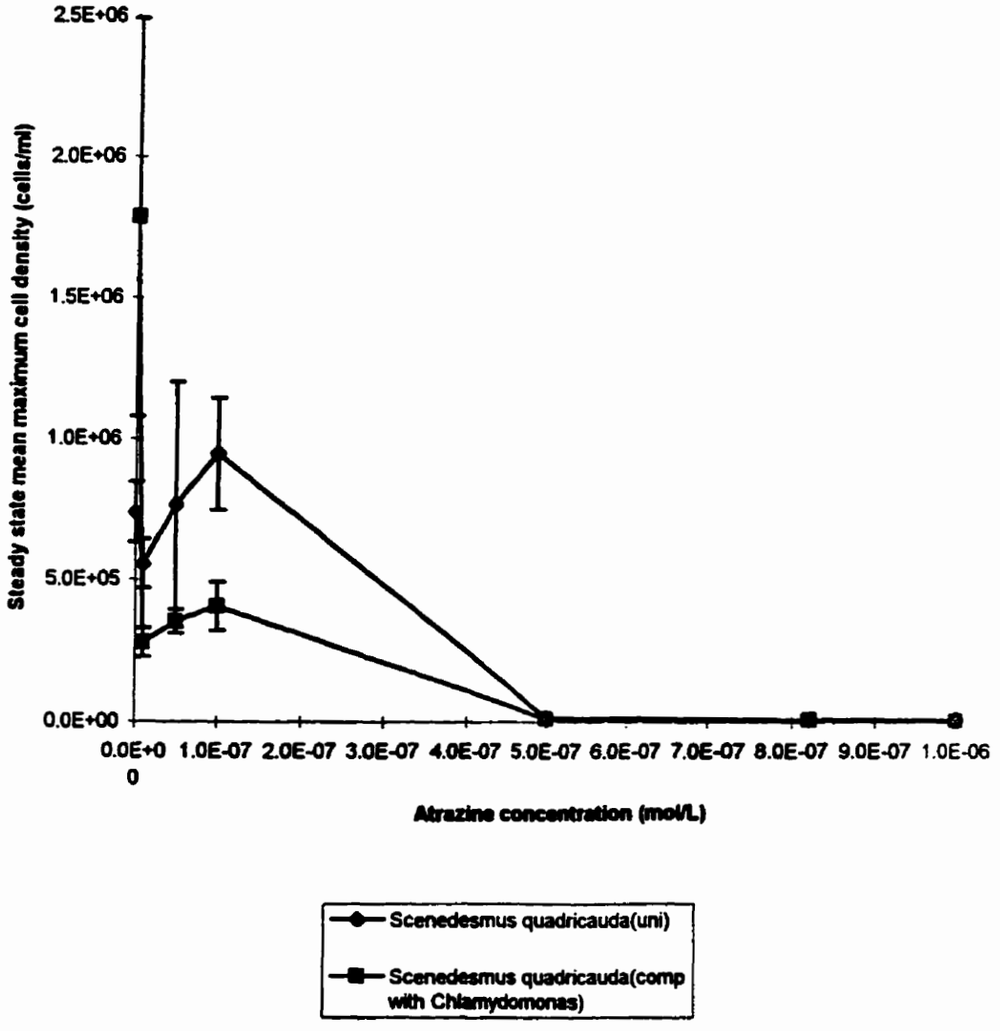


Figure 4.7 Dose responses of *Scenedesmus quadricauda* to the herbicide atrazine in unialgal culture and in competition with *Chlamydomonas musicola*. Error bars represent the standard deviations about the mean maximum steady state cell densities.

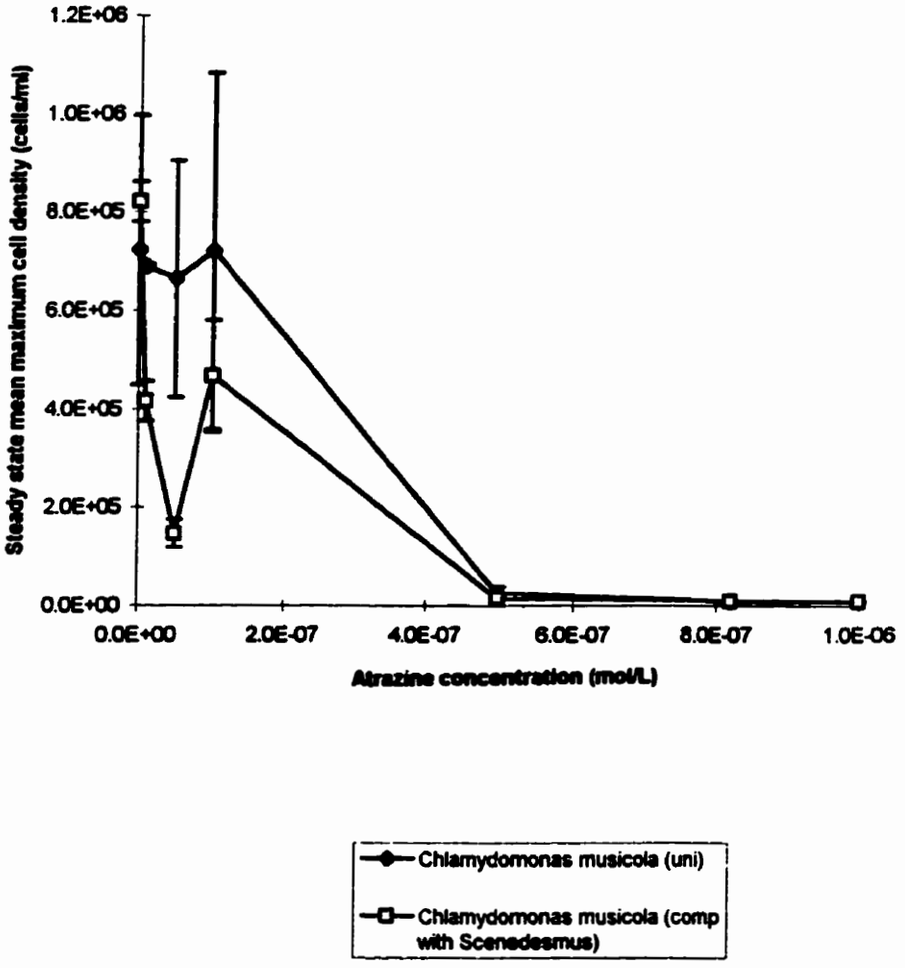


Figure 4.8 Dose responses of *Chlamydomonas musicola* to the herbicide atrazine in unialgal culture and in competition with *Scenedesmus quadricauda*. Error bars represent the standard deviations about the mean maximum steady state cell densities.

steady state cell density of each species dropped below the unialgal levels. Table 4.1 summarizes the competition coefficients between *Scenedesmus quadricauda* and *Chlamydomonas musicola* at the different concentrations of atrazine. Note the $\alpha_{2,1}$ and $\alpha_{1,2}$ values for the no herbicide case are greater than one, indicating a "stimulation" effect. That is each species actually raises the carrying capacity of the environment for the other species. I have no experimental evidence which would support an explanation of this stimulation however it is possible that these species may produce some sort of "growth factor" which could allow each species to acquire or utilize the limiting resource more efficiently. There appears to be a similar stimulation of growth for *Chlorella vulgaris* by *Scenedesmus quadricauda* as indicated by the $\alpha_{2,1} = 1.258$ at $8.2X10^{-7}$ mol/L atrazine concentration. As will be seen in the results from the *Chlamydomonas musicola* vs. *Chlorella vulgaris* and *Scenedesmus quadricauda* vs. *Chlorella vulgaris* competition experiments, this stimulation effect occurs only at the higher atrazine levels.

Table 4.1 Summary of the competition coefficient between the green algae *Chlamydomonas musicola* and *Scenedesmus quadricauda*, at different concentrations of atrazine.

Species	Atrazine concentration (mol/L)	Mean $\alpha_{2,1}$	Standard deviation $\alpha_{2,1}$	Mean $\alpha_{1,2}$	Standard deviation $\alpha_{1,2}$
Chlamydomonas(1) vs. Scenedesmus(2)					
	0	1.135	0.052	2.415	0.885
	$1.0X10^{-8}$	0.604	0.055	0.501	0.083
	$5.0X10^{-8}$	0.616	0.442	1.211	0.814
	$1.0X10^{-7}$	0.427	0.258	0.362	0.095
	$5.0X10^{-7}$	0.787	0.266	0.894	0.302
	$8.2X10^{-7}$	1.258	0.190	0.875	0.268
	$1.0X10^{-6}$	1.060	0.311	0.675	0.284

In examining table 4.1, the first temptation is to individually examine the relationships between each of these 6 α values and increasing herbicide concentration. However, if the goal of the analysis is to ultimately determine a relationship between competitive outcome and herbicide concentration, then we will need to account for the changes in the relative competitive abilities of each species with increasing herbicide concentrations. For example, if the competitive abilities of one species are reduced, the other species automatically has gained an advantage, even if its relative competitive ability have not changed. In order to make any predictions about the response of the whole system to the addition of the herbicide, it is important to examine the combined responses of the herbicide stress. The culture and analysis methods used in this study have in fact incorporated this connectedness. The estimation of each α value pair (ie. $\alpha_{2,1}$ and $\alpha_{1,2}$), was done from growth responses of each species grown together. If for example, the addition of a herbicide reduced the ability of species 1 to lower the carrying capacity of its' competitor species 2, but the ability of species 2 to lower the carrying capacity of its' competitor species 1 remained unchanged, the equilibrium cell density of species 2 would therefore be higher. The "race" can be won even if you do not run any faster, as long as your opponent runs slower. The most important factor is the relative difference in the changes in alpha values due to herbicide stress, compared to the competitive abilities of each species with no herbicide present. "The race can still be won even if you run more slowly, as long as your opponent runs even more slowly than you". One final important consideration in the analysis, (if the running analogy can be used one more time), is how

fast could each of you run in the first place? Changes in competitive abilities as a result of the herbicide stress must be related to the original competitive abilities of the two species.

An interesting question might be; which species has the competitive advantage under any given herbicide stress? This question can be examined by calculating and comparing the ratios of the α values at each herbicide level. The ratio ($\alpha_{2,1}$ / $\alpha_{1,2}$) gives an estimation of the relative abilities of each species to lower the carrying capacity of the other. Figure 4.9 illustrates the graphical form of this relative advantage estimate. Each axis represents the range of alpha values for each species. The 45° line which bisects the graph represents all combinations of the α values such that $\alpha_{2,1}$ / $\alpha_{1,2}$ = 1. The region of the graph below this line represents all combinations of alpha values such that $\alpha_{2,1}$ / $\alpha_{1,2}$ <1, and the region above the line all values of alphas for which $\alpha_{2,1}$ / $\alpha_{1,2}$ >1. Figure 4.10 illustrates the ordered pairs coordinates ($\alpha_{2,1}$, $\alpha_{1,2}$) corresponding to each of the herbicide levels tested for the competing pair *Scenedesmus quadricauda* and *Chlamydomonas musicola*.

It can be seen from figure 4.10 that in the competition experiment with *Scenedesmus quadricauda* vs. *Chlamydomonas musicola*, the alpha ratios fluctuate from less than one (i.e. below the 45° line) to greater than one (above the 45° line), with all but the last two increases in the atrazine level. Only at these highest two herbicide concentrations does *Scenedesmus quadricauda* acquire a slight competitive edge.

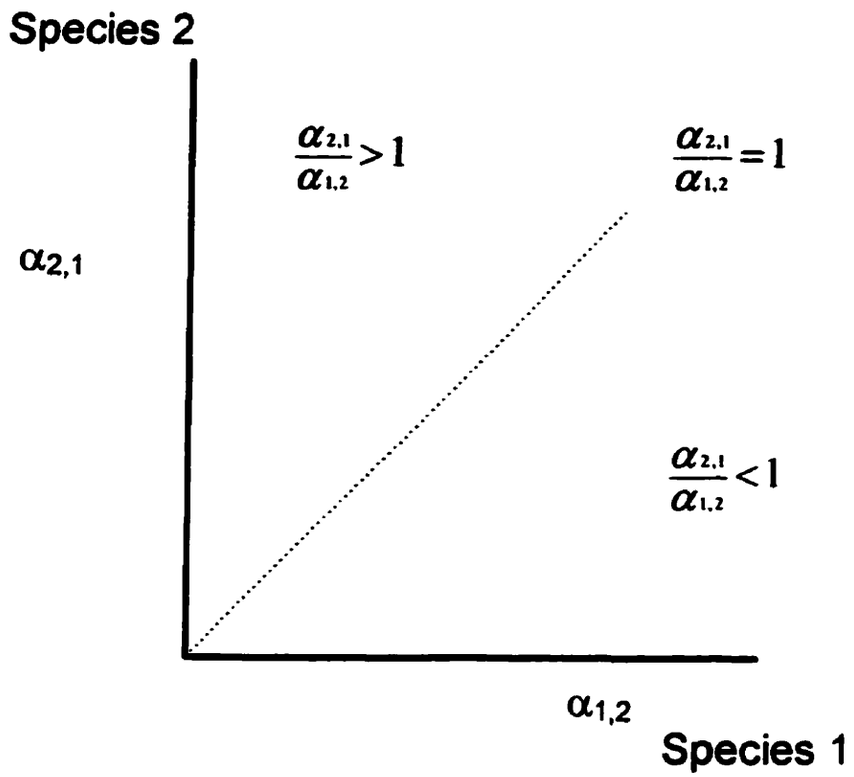


Figure 4.9 A Model of relative competitive advantage. $\alpha_{2,1}$ is the coefficient of competition for the effect of species 2 on species 1, and $\alpha_{1,2}$ is the coefficient of competition for the effect of species 1 on species 2.

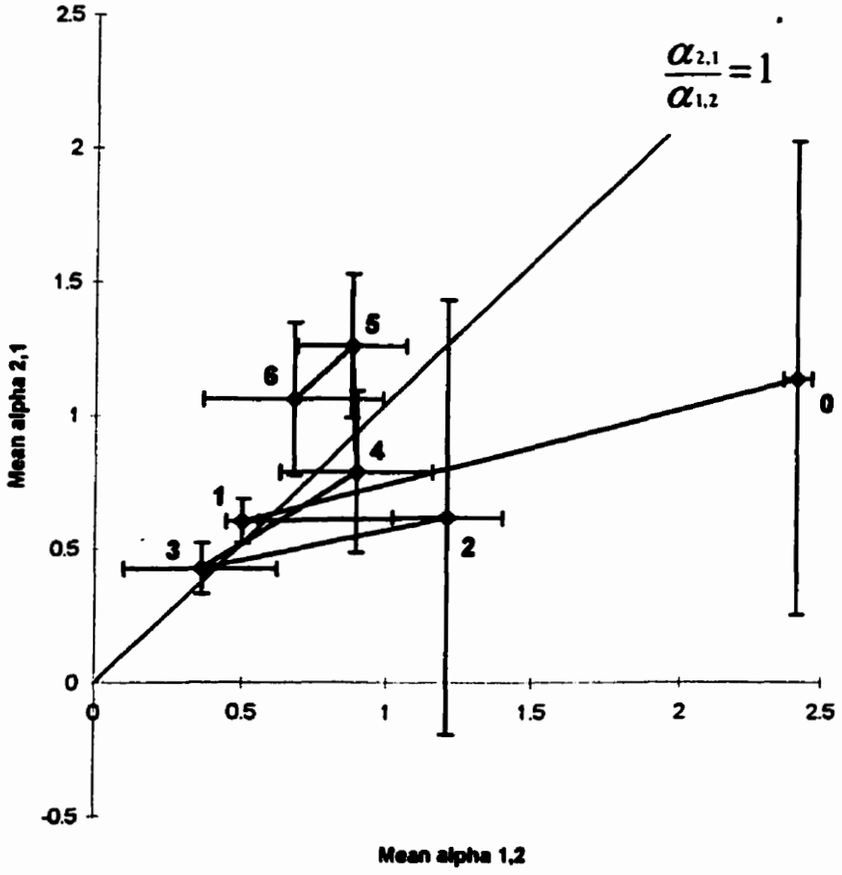


Figure 4.10 Trajectory of alpha values with increasing atrazine concentrations for *Chlamydomonas musicola* (1) vs. *Scenedesmus quadricauda* (2). Numbers indicate increasing herbicide levels (0 = no herbicide, 1=$1.0X10^{-8}$ mol/L, 2=$5.0X10^{-8}$ mol/L, 3=$1.0X10^{-7}$ mol/L, 4=$5.0X10^{-7}$ mol/L, 5=$8.2X10^{-8}$ mol/L and 6=$1.0X10^{-6}$ mol/L.). Error bars equal one standard deviation from the mean.

We can now look at the changes in the alpha values with increasing herbicide levels from the no herbicide case, what I have termed the $\Delta\alpha$ values. Table 4.2 presents a summary of these $\Delta\alpha$ values for the with *Scenedesmus quadricauda* vs. *Chlamydomonas musicola* competition experiments.

Table 4.2 Summary of the $\Delta\alpha$ responses, Euclidean distance (length), and ratios ($\Delta\alpha_{2,1}/\Delta\alpha_{1,2}$) of response vectors for *Scenedesmus quadricauda* vs. *Chlamydomonas musicola* at differing herbicide concentrations.

Species	Atrazine concentration (mol/L)	Delta $\alpha_{2,1}$	Delta $\alpha_{1,2}$	Response Vector Length	Ratio ($\Delta\alpha_{2,1}$ /$\Delta\alpha_{1,2}$)
Scenedesmus (2) vs. Chlamydomonas(1)					
	$1.0X10^{-8}$	-0.531	-1.914	1.986	0.277
	$5.0X10^{-8}$	-0.519	-1.203	1.310	0.431
	1.0X10-^{7}	-0.708	-2.053	2.171	0.345
	$5.0X10^{-7}$	-0.348	-1.520	1.560	0.228
	$8.2X10^{-7}$	0.122	-1.539	1.545	-0.079
	$1.0X10^{-6}$	-0.075	-1.739	1.741	0.044

If we take the $\Delta\alpha$ values from table 4.2, and plot them as ordered pairs ($\alpha_{2,1}$, $\alpha_{1,2}$) we obtain a trajectory of the changes in the competition coefficients with changing atrazine concentrations. Such graphical representations of these responses can often be useful in detecting any response patterns. Figure 4.1 illustrates the trajectory of the $\Delta\alpha$ values for the *Scenedesmus quadricauda* vs. *Chlamydomonas musicola* competition experiments.

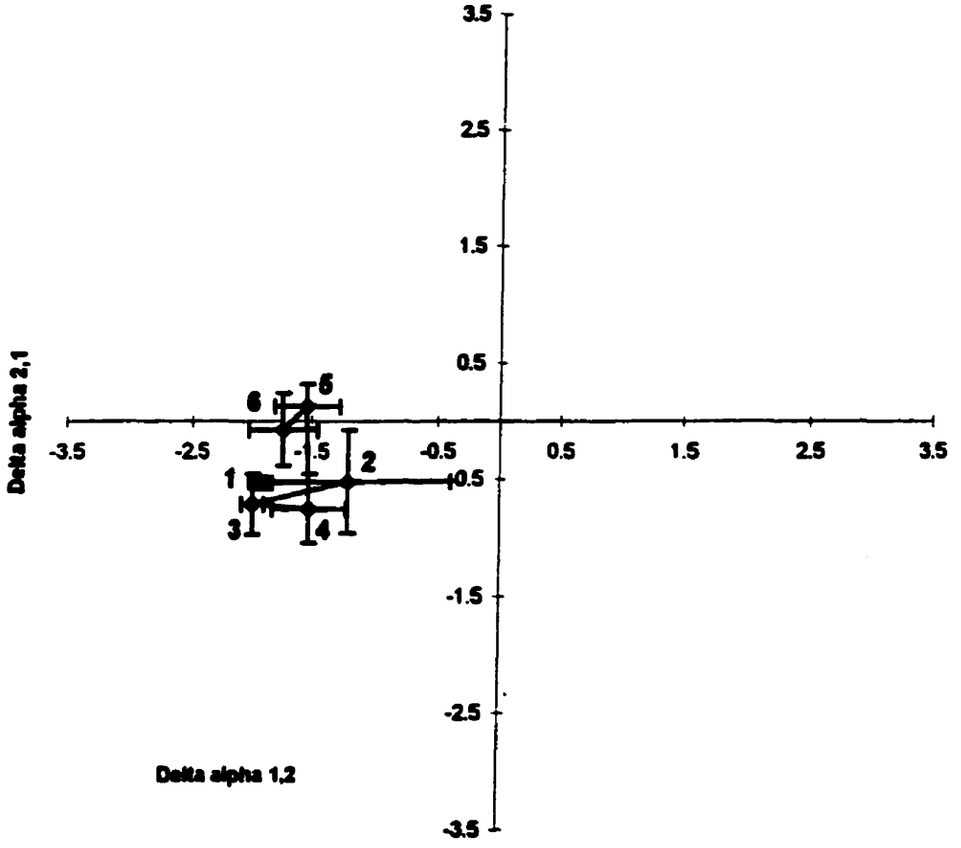


Figure 4.11 Trajectory of the $\Delta\alpha$ values with increasing atrazine concentration, for *Scenedesmus quadricauda* (species 2) and *Chlamydomonas musicola* (species 1). Numbers represent the following atrazine levels; 1=$1.0X10^{-8}$ mol/L, 2=$5.0X10^{-8}$ mol/L, 3=$1.0X10^{-7}$ mol/L, 4=$5.0X10^{-7}$ mol/L, 5=$8.2X10^{-8}$ mol/L and 6=$1.0X10^{-6}$ mol/L. Error bars represent one standard deviation from the mean. Horizontal error bars indicate the variation of the $\Delta\alpha_{2,1}$ estimates, and the vertical error bars indicate the variation in the $\Delta\alpha_{1,2}$ estimates.

From an examination of this $\Delta\alpha$ response trajectory, one can detect two distinct clusters of points. In order to determine whether these clusters represent distinct responses, and to determine which species has been most severely effected by the change in herbicide concentration, each element of each ordered number pair was compared to the same elements of the other ordered number pairs using the approximate randomization method. Tables 4.3 presents the results of this analysis for the *Scenedesmus quadricauda* vs. *Chlamydomonas musicola* competition experiments.

Table 4.3 A comparison of the $\Delta\alpha$ response patterns for the appearance of clustered responses to differing concentrations of atrazine, for all competing species pairs. The coordinates of each point are ordered numbered pairs consisting of the values ($\Delta\alpha_{2,1}$, $\Delta\alpha_{1,2}$). Therefore "$5.0X10^{-7}$ mol/L $\Delta\alpha_{1,2}$ " refers to the change in the competition coefficient (effect of species 1 on the carrying capacity of species 2) produced at an atrazine concentration of $5.0X10^{-7}$ mol/L. as compared to the $\alpha_{1,2}$ value produced by that species pair when no herbicide is present. The null hypothesis being tested is that there is no difference between any two elements. Each element pair was compared using approximate randomization test. Level of significance was p=0.01. Elements with the same lower case letter were not significantly different whereas elements with different lower case letters were.

Species	Ordered pair element	Significance
Chlamydomonas musicola(1)	$1.0X10^{-8}$ mol/L $\Delta\alpha_{1,2}$	a
vs.	$5.0X10^{-8}$ mol/L $\Delta\alpha_{1,2}$	a
Scenedesmus quadricauda(2)	$1.0X10^{-7}$ mol/L $\Delta\alpha_{1,2}$	
	$5.0X10^{-7}$ mol/L $\Delta\alpha_{1,2}$	b
	$8.2X10^{-8}$ mol/L $\Delta\alpha_{1,2}$	b
	$1.0X10^{-6}$ mol/L $\Delta\alpha_{1,2}$	b
	$1.0X10^{-8}$ mol/L $\Delta\alpha_{2,1}$	c
	$5.0X10^{-8}$ mol/L $\Delta\alpha_{2,1}$	c
	$1.0X10^{-7}$ mol/L $\Delta\alpha_{2,1}$	c
	$5.0X10^{-7}$ mol/L $\Delta\alpha_{2,1}$	c
	$8.2X10^{-8}$ mol/L $\Delta\alpha_{2,1}$	d
	$1.0X10^{-6}$ mol/L $\Delta\alpha_{2,1}$	d

If we examine the response trajectory depicted in Figure 4.17 there appear to be two clusters, one distinctly in quadrant 4, and the other straddling the x-axis between quadrants 2 and 3. An examination of Table 4.3 shows that there is no difference in the $\Delta\alpha$ response of the effect of *Chlamydomonas musicola* on *Scenedesmus quadricauda* at either $1.0X10^{-8}$ or $5.0X10^{-8}$ mol/L atrazine. There are however, significant differences in the $\Delta\alpha_{1,2}$ responses at exposures of $1.0X10^{-7}$, and $5.0X10^{-7}$ moles of atrazine. When one compares the $\Delta\alpha_{2,1}$ responses for these two species we see no significant difference between any of the changes in the effects of *Scenedesmus quadricauda* on *Chlamydomonas musicola* at $1.0X10^{-8}$, $5.0X10^{-8}$, $1.0X10^{-7}$, or $5.0X10^{-7}$ moles of atrazine. This cluster is therefore primarily the result of the consistent response in the competitive abilities of *Scenedesmus quadricauda* to these low herbicide levels. The second cluster is made up of the $\Delta\alpha$ responses of these two species to the two highest levels of atrazine exposure, $8.2X10^{-7}$ and $1.0X10^{-6}$ moles. There was no significant differences in either of the $\Delta\alpha$ responses of these two species to these two levels of atrazine. There were however significantly different responses between all the $\Delta\alpha$ points in the low herbicide cluster and those in the high herbicide cluster. This two-species system seems to have two different responses, one to low level exposure, and one at the higher herbicide level. The clustering seems to be controlled for the most part by the responses of *Scenedesmus quadricauda*, with *Chlamydomonas musicola* showing a more graded response pattern through the lower atrazine levels. At lower atrazine exposures both species seem to be able to improve there competitive abilities with each other. From the ratios of ($\Delta\alpha_{2,1}$ / $\Delta\alpha_{1,2}$) reported in Table 4.3 it can be seen that for the low herbicide cluster all the ratios

are less than one. This would indicate that *Chlamydomonas musicola* has gained an advantage over *Scenedesmus quadricauda* under those low levels of herbicide. The other cluster straddles the x-axis which would indicate that the effect *of Scenedesmus quadricauda* on *Chlamydomonas musicola* has changed little as compared to the zero herbicide control. On the other hand, the effect of *Chlamydomonas musicola* on *Scenedesmus quadricauda* has increased substantially, giving *Chlamydomonas musicola* a distinct competitive advantage at these higher atrazine levels. From this pattern of response, one would expect *C. musicola* to gain a slight competitive advantage over *Scenedesmus quadricauda* at lower atrazine levels, and to gain a greater edge when the herbicide levels rise above $5.0X10^{-7}$ mol/L.. It would seem that the pattern in the change in the competition coefficients could be used as a predictive tool in determining the potential changes in the relative abundance's of these two species in the presence of different levels of atrazine.

Scenedesmus quadracauda* and *Chlorella vulgaris

Figures 4.12 and 4.13 illustrate the dose responses of between *Scenedesmus quadracauda* and *Chlorella vulgaris* to the herbicide atrazine when each is grown separately and together in semi-continuous culture.

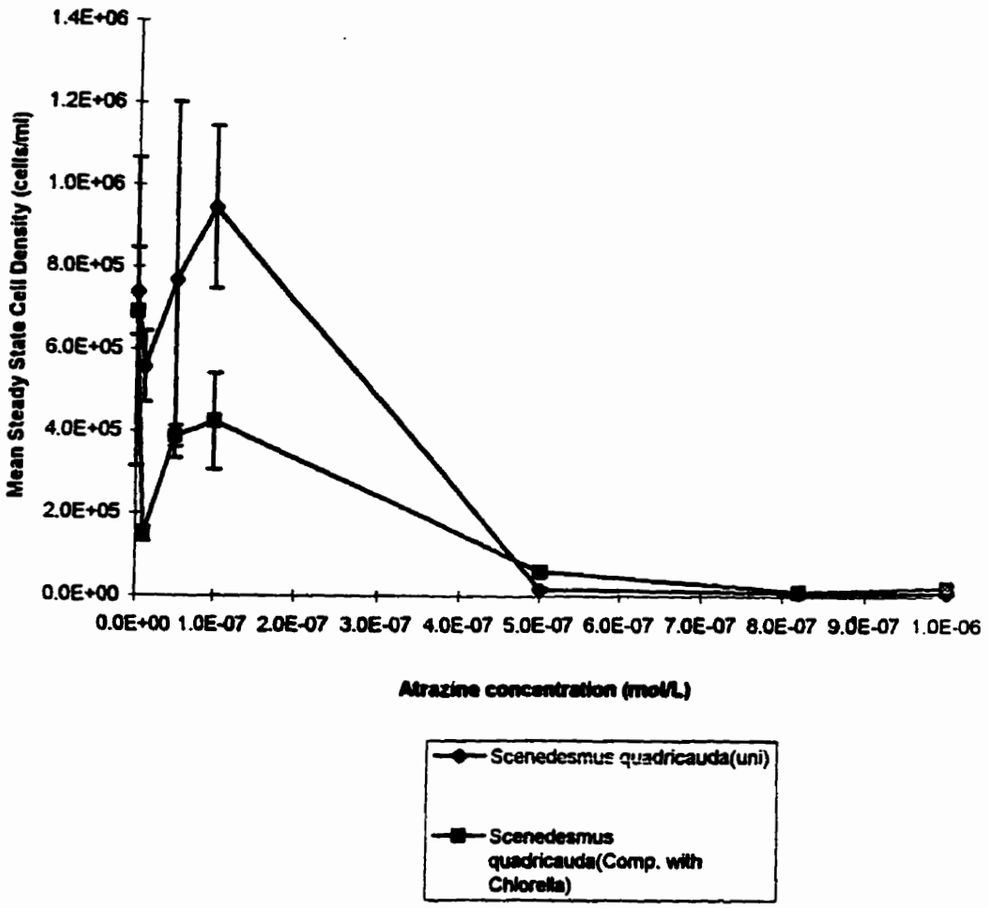


Figure 4.12 Dose responses of *Scenedesmus quadricauda* to the herbicide atrazine in unialgal culture and in competition with *Chlorella vulgaris*. Error bars represent the standard deviations about the mean maximum steady state cell densities.

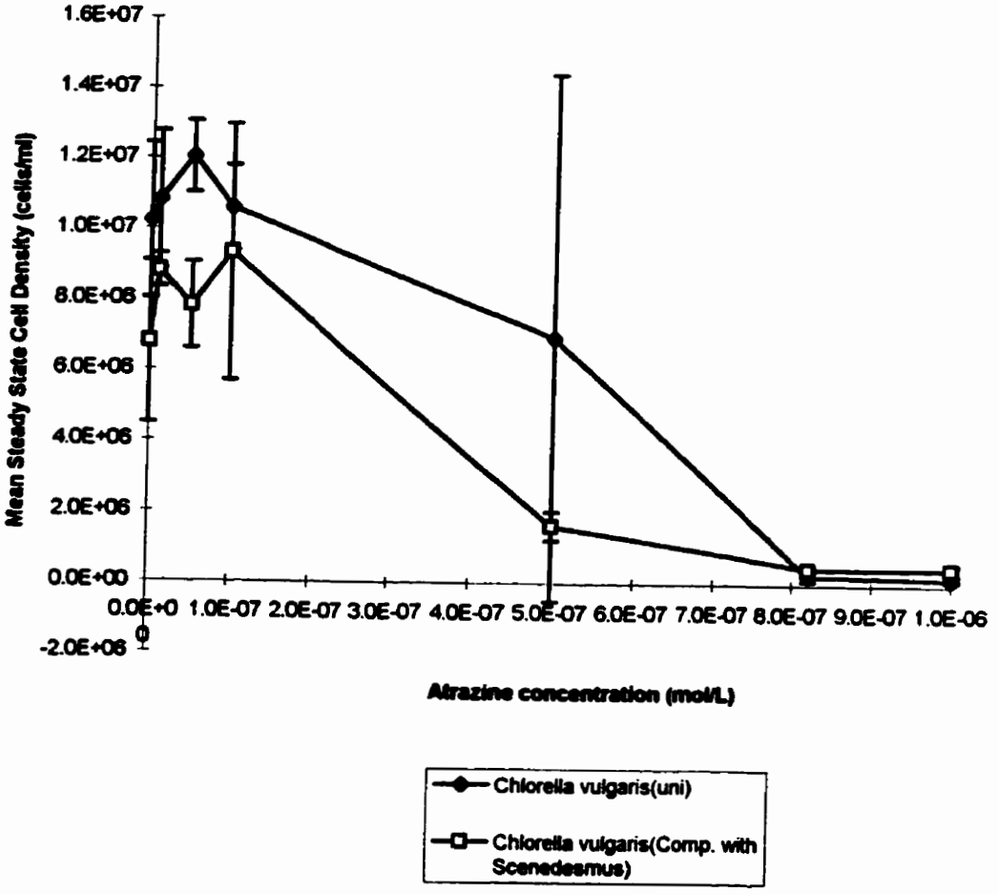


Figure 4.13 Dose responses of *Chlorella vulgaris* to the herbicide atrazine in unialgal culture and in competition with *Scenedesmus quadricauda*. Error bars represent the standard deviations about the mean maximum steady state cell densities.

A very unexpected result can be seen in figure 4.12. At $5.0X10^{-7}$ mol/L atrazine, when mean steady state cell densities were compared using an approximate randomization test, *Scenedesmus quadricauda* showed greater cell density in competition with *Chlorella vulgaris* than when exposed to this same herbicide level in unialgal culture (p=0.223). Each of these results suggests that there are more complex interactions at work in the competitive cultures than have been detected by previous toxicological protocols. With these phenomena in mind, I set out to determine how exposure to atrazine would effect the competitive interactions between these three test species

The results of the competition experiments between *Scenedesmus quadracauda* and *Chlorella vulgaris* showed a different pattern than was seen in the *Scenedesmus quadricauda* vs. *Chlamydomonas musicola* experiments. Table 4.4 summarizes these results.

Table 4.4 Summary of the competition coefficient between the two green algae *Scenedesmus quadricauda*, and *Chlorella vulgaris* at different concentrations of atrazine.

Species	Atrazine concentration (mol/L)	Mean $\alpha_{2,1}$	Standard deviation $\alpha_{2,1}$	Mean $\alpha_{1,2}$	Standard deviation $\alpha_{1,2}$
Chlorella(1) vs. Scenedesmus(2)					
	0	0.058	0.032	0.802	0.444
	$1.0X10^{-8}$	0.814	0.039	0.266	0.028
	$5.0X10^{-8}$	0.560	0.125	0.568	0.097
	$1.0X10^{-7}$	0.837	0.370	0.458	0.187
	$5.0X10^{-7}$	0.249	0.051	4.108	1.050
	$8.2X10^{-7}$	1.727	0.365	1.597	0.581
	$1.0X10^{-6}$	2.421	0.818	2.670	0.703

The $8.2X10^{-7}$, $1.0X10^{-6}$ mol/L alpha values for each species are all greater than one. This would tend to indicate that each species benefits from the presence of the other at these higher atrazine levels. Also, *Scenedesmus quadricauda* seems to be considerably better off at $5.0X10^{-7}$ mol/L atrazine the presence of *Chlorella vulgaris* than it was in unialgal culture at that concentration. In order to look for a pattern in the alpha values with increasing atrazine concentration the mean alpha values for each species at each atrazine concentration were graphed against each other (figure 4.14).

In the *Scenedesmus quadricauda* vs. *Chlorella vulgaris* competition experiments, (Figure 4.14), it is at this same atrazine level ($5.0X10^{-7}$ mol/L), that *Chlorella vulgaris'* ability to compete with *Scenedesmus quadricauda* was dramatically greater than that under no atrazine stress. At $5.0X10^{-7}$ mol/L the competitive ability of *Scenedesmus quadricauda* did increase above the level seen under no herbicide stress. These results would indicate that under phosphate limitation, and at a herbicide concentration of $5.0X10^{-7}$ mol/L, *Chlorella vulgaris* should exclude *Scenedesmus quadricauda.*

The $\Delta\alpha$ values for each species at each herbicide level from those at the no herbicide level for the *Scenedesmus quadricauda* vs. *Chlorella vulgaris* competition experiments are presented in table 4.5. Figure 4.15 graphically presents these changes in the alpha values ($\Delta\alpha$) at each atrazine level for the *Scenedesmus quadricauda* vs. *Chlorella vulgaris* competition experiments.

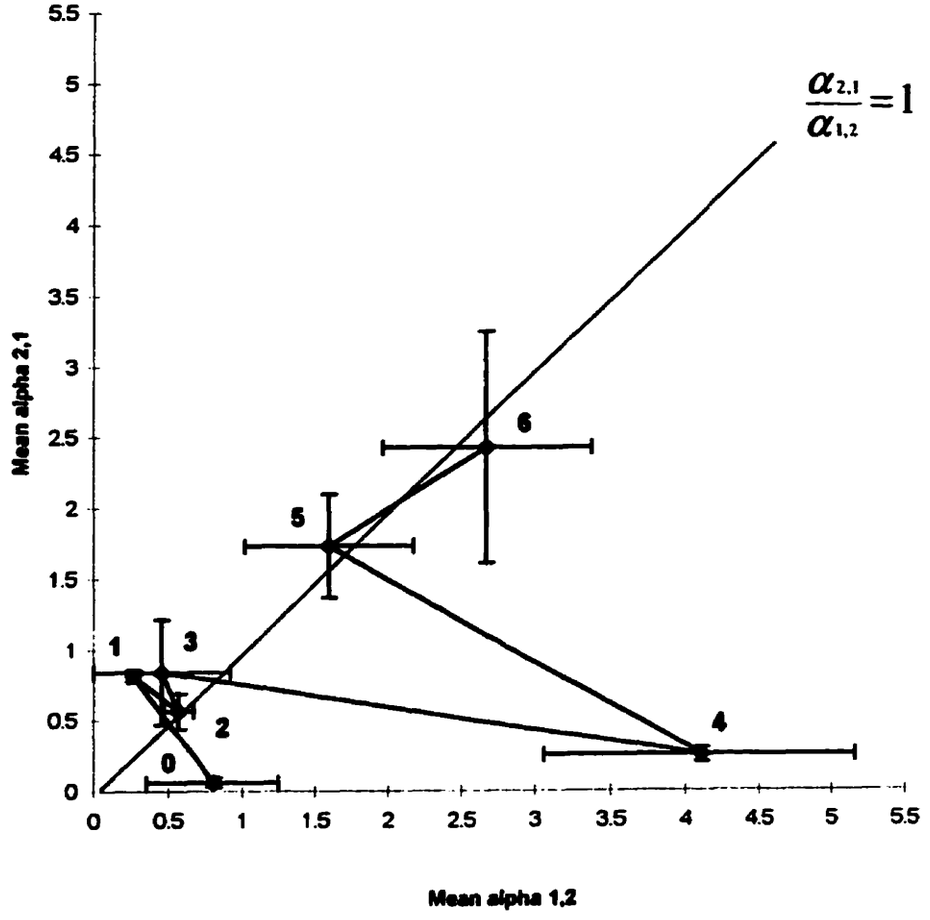


Figure 4.14 Trajectory of alpha values with increasing atrazine concentrations for *Chlorella vulgaris* (1) vs. *Scenedesmus quadricauda* (2). Numbers indicate increasing herbicide levels (0 = no herbicide). Error bars equal one standard deviation from the mean.

Table 4.5 Summary of the $\Delta\alpha$ responses, Euclidean distance (length), and ratios ($\Delta\alpha_{2,1}/\Delta\alpha_{1,2}$) of response vectors for each species pair at differing herbicide concentrations.

Species	Atrazine concentration (mol/L)	Delta $\alpha_{2,1}$	Delta $\alpha_{1,2}$	Response Vector Length	Ratio ($\Delta\alpha_{2,1}$ /$\Delta\alpha_{1,2}$)
Scenedesmus (2) vs. Chlorella (1)					
	$1.0X10^{-8}$	0.756	-0.535	0.926	-1.411
	$5.0X10^{-8}$	0.502	-0.234	0.554	-2.142
	1.0X10-^{7}	0.779	-0.344	0.851	-2.260
	$5.0X10^{-7}$	0.191	3.305	3.311	0.057
	$8.2X10^{-7}$	1.669	0.795	1.849	2.099
	$1.0X10^{-6}$	2.363	1.867	3.012	1.265

From an examination of these $\Delta\alpha$ response trajectory in figure 4.15, once again we see two distinct clusters of points. In order to determine whether these clusters represent distinct responses, and to determine which species has been most severely effected by the change in herbicide concentration, each element of each ordered number pair was compared to the same elements of the other ordered number pairs using the approximate randomization method. Tables 4.6 presents the results of this analysis.

From the graphical representation in figure 4.15 there is a tight cluster of $\Delta\alpha$ response points in quadrant 3, corresponding to the three lowest herbicide concentrations tested ($1.0X10^{-8}$ $5.0X10^{-8}$ and $1.0X10^{-7}$ mol/L). There is a less clustered but distinctly different set of $\Delta\alpha$ response points in quadrant 2, corresponding to the three highest herbicide concentrations tested ($5.0X10^{-7}$ $8.2X10^{-7}$ and $1.0X10^{-6}$ mol/L). Within the two response clusters in Figure 4.15 there is also a difference in the strength of the responses.

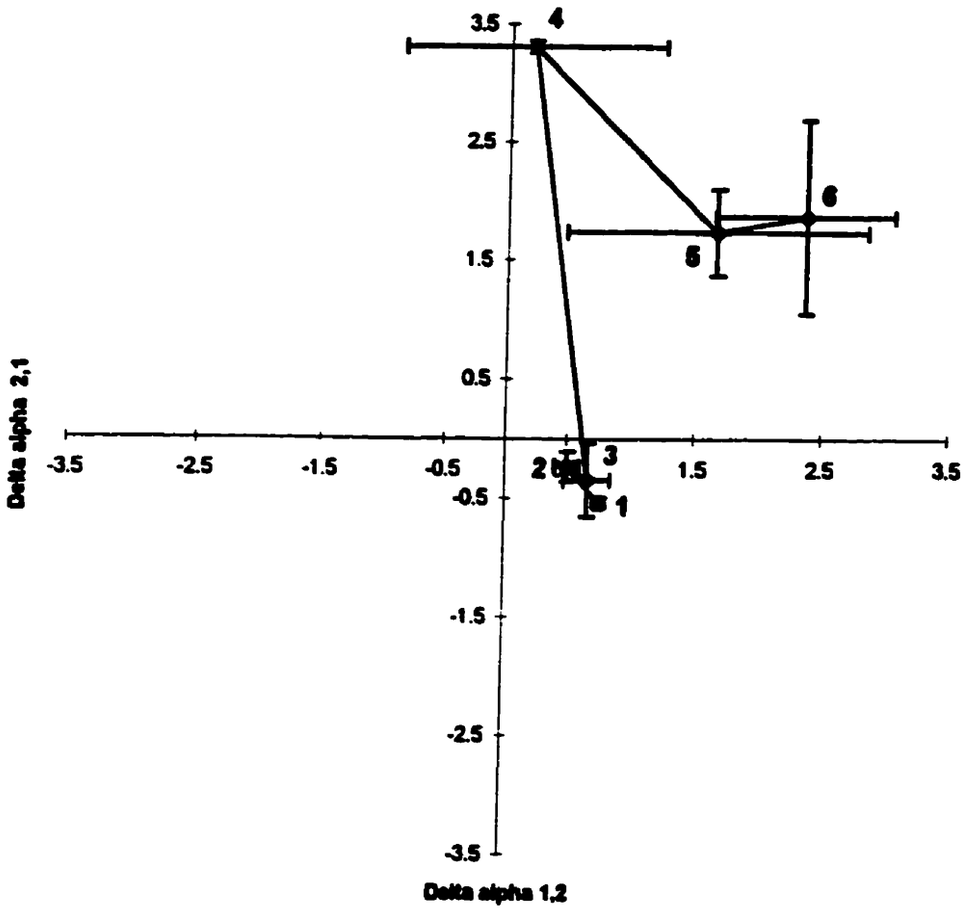


Figure 4.15 Trajectory of the $\Delta\alpha$ values with increasing atrazine concentration, for *Scenedesmus quadricauda* (species 2) and *Chlorella vulgaris* (species 1). Numbers represent the following atrazine levels; 1=1.0X10^{-8} mol/L, 2=5.0X10^{-8} mol/L, 3=1.0X10^{-7} mol/L, 4=5.0X10^{-7} mol/L, 5=8.2X10^{-8} mol/L and 6=1.0X10^{-6} mol/L. Error bars represent one standard deviation from the mean. Horizontal error bars indicate the variation of the $\Delta\alpha_{2,1}$ estimates, and the vertical error bars indicate the variation in the $\Delta\alpha_{1,2}$ estimates.

Table 4.6 A comparison of the $\Delta\alpha$ response patterns for the appearance of clustered responses to differing concentrations of atrazine, for all competing species pairs. The coordinates of each point are ordered numbered pairs consisting of the values ($\Delta\alpha_{2,1}$, $\Delta\alpha_{1,2}$). Therefore "C $\Delta\alpha_{1,2}$ " refers to the change in the competition coefficient (effect of species 1 on the carrying capacity of species 2) produced at an atrazine concentration of $5.0X10^{-7}$ mol/L. as compared to the $\alpha_{1,2}$ value produced by that species pair when no herbicide is present. The null hypothesis being tested is that there is no difference between any two elements. Each element pair was compared using approximate randomization test. Level of significance was p=0.01. Elements with the same lower case letter were not significantly different whereas elements with different lower case letters were.

Species	Ordered pair element	Significance
Scenedesmus quadricauda(2)	$1.0X10^{-8}$ mol/L $\Delta\alpha_{1,2}$	
vs.	$5.0X10^{-8}$ mol/L $\Delta\alpha_{1,2}$	a
Chlorella vulgaris(1)	$1.0X10^{-7}$ mol/L $\Delta\alpha_{1,2}$	a
	$5.0X10^{-7}$ mol/L $\Delta\alpha_{1,2}$	
	$8.2X10^{-8}$ mol/L $\Delta\alpha_{1,2}$	
	$1.0X10^{-6}$ mol/L $\Delta\alpha_{1,2}$	
	$1.0X10^{-8}$ mol/L $\Delta\alpha_{2,1}$	b
	$5.0X10^{-8}$ mol/L $\Delta\alpha_{2,1}$	c
	$1.0X10^{-7}$ mol/L $\Delta\alpha_{2,1}$	bc
	$5.0X10^{-7}$ mol/L $\Delta\alpha_{2,1}$	
	$8.2X10^{-8}$ mol/L $\Delta\alpha_{2,1}$	
	$1.0X10^{-6}$ mol/L $\Delta\alpha_{2,1}$	

The low herbicide response shows very little shift from the origin, while the high herbicide levels produce a very strong response. Once again, it would appear that below a certain concentration of toxin the system behaves in one way, and above that concentration of toxin the system behaves quite differently.

The quadrant 3 low herbicide response cluster in figure 4.15 shows that under these low herbicide levels, the effect that *Scenedesmus quadricauda* had on the carrying capacity of *Chlorella vulgaris* has decreased, while the effect *Chlorella vulgaris* had on

the carrying capacity of *Scenedesmus quadricauda* increases. At high atrazine levels the responses have shifted into quadrant 2 in the model. This indicates that under this high herbicide stress, each of these two species is more capable of reducing the others' carrying capacity. However each species is still having some negative impact on the other, so there is still a question of which (if either) has a competitive advantage. At an atrazine concentration of $8.2X10^{-7}$ and $1.0X10^{-6}$ mol/L., the ratio of $\Delta\alpha_{2,1}/\Delta\alpha_{1,2}$ is greater than 1 (Table 4.4), which puts the ordered pair below the 45° line, indicating in this case that *Scenedesmus quadricauda* has gained the substantial competitive advantage. However at an atrazine concentration of $5.0X10^{-7}$ mol/L., the $\Delta\alpha_{2,1}/\Delta\alpha_{1,2}$ was substantially below one. At this concentration of herbicide *Chlorella vulgaris* has the competitive advantage.

Chlamydomonas musicola and *Chlorella vulgaris*

Figures 4.16 and 4.17 illustrate the dose responses of between *Scenedesmus quadracauda* and *Chlorella vulgaris* to the herbicide atrazine when each is grown separately and together in semi-continuous culture.

Table 4.7 presents the competition coefficients between *Chlamydomonas musicola* and *Chlorella vulgaris* at the different herbicide levels. Figure 4.18 presents the trajectory of these alpha values with increasing atrazine concentrations for the *Chlamydomonas musicola* and *Chlorella vulgaris* competition experiments.

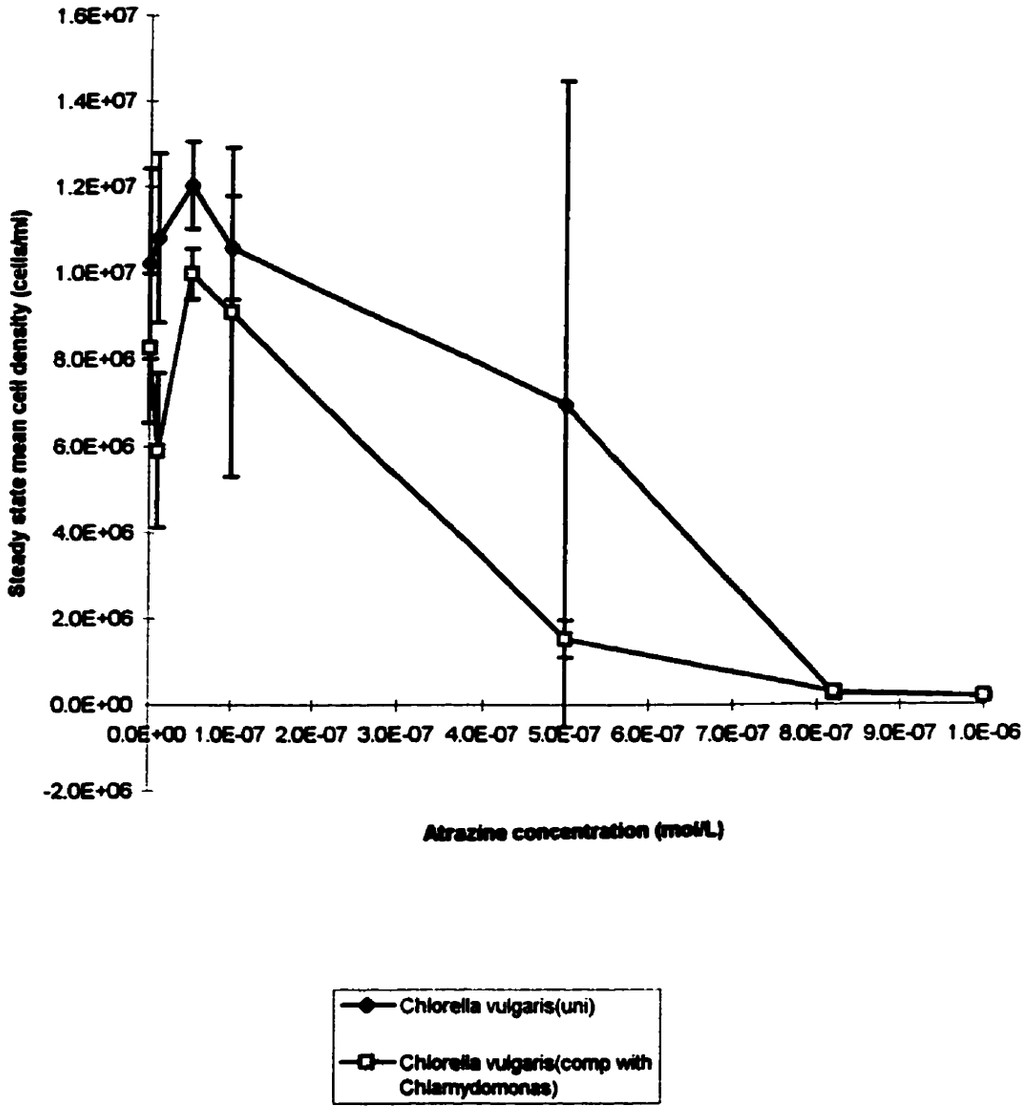


Figure 4.16 Dose responses of *Chlorella vulgaris* to the herbicide atrazine in unialgal culture and in competition with *Chlamydomonas musicola*. Error bars represent the standard deviations about the mean maximum steady state cell densities.

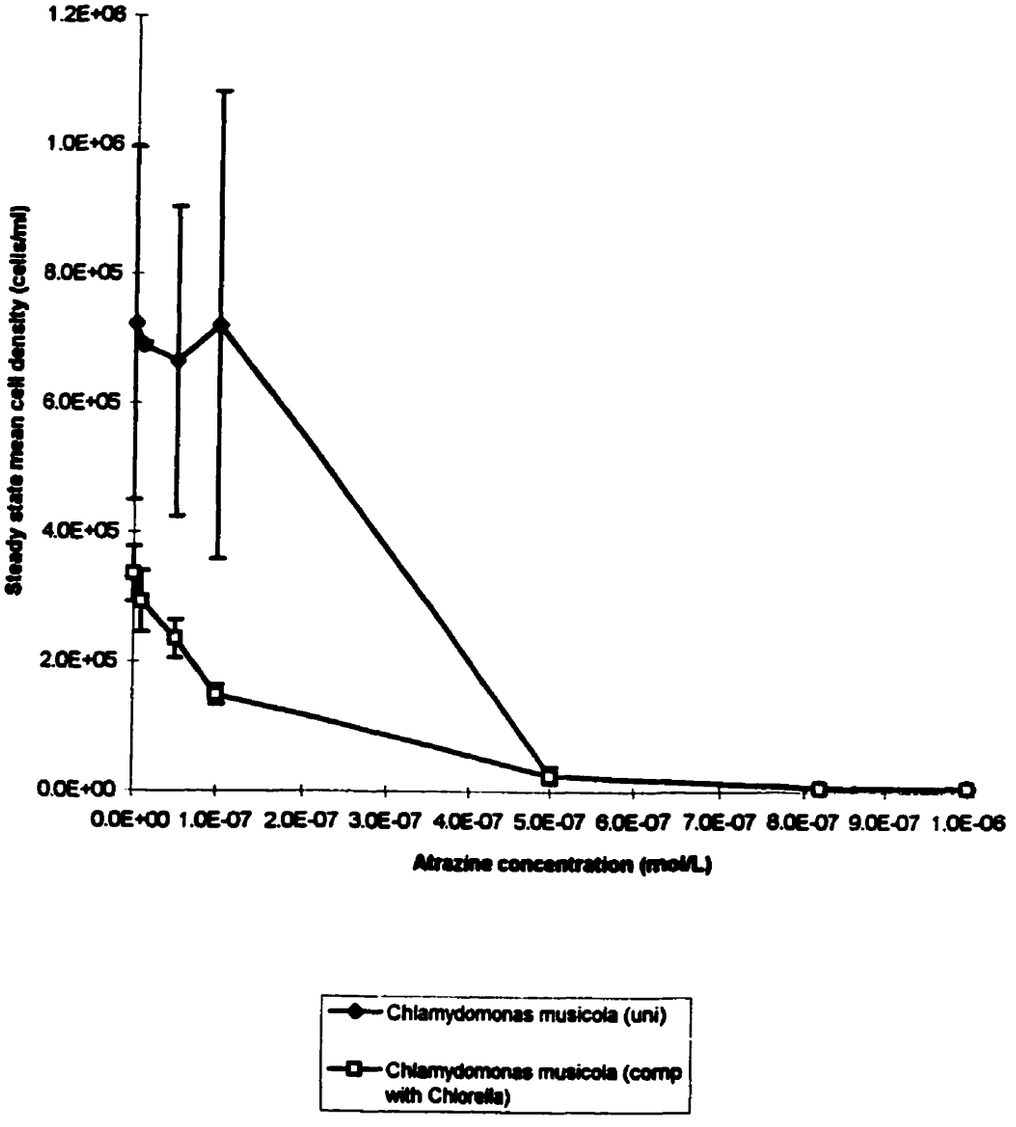


Figure 4.17 Dose responses of *Chlamydomonas musicola* to the herbicide atrazine in unialgal culture and in competition with *Chlorella vulgaris*. Error bars represent the standard deviations about the mean maximum steady state cell densities.

Table 4.7 Summary of the competition coefficient between the two green algae *Chlamydomonas musicola*, and *Chlorella vulgaris* at different concentrations of atrazine.

Species	Atrazine concentration (mol/L)	Mean $\alpha_{2,1}$	Standard deviation $\alpha_{2,1}$	Mean $\alpha_{1,2}$	Standard deviation $\alpha_{1,2}$
Chlamydomonas(1) vs. Chlorella(2)					
	0	0.464	0.055	0.808	0.156
	$1.0X10^{-8}$	0.424	0.069	0.545	0.165
	$5.0X10^{-8}$	0.244	0.120	0.742	0.138
	$1.0X10^{-7}$	0.297	0.114	0.903	0.348
	$5.0X10^{-7}$	0.809	0.340	0.136	0.099
	$8.2X10^{-7}$	0.741	0.128	0.862	0.313
	$1.0X10^{-6}$	0.650	0.144	1.297	0.648

In the competition experiments between *Chlamydomonas musicola* and *Chlorella vulgaris*, (Figure 4.18), *C. musicola* appears to have the competitive edge at all atrazine levels except $5.0X10^{-7}$ mol/L. Such shifts in competitive equilibria would not be predicted from standard single-species bioassays. However these results do have some interesting implications for population dynamics. At intermediate levels of atrazine contamination these results indicate that there could be a dramatic shift in the relative abundance of these two species.

Table 4.8 presents the $\Delta\alpha$ values for the between *Chlamydomonas musicola* and *Chlorella vulgaris*, competition experiments. If we take the $\Delta\alpha$ values from table 4.8, and plot them as ordered pairs ($\alpha_{2,1}$, $\alpha_{1,2}$) we obtain a trajectory of the changes in the competition coefficients with changing atrazine concentrations. Figure 4.19 shows the trajectory of $\Delta\alpha$ values for the *Chlamydomonas musicola* and *Chlorella vulgaris*, competition experiments.

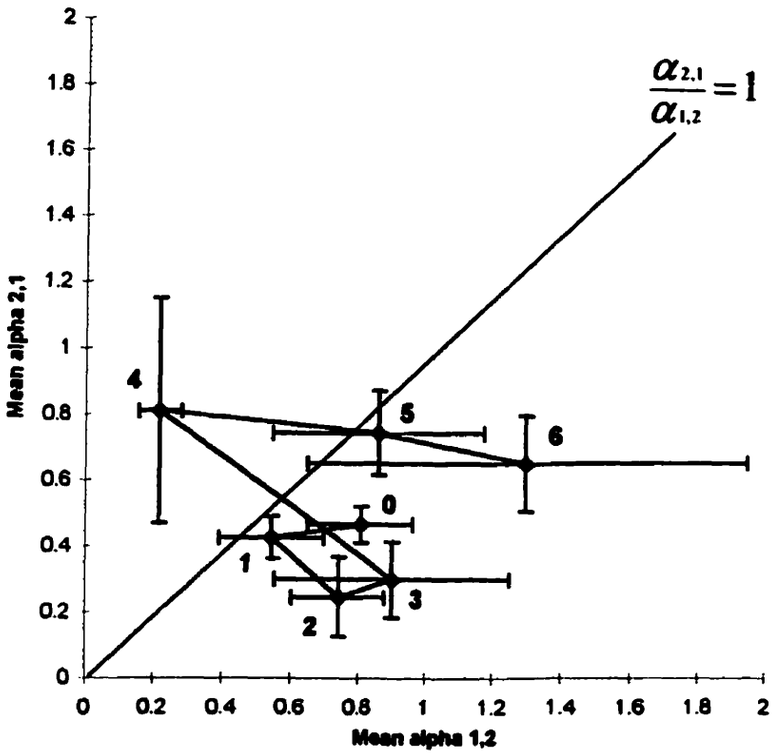


Figure 4.18 Trajectory of alpha values with increasing atrazine concentrations for *Chlamydomonas musicola* (1) vs. *Chlorella vulgaris* (2). Numbers indicate increasing herbicide levels (0 = no herbicide). Error bars equal one standard deviation from the mean.

Table 4.8 Summary of the $\Delta\alpha$ responses, Euclidean distance (length), and ratios ($\Delta\alpha_{2,1}/\Delta\alpha_{1,2}$) of response vectors for the *Chlamydomonas musicola* and *Chlorella vulgaris* species pair at differing herbicide concentrations.

Species	Atrazine concentration (mol/L)	Delta $\alpha_{2,1}$	Delta $\alpha_{1,2}$	Response Vector Length	Ratio ($\Delta\alpha_{2,1}$ /$\Delta\alpha_{1,2}$)
Chlorella (2) vs. Chlamydomonas(1)					
	$1.0X10^{-8}$	-0.039	-0.262	0.264	0.148
	$5.0X10^{-8}$	-0.218	-0.066	0.227	3.303
	1.0X10-^{7}	-0.166	0.095	0.191	-1.744
	$5.0X10^{-7}$	0.345	-0.672	0.755	-0.514
	$8.2X10^{-7}$	0.278	0.054	0.283	5.150
	$1.0X10^{-6}$	0.186	0.488	0.522	0.3.83

Once again from an analysis of these $\Delta\alpha$ response trajectories (figure 4.19), one can detect two distinct clusters of points. In order to determine whether these clusters represent distinct responses, and to determine which species has been most severely effected by the change in herbicide concentration, each element of each ordered number pair was compared to the same elements of the other ordered number pairs using the approximate randomization method. Tables 4.9 presents the results of this analysis.

From the graphical representation in Figure 4.19 we can see that $\Delta\alpha$ trajectory of increasing herbicide concentrations tracks from quadrant 4 back into quadrant 3. The trajectory then reverses and moves into quadrant 1, then tracks on into quadrant 2. This response pattern is unlike that of either of the other two test species pairs. At the two lowest atrazine concentrations, ($1.0X10^{-8}$ and $5.0X10^{-8}$ mol/L), both species show a slight decrease in their abilities to reduce the carrying capacity of the other, with *Chlamydomonas musicola* having the advantage at the lowest atrazine concentration.

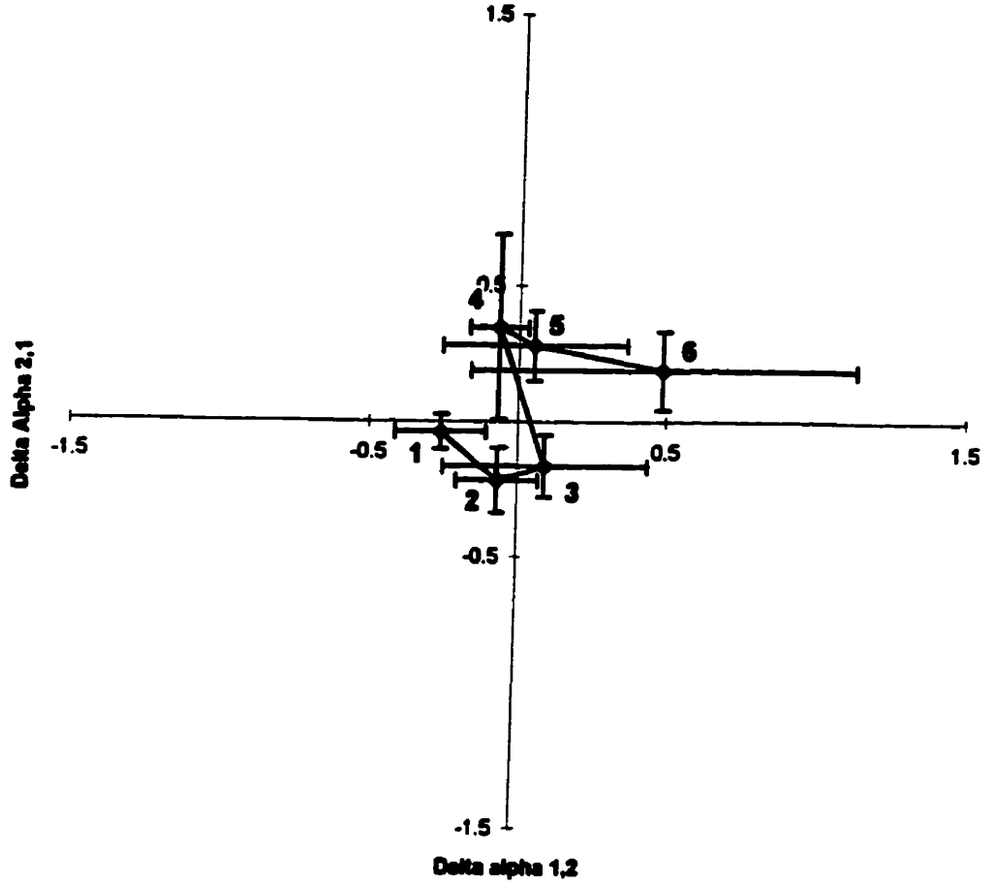


Figure 4.19 Trajectory of the $\Delta\alpha$ values with increasing atrazine concentration, for *Chlorella vulgaris* (species 2) and *Chlamydomonas musicola* (species 1). Numbers represent the following atrazine levels; 1=$1.0X10^{-8}$ mol/L, 2=$5.0X10^{-8}$ mol/L, 3=$1.0X10^{-7}$ mol/L, 4=$5.0X10^{-7}$ mol/L, 5=$8.2X10^{-8}$ mol/L and 6=$1.0X10^{-6}$ mol/L. Error bars represent one standard deviation from the mean. Horizontal error bars indicate the variation of the $\Delta\alpha_{2,1}$ estimates, and the vertical error bars indicate the variation in the $\Delta\alpha_{1,2}$ estimates.

Table 4.9 A comparison of the $\Delta\alpha$ response patterns for the appearance of clustered responses to differing concentrations of atrazine, for all competing species pairs. The coordinates of each point are ordered numbered pairs consisting of the values ($\Delta\alpha_{2,1}$, $\Delta\alpha_{1,2}$). Therefore "C $\Delta\alpha_{1,2}$" refers to the change in the competition coefficient (effect of species 1 on the carrying capacity of species 2) produced at an atrazine concentration of $5.0X10^{-7}$ mol/L. as compared to the $\alpha_{1,2}$ value produced by that species pair when no herbicide is present. The null hypothesis being tested is that there is no difference between any two elements. Each element pair was compared using approximate randomization test. Level of significance was p=0.01. Elements with the same lower case letter were not significantly different whereas elements with different lower case letters were.

Species	Ordered pair element	Significance
Chlorella vulgaris(2)	$1.0X10^{-8}$ mol/L $\Delta\alpha_{1,2}$	a
vs.	$5.0X10^{-8}$ mol/L $\Delta\alpha_{1,2}$	ad
Chlamydomonas musicola(1)	$1.0X10^{-7}$ mol/L $\Delta\alpha_{1,2}$	ad
	$5.0X10^{-7}$ mol/L $\Delta\alpha_{1,2}$	
	$8.2X10^{-8}$ mol/L $\Delta\alpha_{1,2}$	d
	$1.0X10^{-6}$ mol/L $\Delta\alpha_{1,2}$	d
	$1.0X10^{-8}$ mol/L $\Delta\alpha_{2,1}$	
	$5.0X10^{-8}$ mol/L $\Delta\alpha_{2,1}$	b
	$1.0X10^{-7}$ mol/L $\Delta\alpha_{2,1}$	b
	$5.0X10^{-7}$ mol/L $\Delta\alpha_{2,1}$	c
	$8.2X10^{-8}$ mol/L $\Delta\alpha_{2,1}$	c
	$1.0X10^{-6}$ mol/L $\Delta\alpha_{2,1}$	c

At $5.0X10^{-8}$ mol/L the ratio of $\Delta\alpha_{2,1}/\Delta\alpha_{1,2}$ is less than 1 indicating that *Chlorella vulgaris* now has the competitive edge. At $1.0X10^{-7}$ mol/L atrazine the $\Delta\alpha$ response of this species pair shifts into quadrant 3. This shift is due to a increase in the ability of *Chlamydomonas musicola* to affect the carrying capacity of *Chlorella vulgaris*. At the same time, *Chlorella vulgaris* shows a similar decrease in its $\alpha_{2,1}$ value at this atrazine concentration, compared to the no herbicide control, as was seen at the two previous atrazine levels. At the next highest atrazine level, $5.0X10^{-7}$ mol/L, the effect of

Chlamydomonas musicola on *Chlorella vulgaris* has significantly decreased over the no herbicide control, while *Chlorella vulgaris* has had its competitive ability increased above that of the no herbicide control level. At $5.0X10^{-7}$ mol/L, the ratio of $\Delta\alpha_{2,1}/\Delta\alpha_{1,2}$ is greater than -1. At this atrazine level that *Chlorella vulgaris* achieves a competitive advantage over *Chlamydomonas musicola*. As the herbicide levels rise to $8.0X10^{-7}$ the response shifts into quadrant 1 with *Chlorella vulgaris* maintaining its competitive edge. At the highest herbicide level, $1.0X10^{-6}$ mol/L, *Chlamydomonas musicola*, once again reestablishes its competitive superiority due to the reduction in the competitive abilities of *Chlorella vulgaris*. This $\Delta\alpha$ response pattern seems to be due primarily to the two clusters of $\Delta\alpha_{2,1}$ values of *Chlorella vulgaris*. While there is little significant change in the effects of *Chlamydomonas musicola* on *Chlorella vulgaris* over the range of atrazine concentrations examined.

Conclusions

From these competition experiments there appear to be two important characteristics in the way the competition coefficients change with respect to herbicide concentration. One interesting characteristic is the apparent "threshold" of the responses in some species pairs. The competitive abilities of the species pairs respond in much the same way over a range of lower herbicide stresses. However when the atrazine level exceed a certain concentration, the competitive abilities of the test species shift to distinctly different equilibria. This high level response pattern remains relatively consistent over the range of these high herbicide levels. This result may not seem particularly

startling. This type of threshold response is quite common at the organismal level. In Figure 3.1 of this thesis we saw that the organismal threshold response of *Chlamydomonas musicola* to atrazine expressed as % inhibition was between $1.0X10^{-7}$ and $5.0X10^{-7}$ mol/L. Figure 3.2 shows a similar physiological threshold for *Scenedesmus quadricauda* while Figure 3.3 indicates that the threshold may be slightly higher for *Chlorella vulgaris*. It is interesting to note that the threshold responses seen in competition (Figures 4.11, 4.15 and 4.19) show a similar pattern. The % inhibition curves in Figures 3.1, 3.2 and 3.3 have the classic sigmoidal shape. The sigmoidal shaped dose response curve is probably the most common response seen in organismal toxicology. The results of these experiments serve to illustrate that this type of response may be an inherent characteristic of living systems at higher levels of organization as well. I would submit that if the threshold response is such a pervasive characteristic of living systems, and if we are ever going to understand how these systems behave under the assault of xenobiotics, then we must include this phenomenon into our analysis and models.

In searching for an explanation of this threshold effect I begin to wonder if at high atrazine levels competition is occurring in these systems. One can see from figures 4.7 , 4.8 and 4.13 that for each species grown in unialgal semi-continuous culture, at the highest atrazine levels there is a decrease in the carrying capacity by approximately 4 orders of magnitude. At the low concentrations of atrazine the carrying capacity is quite high, and presumably phosphorus is the limiting resource. These species should then be competing for phosphorus at the lower atrazine concentrations. However at the highest concentrations of atrazine, its direct toxic effects reduces the population densities of each

species to such low levels, it is unlikely that the demand of phosphorus would exceed its supply. Therefore at high atrazine concentrations, competition for phosphorus would not occur.

In these experiments I used competition coefficients to measure the interactions between the species. If competition is not occurring at the high herbicide levels, then what are these "competition coefficients" measuring? Clearly there is some sort of interaction between the algae, but if it is not competition for phosphorus then what type of interaction is it? One possibility is the interaction is one of detoxification of the atrazine by one species which serves to lower the exposure level of the other species. It may be possible that the detoxification of the herbicide by one species may be beneficial to the other species. It is also possible that if these species have been exposed to atrazine for an extended period of time such a "detoxification interaction" may have evolved as an important component in this ecosystem. It might be better to refer to the "competition coefficients" measured in these experiments as "interaction coefficients".

A second important result of these experiments was the illustration of how different were the changes in competitive abilities of a given species under herbicide stress, in one competitive system compared to another. The fluctuations in the $\Delta\alpha$ values of *Chlamydomonas musicola* in competition with *Chlorella vulgaris* are very different from the fluctuations in the $\Delta\alpha$ values of *Chlamydomonas musicola* in competition with *Scenedesmus quadricauda.* As well the $\Delta\alpha$ values of *Chlorella vulgaris* in competition with *Chlamydomonas musicola* changed very little over the range of atrazine concentrations examined, whereas they change to a much greater degree in competition

with *Scenedesmus quadricauda*. Again this result should not be surprising. Each species will have different abilities to acquire and utilize a limiting resource, and each species may also show different susceptibilities to the toxic effects of any given xenobiotic. What is important, is that these experiments illustrate that the influence of toxic materials in an ecosystems on the competitive processes can produce outcomes that are entirely different from those predicted from unialgal bioassay methods.

This brings us to the heart of this analysis, the "Delta alphas" ($\Delta\alpha$) or change in the competition coefficients in response to a herbicide stress, as compared to their competitive abilities with no herbicide present. From an examination of Figures 4.11, 4.15 and 4.19, it would appear that the change in the competitive coefficients between two species due to the stress of a particular herbicide will be unique to that pair of species, and possibly unique to that herbicide. Changes in the competitive coefficient between species 1 and species 2, have no influence on changes in the competitive coefficients between species 1 and species 3. After the $\Delta\alpha$'s of a great many species pairs have been investigated, we may find that there are groups of species that all respond in much the same way to herbicide stress, when paired with members of other groups.

Changes in the steady state equilibrium cell densities of the two competing algal species, as a result of an added herbicide, are due in part to the direct effects of the herbicide on each species, i.e. direct reduction of a species' carrying capacity, and also due in part to the effects of the herbicide on the relative ability of each species to reduce the carrying capacity of the other. Traditional unialgal bioassay systems have adequately

measured the first component of this equilibrium change, the $\Delta\alpha$'s from this competitive bioassay protocol can now measure the second.

These experiments illustrate the responses of only three phytoplankton species, to the effects of one herbicide. Obviously if we are ever to understand the effects of xenobiotics on natural ecosystems, we need to examine the competitive responses of more species from across many taxonomic groups, to toxins of many different modes of action.

Chapter 5

A model of phytoplankton population dynamics in a two species competitive system under atrazine stress

A. Abstract

A correlative model which relates the toxic effects of various concentrations of the herbicide Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine), on the competitive abilities of three Chlorophycean phytoplankton was developed. The dose responses of the competition coefficients (α) showed a distinct biphasic response. As a result of this threshold effect, the model established and used different relationships for the changes in the α values above and below the threshold atrazine concentration. Using this threshold model, simulation experiments were conducted, and it was found that the initial population density of the competing species had a dramatic effect on the final outcome of the competition under different herbicide concentrations.

B. Introduction

The classic Lotka-Volterra competition models have always assumed a constant set of competitive coefficients (α) at all population levels. From the results of the experiments described in chapter 5, we have seen that a change in the concentration of atrazine can dramatically change the competition coefficients, thus altering the competitive equilibrium of the system. In this chapter I will endeavor to take the results of chapter 5 and incorporate the relationship between changes in the competition coefficients ($\Delta\alpha$), and changes in atrazine concentration, for each species pair, into the Lotka-Volterra equations for two species competition. The purpose of the model will be to predict the outcome of the competition between two test species at any given atrazine level.

The model that I will develop in this chapter would best be described as a correlative model, as this term is defined in Gold (1977). By definition, this type of model is required only to reflect an observed relationship between two (or more) variables. Such a model functions to summarize that relationship, often so that the relationship can be verified. The model then could be used as a basis for prediction and control. In a sense, the two species, synthetic microcosms I have used in these experiments are simplified subsystems of a natural ecosystem. The correlative model of this individual subsystem becomes a part of the structure of an explanatory model of the entire system. This is the usual procedure when one wishes to explain the behaviour of a system in terms of the behaviour of its components. At this point we are only interested in how the behaviour of the individual components combine to produce the behaviour of the system. The ecologist may not be concerned (at first) with the question of why the organisms exhibit the

behaviour they do, but only with the question of how organisms with a given kind of behaviour interact. However, ecologists must eventually concern themselves with questions of why these subsystems behave the way they do.

It might be useful at this point to review some of the assumptions and components of this model. The basic question that this model was designed to address has to do with how the population dynamics of two algal species, competing for a limiting resource, would change with the addition of a herbicide. Figure 5.1 breaks the system down into its important components.

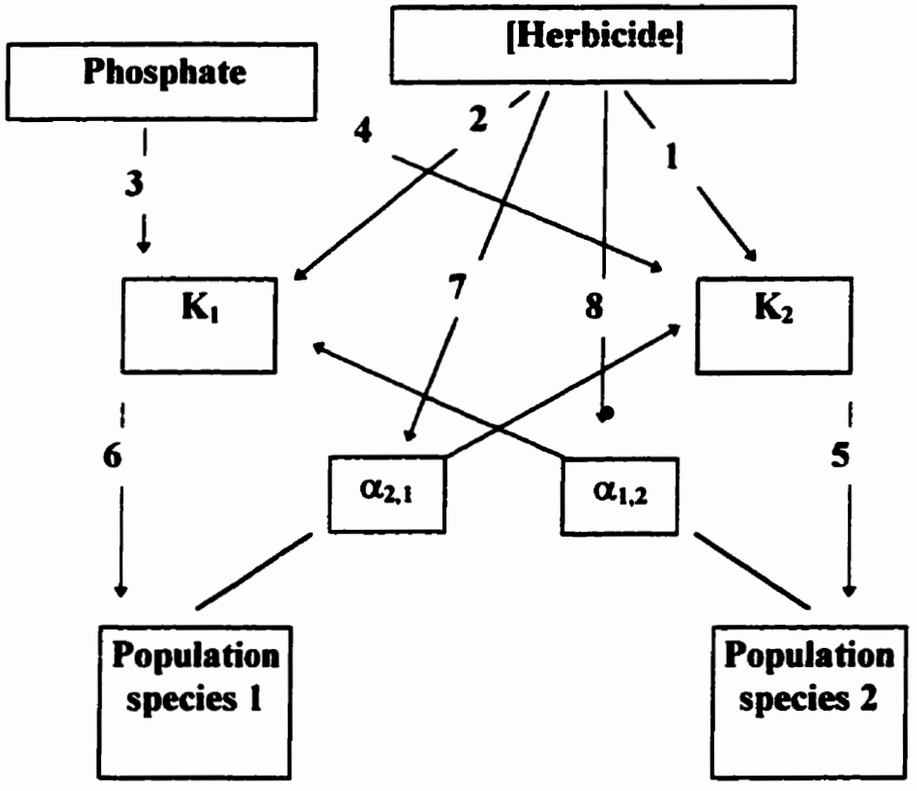


Figure 5.1 Graphic representation of a two species competitive system under the influence of a herbicide. See the following text for further explanation.

C. Materials and Methods

The mathematical representation of the model illustrated in Figure 5.1 is an extension of the Lotka-Volterra equations as described in an earlier chapter. These equations can be written in the form of a per capita rate of population change as follows:

$$\frac{dN_1}{N_1 dt} = r_1\left(\frac{K_1 - N_1 - \alpha_{2,1}N_2}{K_1}\right) \qquad (1)$$

$$\frac{dN_2}{N_2 dt} = r_2\left(\frac{K_2 - N_2 - \alpha_{1,2}N_1}{K_2}\right) \qquad (2)$$

As we saw in the last chapter the population density of each species is subtracted from the carrying capacity of that species to give a density dependent feedback term. As well, the population of the competitor is weighted by the competition coefficient $\alpha_{2,1}$ or $\alpha_{1,2}$ (the effect of species 2 on species 1, and the effect of species 1 on species 2 respectively). These alpha values are proportionality "constants" defining the amount of K_1 used by every individual of species 2 ($\alpha_{2,1}$), and the amount of K_2 used by each member of species 1 ($\alpha_{1,2}$). What we have seen from the results of chapter 4, is that these alpha values are not constant at all. They vary with atrazine levels, and most likely with many other environmental variables as well. In this model, we will include a series of equations derived from the empirical data from the two species semicontinuous culture herbicide experiments, which reflect the changes in these alpha values with changing atrazine levels. For each set of competition equations the first step will be to calculate the appropriate α

$$(N_1)_t + \Delta t = (N_1)_t + (\Delta N_1)_t \quad (7)$$

$$(N_2)_t + \Delta t = (N_2)_t + (\Delta N_2)_t \quad (8)$$

Within equations (5) and (6) there are three parameters that need to estimated. The growth rate (r) of the culture under the specific set of culture conditions, the carrying capacities (K_1, K_2) for each species under the specific culture conditions, and the competition coefficients ($\alpha_{2.1}$) and ($\alpha_{1.2}$). The growth rate (r) estimate should be made from the no herbicide, unialgal continuous culture experiments, using the mean of the steady state growth rates estimated from the day 5 to the end of the experiment data. Recall that once the continuous cultures have reached steady state on day 5, the growth rate will equal the dilution rate. Dilution rate is determined by the experimenter, so it is possible to establish any growth rate that the experimenter might wish to examine. In our experiments the mean steady state growth rate (r) was set at 0.2, by a 20% dilution rate. Remember that under steady state conditions the growth rate will be 0.2 regardless of the presence of the herbicide. Therefore growth rate will not vary with changing atrazine levels. The only potential complication with this continuous culture system might be at very high atrazine levels, growth could be so severely effected by the toxic effects of the herbicide that the growth rate would decline below the dilution rate and the culture could wash out. This was never the case at any of the atrazine concentration that were tested in these experiments. If one wished to use a continuous culture system to test the effects of an extremely high concentration of toxin, a two-stage chemostat could be used as was described in chapter two. Recall that our semicontinuous culture did not reach steady

atrazine concentration from the unialgal semicontinuous culture experiments for each test species.

The last parameters that we will need to estimate are the competition coefficients ($\alpha_{2,1}$) and ($\alpha_{1,2}$). As was seen in chapter 4, there is a relationship between the concentration of atrazine and the values of these competition coefficients for each pair of test species. It was also shown in chapter 4 that there was a threshold response involved in these relationships. In order to investigate the importance of this threshold effect, and to better understand the dynamics of this system, two separate approaches will be used to develop the relationships of alpha values vs. atrazine concentrations in this model. Two separate simulation models will be developed. In one, a curve will be fitted through the entire data set for lowest atrazine concentration to the highest. The second approach will be to divide the data set into "below the threshold" and "above the threshold". To each of these regions of the data plot we will fit a separate curve. This approach should more accurately simulate the differing responses of the system at low vs. high atrazine levels.

The three test species vary greatly in their cell volumes. Therefore a small increase in the cell density of a large species may actually generate a larger "biomass" increase, than a large cell density increase of a species with small volume. In order to investigate the relationship of the change in biomass of each species in competition measurements of cell length and width were made for each test species at each atrazine concentration in both unialgal and competitive experiments. These dimension measurements were made using an ocular micrometer. With these measurements the volumes of each test species were estimated assuming a standard geometric shape for each species. *Chlorella vulgaris*

was assumed to be a sphere, *Chlamydomonas musicola* was assumed to be an ellipsoid, and *Scenedesmus quadricauda* was assumed to be a cylinder. Where there appeared to be a change in cell volume with changing atrazine levels, the equation describing that relationship was incorporated into the biomass version of the model.

The model equations were entered into the a Microsoft Excel ®, spreadsheet. The simulations were designed to output four graphs. The first plot was of simulation data of cell densities of each competing species in cells per ml vs. time in days. The second graph plotted the same cell density data species 1 vs. species 2 species space. The final two graphs were the same as the first two with biomass, expressed as a volume in cubic meters, substituted for cell density. The simulations were set to run for 40 days, about four times as long as the actual experimental runs.

The behaviour of the two models (continuous and threshold approach) were evaluated at different inoculum levels and different herbicide levels. In order to evaluate the performance of these models they were compared to the results of the actual competition experiments using a comparative index. This index was a ratio of the mean steady state (day 5-day 11) cell density of species 1 divided by species 2. This ratio was calculated for the simulation runs as well as from the results of the actual competition experiments. These two ratios were then compared. The closer the agreement between these ratios the better the performance of the simulation.

D. Results and Discussion

The data derived from the unialgal continuous culture experiments described in chapter 4 allowed for the development of the relationship between the carrying capacity of the culture system with increasing atrazine concentration. Figures 5.2 through 5.4 show the plots of these data, and the curves that were used to describe them. It was determined that an exponential function yielded the best Pearsons Product Moment Correlation coefficient (R^2). Table 5.1 lists the best fit equations and their R^2 values that were used in the competition models to set the carrying capacity for the simulation for a given atrazine concentration.

Table 5.1 Exponential best fit equations and R^2 values for unialgal steady state cell density vs. atrazine concentration data. In the equations X= atrazine concentration (mol/L), and Y= cell density(cells/ml)

Species	Exponential relationship	R^2
Scenedesmus quadricauda	$Y= 758960e^{-5.0E+6X}$	0.9122
Chlamydomonas musicola	$Y= 761314e^{-5.0E+06X}$	0.9564
Chlorella vulgaris	$Y= 758960e^{-5.0E+6X}$	0.9122

In order to convert the steady state cell density values to an estimate of biomass (cell volume in m^3) for each species, it was necessary to determine if there was any change in the cell volume with changes in atrazine concentrations. For *Chlorella vulgaris* the cells dimensions were so small no change in cell volume was detectable using the ocular micrometer method of measurement. The mean cell volume for *Chlorella vulgaris* was determined to be $1.41X10^{-14}$ m^3.

For *Scenedesmus quadricauda* and *Chlamydomonas musicola* there were a detectable change in cell volume with changes in atrazine concentration.

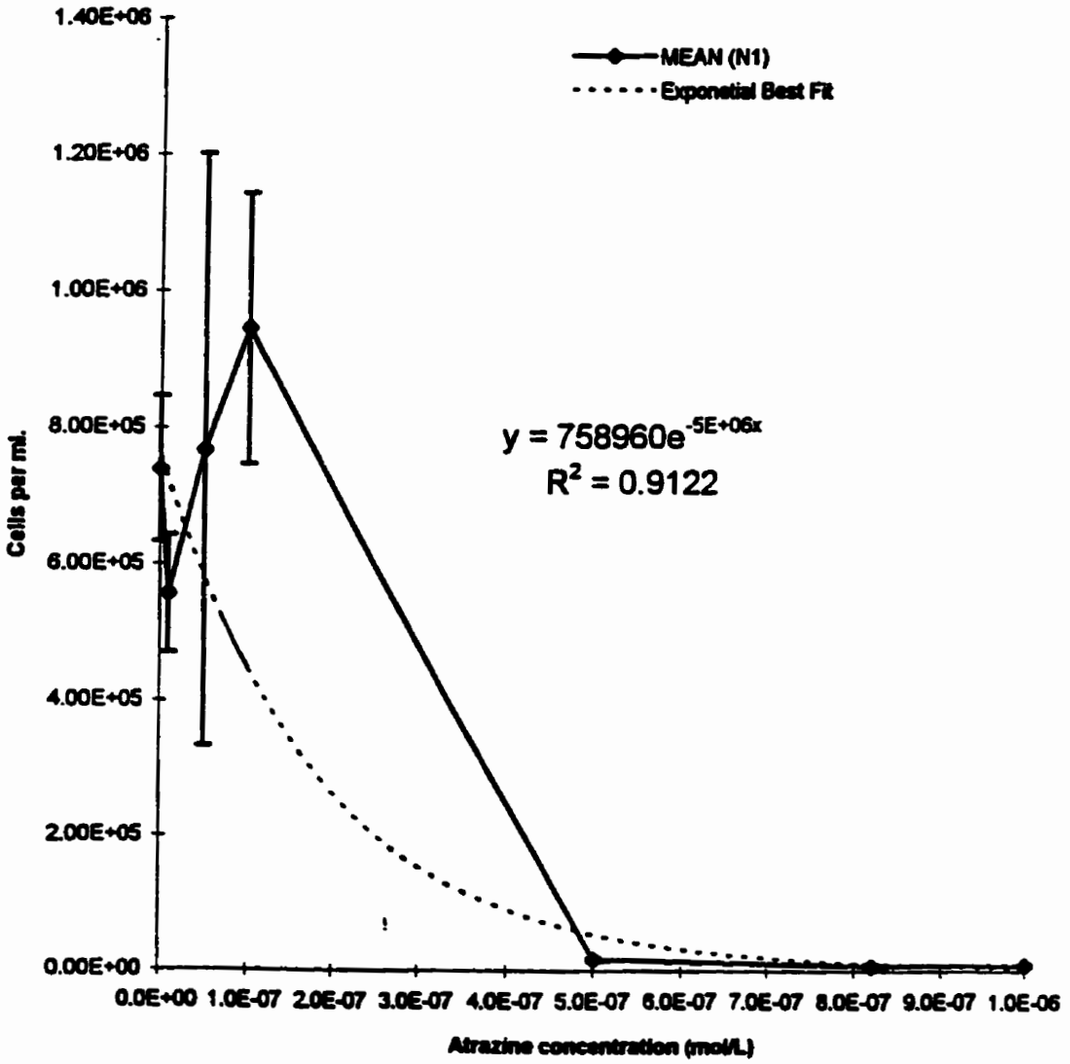


Figure 5.2 Changes in steady state cell density with differing atrazine concentration and exponential best fit for *Scenedesmus quadricauda* grown in unialgal semicontinuous culture. Error bars represent one standard deviation. For each point n=8.

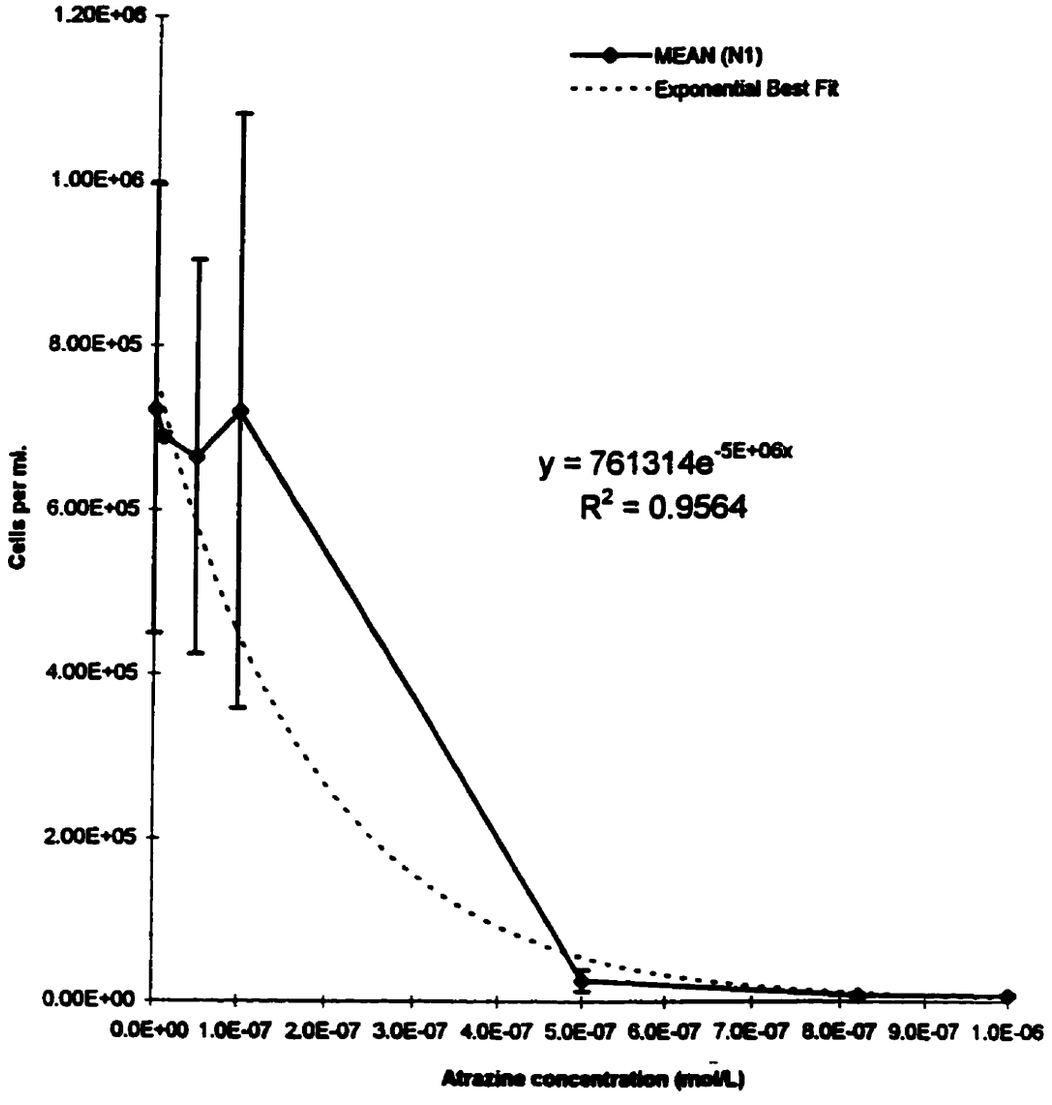


Figure 5.3 Changes in steady state cell density with differing atrazine concentration and exponential best fit for *Chlamydomonas musicola* grown in unialgal semicontinuous culture. Error bars represent one standard deviation. For each point n=8.

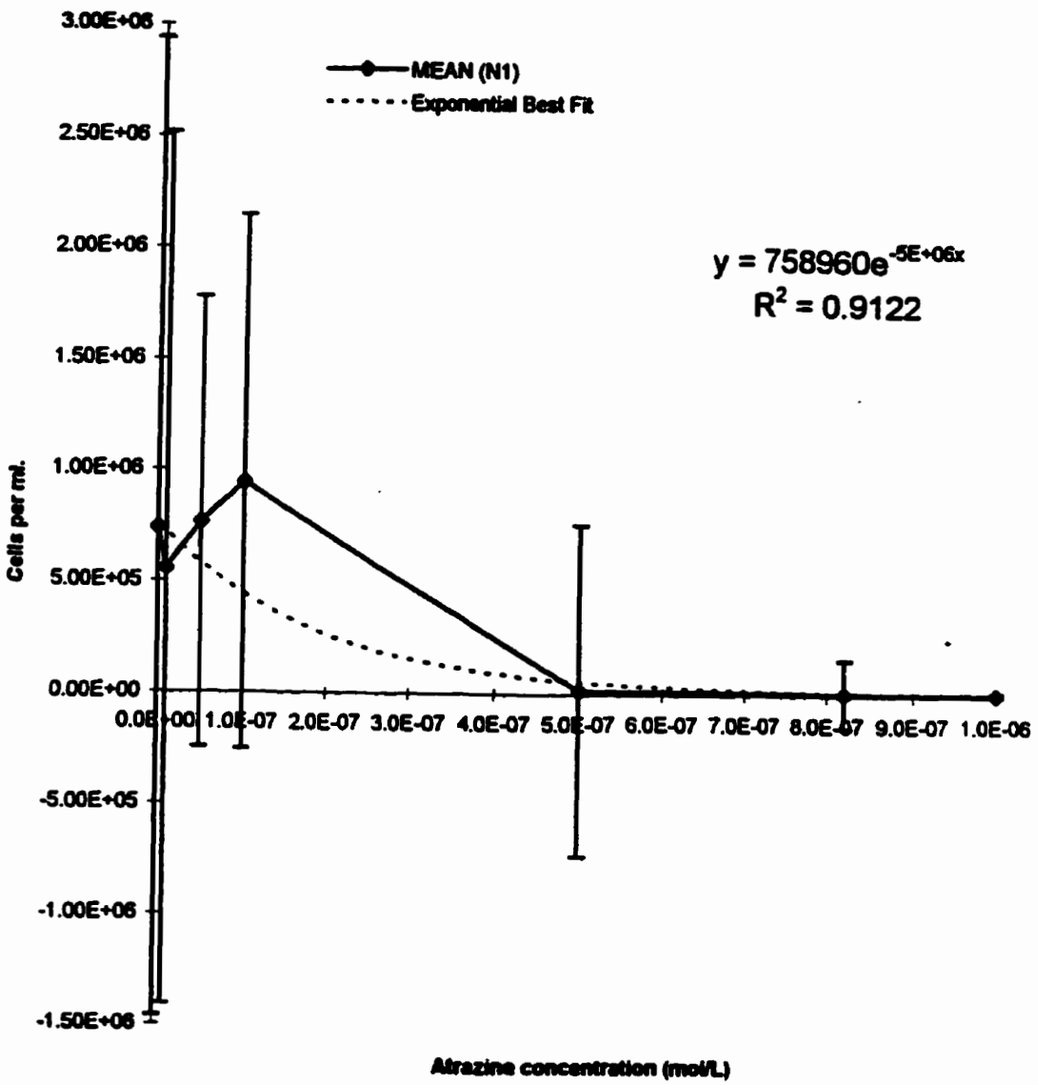


Figure 5.4 Changes in steady state cell density with differing atrazine concentration and exponential best fit for *Chlorella vulgaris* grown in unialgal semicontinuous culture. Error bars represent one standard deviation. For each point n=8.

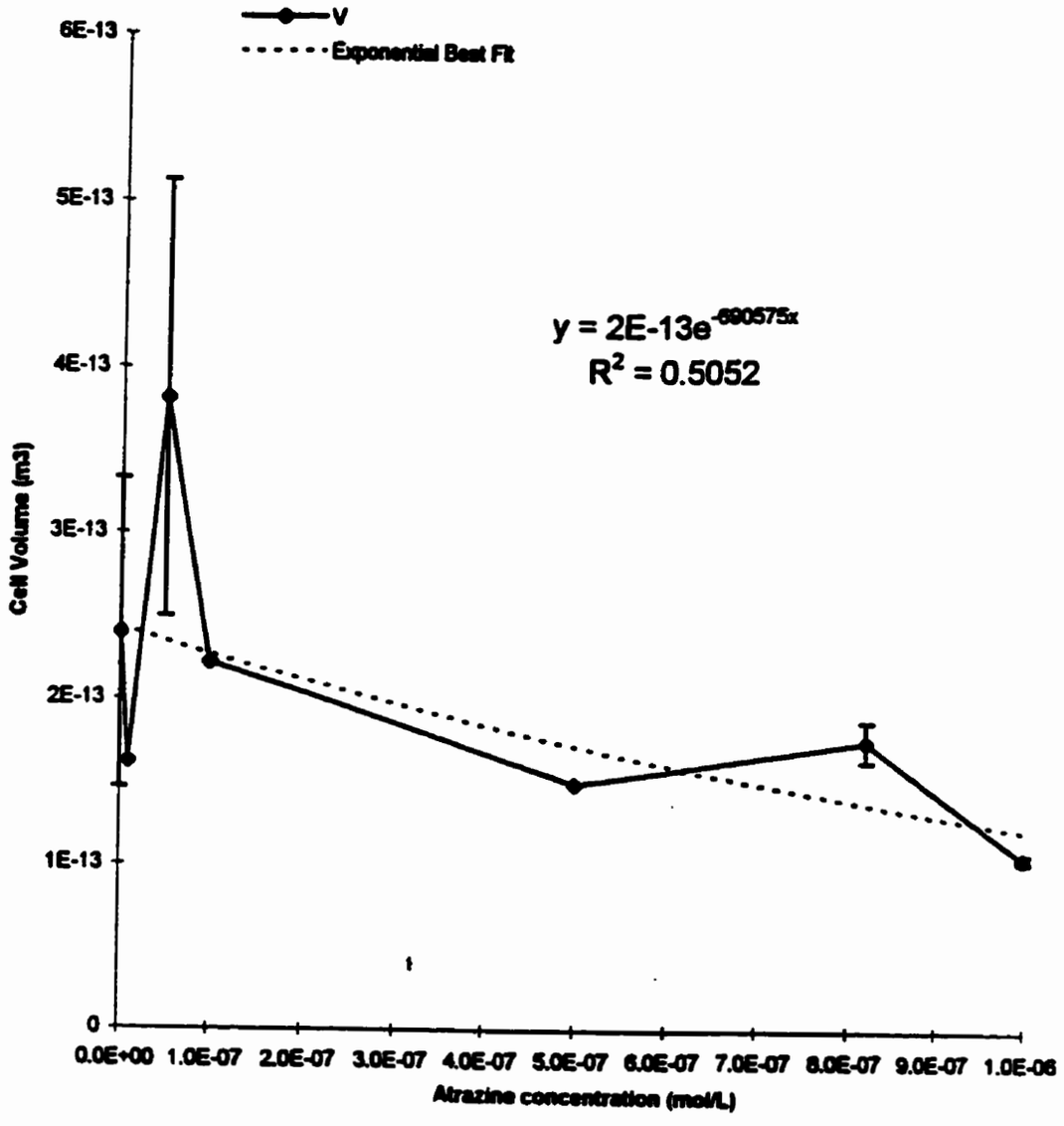


Figure 5.5 Changes in mean cell volume with changes in atrazine concentration and exponential best fit for *Secenedesmus quadricauda*. Error bars represent one standard deviation. For each point n=8.

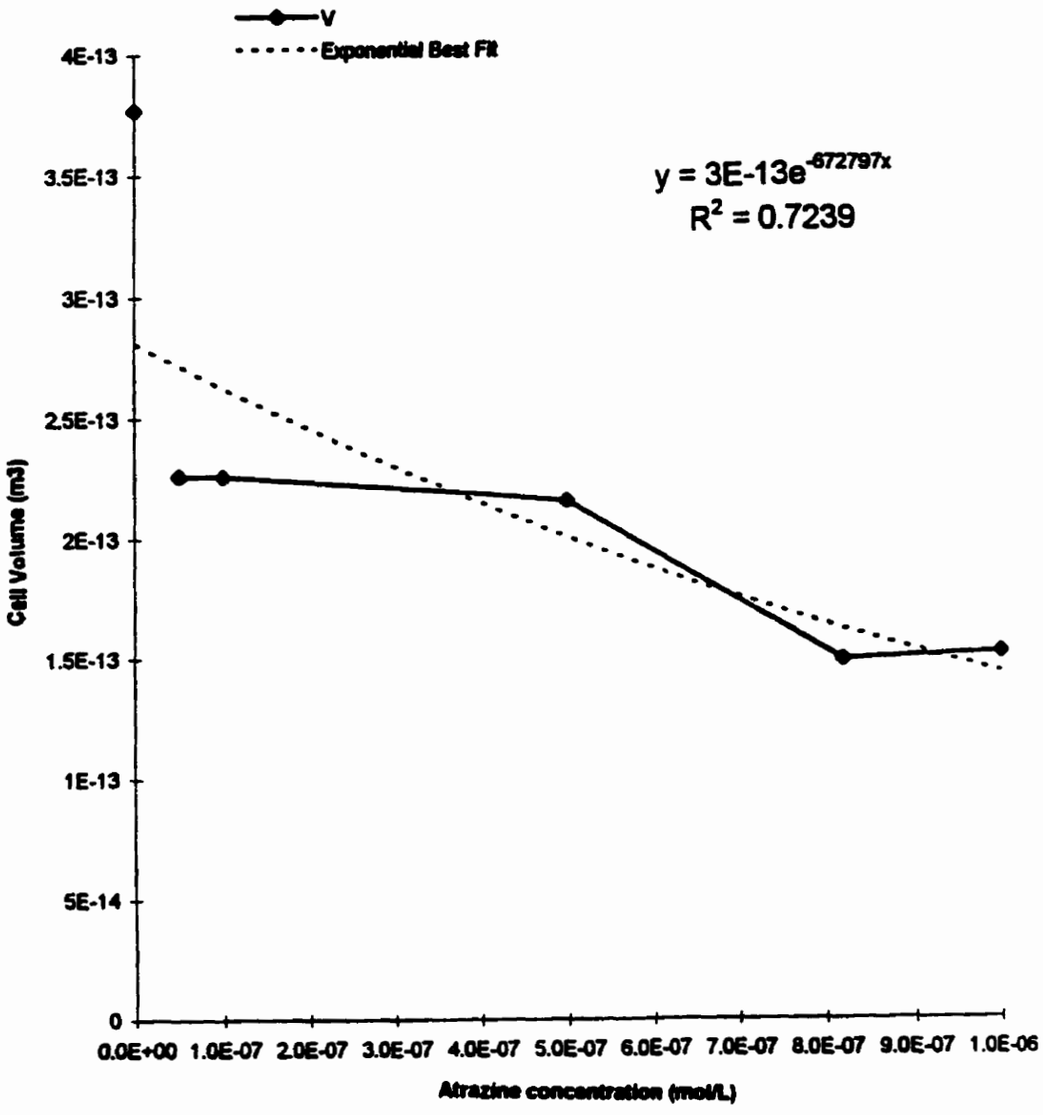


Figure 5.6 Changes in mean cell volume with changes in atrazine concentration and exponential best fit for *Chlamydomonas musicola*. For each point n=8.

Figures 5.5 and 5.6 show the plots of steady state cell volumes in response to atrazine stress in these species. The exponential best fit equations which relate this change in cell volume to atrazine concentration for these two species are given in table 5.2.

Table 5.2 Exponential best fit equations and R^2 values for unialgal steady state cell volume vs. atrazine concentration for *Scenedesmus quadricauda* and *Chlamydomonas musicola*. In the equations X= atrazine concentration (mol/L), and Y= cell volume (m^3).

Species	Exponential relationship	R^2
Scenedesmus quadricauda	$Y= 2.0X10^{-13}\ e^{-690575X}$	0.5052
Chlamydomonas musicola	$Y= 3.0X10^{-13}\ e^{-672797X}$	0.7239

These biomass relationships were built into the competition models in order to investigate the actual changes in "growth" of each species. It was hypothesized that even if cell division was too slow due to the toxic effects of atrazine, the existing cells may be able to continue to increase their cell volumes. The changes in population biomass under herbicide stress may not be the same as population density changes. I was also curious about the competitive responses of large species in competition with smaller species, and whether tracking population biomass changes, which take into account cell size, would better represent the total toxic response of the species. Therefore, each version of the simulations included a biomass output as well as the cell density output.

The final parameter relationship that needed to be estimated was the changes in the competition coefficients $\alpha_{2,1}$ and $\alpha_{1,2}$ with the variations in atrazine level. For each species pair the mean alpha values were calculated and plotted against atrazine concentrations. The curve that best fitted these data were then calculated. Figures 5.7 through 5.9 show these plots.

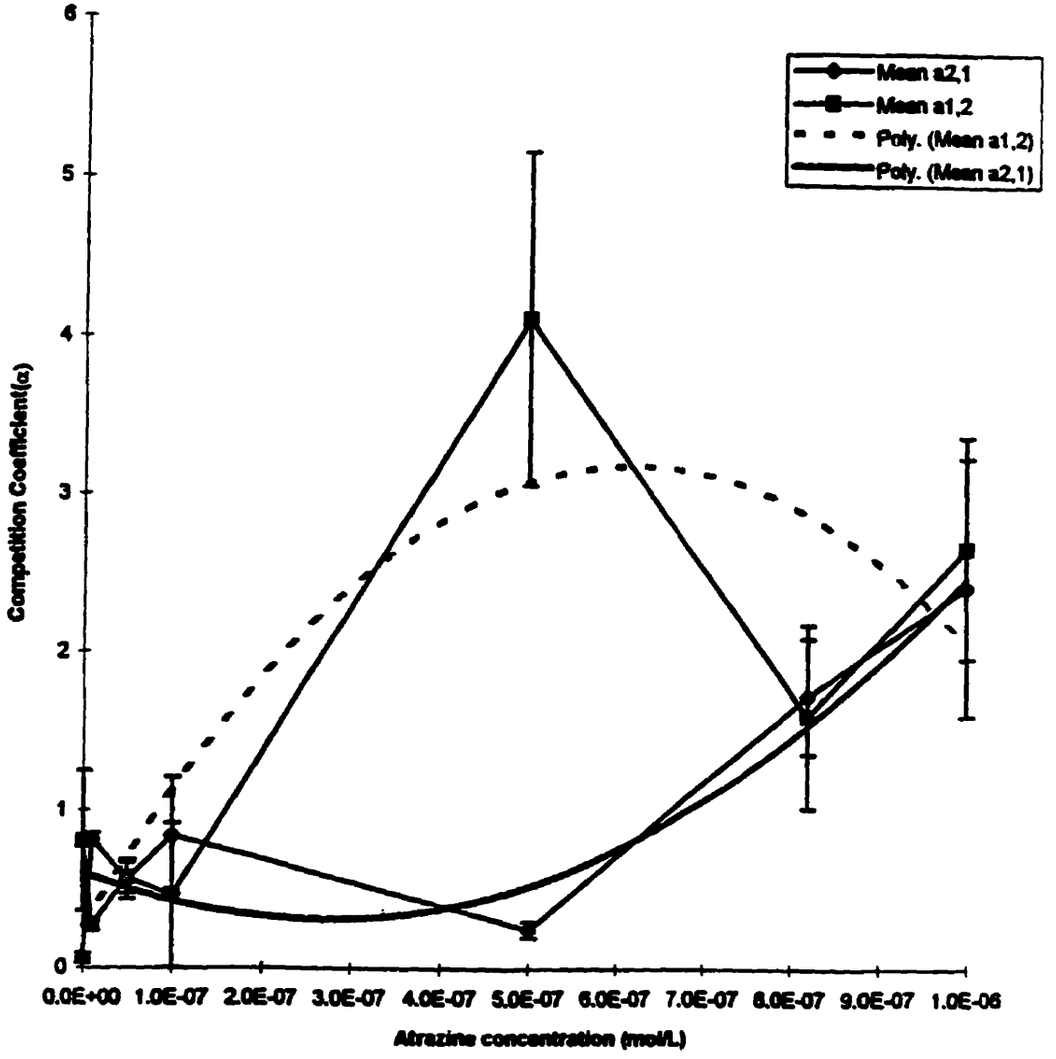


Figure 5.7 Changes in alpha values with increasing atrazine concentration and best fit curves for *Scenedesmus quadricauda* (2) vs. *Chlorella vulgaris* (1) Error bars represent one standard deviation. See Table 5.6 for equations of best fit curves.

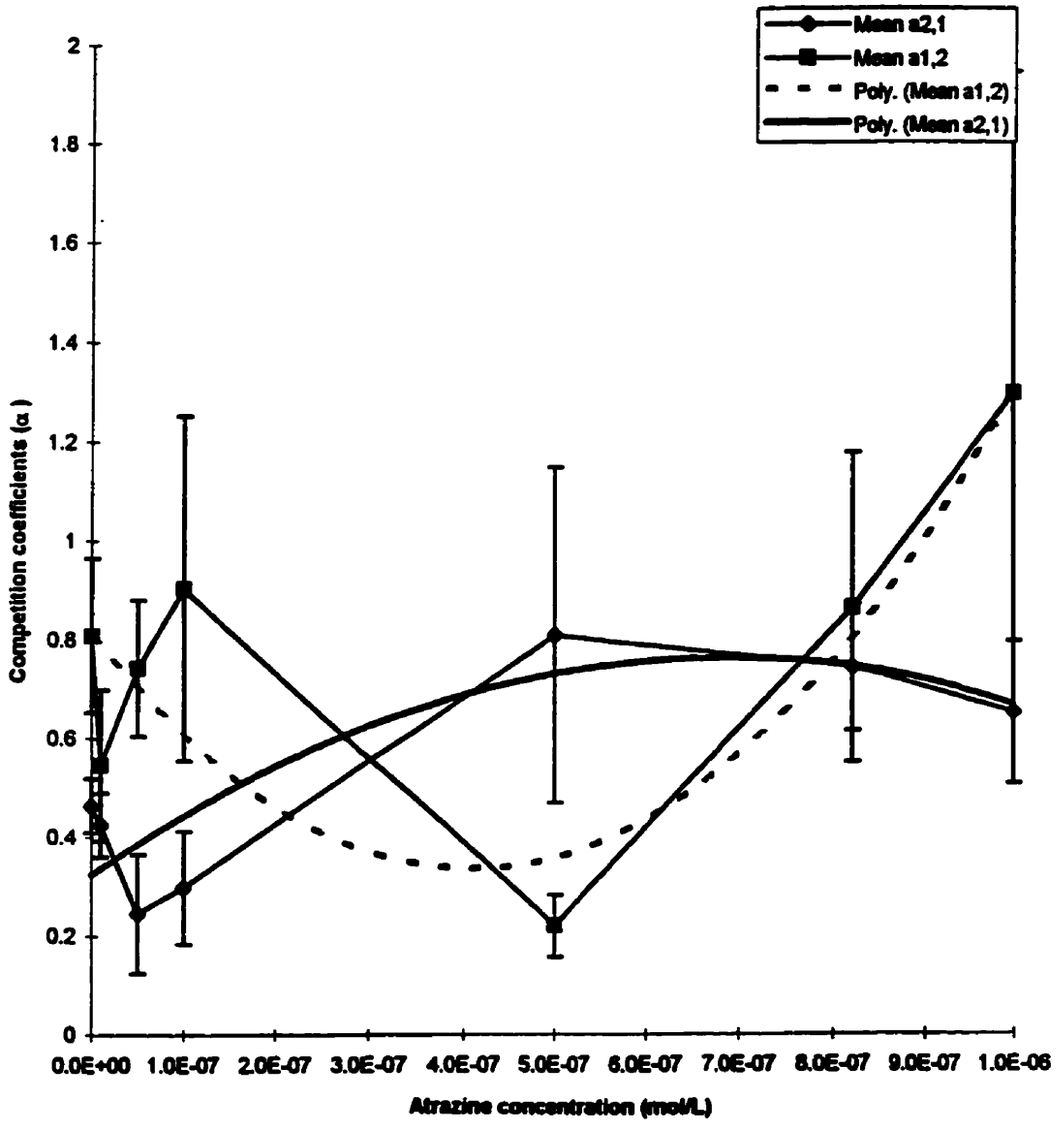


Figure 5.8 Changes in alpha values with increasing atrazine concentration and best fit curves for *Scenedesmus quadricauda* (2) vs. *Chlamydomonas musicola* (1). Error bars represent one standard deviation. See Table 5.6 for equations of best fit curves.

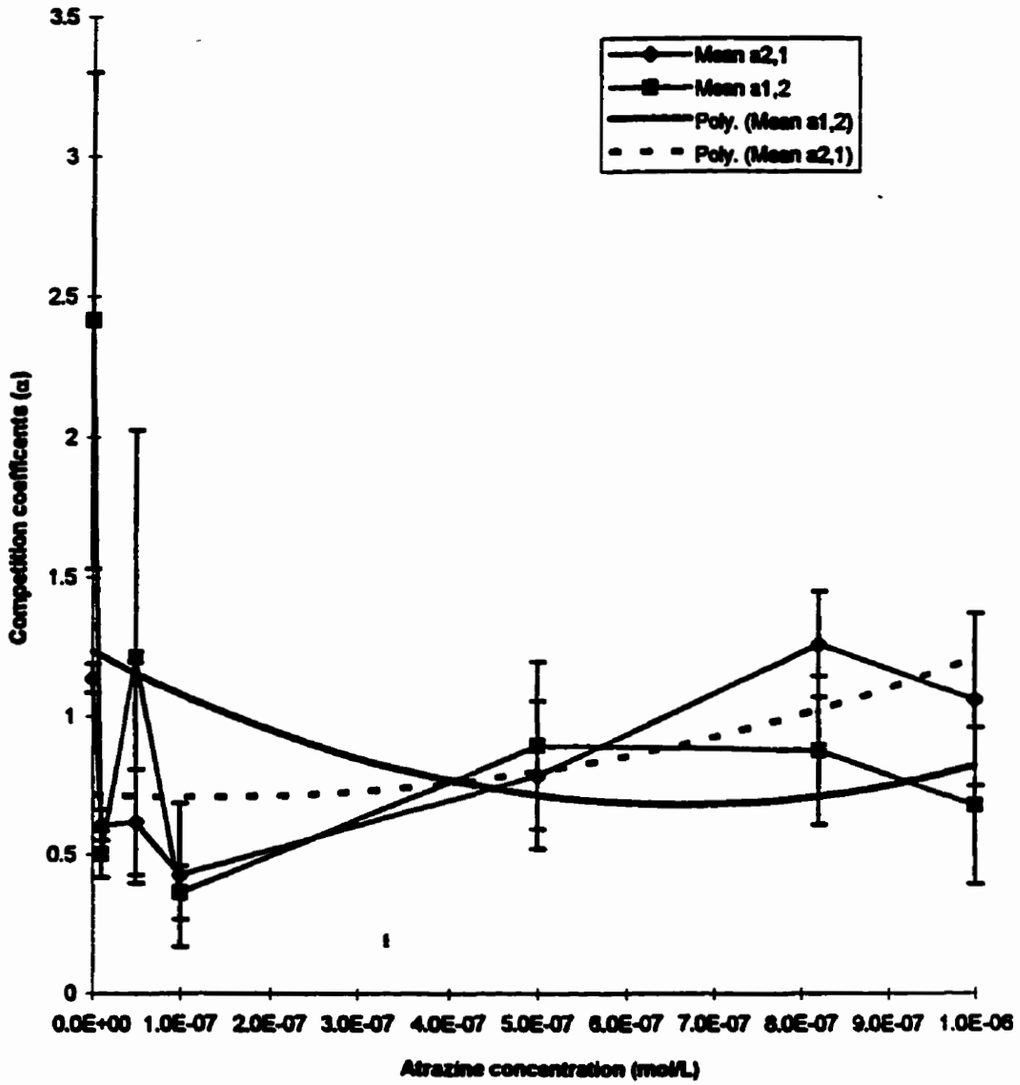


Figure 5.9 Changes in alpha values with increasing atrazine concentration and best fit curves for *Chlorella vulgaris* (2) vs. *Chlamydomonas musicola* (1). Error bars represent one standard deviation. See Table 5.6 for equations of best fit curves.

From an examination of figures 5.7 through 5.9, it became obvious that there were two distinct responses in the changes in alpha values with increasing atrazine concentration demonstrated by the different species pairs. In chapter 4 it was shown that there was a distinct threshold effect in the changes in the alpha values over the range of atrazine concentrations tested. There was one response at low herbicide levels and another at the higher levels. In order to investigate the importance of this threshold effect two simulations were developed. For the first simulation a curve was fitted to the alpha value data set over the entire range of herbicide levels. Table 5.3 lists the best fit equations used in this entire range simulation. In the second simulation separate curves were fitted to the low herbicide range (0 to $1.0X10^{-7}$ mol/L) and to the higher range ($5.0X10^{-7}$ to $1.0X10^{-6}$ mol/L), for each competing species pair. Tables 5.4a and 5.4b list these best fit equations used in the threshold simulation.

Table 5.3 Best fit equations and R^2 values for changes in alpha values vs. atrazine concentration for *Chlorella vulgaris*, *Scenedesmus quadricauda* and *Chlamydomonas musicola*. In the equations x= atrazine concentration (mol/L), and y= competition coefficient. R^2 values calculated from 6 data points.

Species pair	$\alpha_{1,2}$		$\alpha_{2,1}$	
	Equation	R^2	Equation	R^2
Scenedesmus quadricauda (2) vs. *Chlorella vulgaris* (1)	$y=-8.0X10^{12}x^2 + 9.0x10^6 x + 0.2581$	0.6851	$y=4.0X10^{12}x^2 + 2.0X10^6 x + 0.6045$	0.8494
Scenedesmus quadricauda (2) vs. *Chlamydomonas musicola* (1)	$y=1.0X10^{12}x^2 - 2.0X10^6 x + 1.2341$	0.1153	$y=6.0X10^{11}x^2 - 156573x +0.7175$	0.4023
Chlorella vulgaris (2) vs. *Chlamydomonas musicola* (1)	$y=-9.0X10^{11}x^2 + 1.0X10^6 x + 0.3241$	0.7396	$y=3.0X10^{12}x^2 -2.0X10^6 x + 0.8117$	0.7417

Table 5.4a Best fit equations and R^2 values for changes in alpha values vs. atrazine concentration for *Chlorella vulgaris*, *Scenedesmus quadricauda* and *Chlamydomonas musicola* at low herbicide ranges (0 to $1.0X10^{-7}$ mol/L). In the equations x= atrazine concentration (mol/L), and y= competition coefficient. R^2 values calculated from 3 data points.

Species pair	$\alpha_{1,2}$		$\alpha_{2,1}$	
	Equation	R^2	Equation	R^2
Scenedesmus quadricauda (2) vs. *Chlorella vulgaris* (1)	$y= -1.0X10^{6} x + 0.5688$	0.0552	$y= 5.5X10^{6} x + 0.3798$	0.3472
Scenedesmus quadricauda (2) vs. *Chlamydomonas musicola* (1)	$y= 9.0X10^{13} x^2 - 2.0X10^{7} x + 1.6994$	0.3811	$y= 7.0X10^{13} x^2 - 1.0X10^{7} x + 0.9613$	0.6534
Chlorella vulgaris (2) vs. *Chlamydomonas musicola* (1)	$y= 5.0X10^{13} x^2 - 3.0X10^{6} x + 0.7078$	0.5299	$y= 5.0X10^{13} x^2 - 7.0X10^{6} x + 0.4756$	0.987

Table 5.4b Best fit equations and R^2 values for changes in alpha values vs. atrazine concentration for *Chlorella vulgaris*, *Scenedesmus quadricauda* and *Chlamydomonas musicola* at high herbicide ranges ($5.0X10^{-7}$ to $1.0X10^{-6}$ mol/L). In the equations x= atrazine concentration (mol/L), and y= competition coefficient. R^2 values calculated from 3 data points.

Species pair	$\alpha_{1,2}$		$\alpha_{2,1}$	
	Equation	R^2	Equation	R^2
Scenedesmus quadricauda (2) vs. *Chlorella vulgaris* (1)	$y= 3.0X10^{13} x^2 - 4.0X10^{7} x + 19.351$	1	$Y= 4.0X10^{6} x - 1.9178$	0.998
Scenedesmus quadricauda (2) vs. *Chlamydomonas musicola* (1)	$Y= -394140 x + 1.1202$	0.6768	$Y= 652742 x + 0.5306$	0.4889
Chlorella vulgaris (2) vs. *Chlamydomonas musicola* (1)	$Y= 2.0X10^{6} x - 0.8621$	0.9976	$Y= -304356 x + 0.9692$	0.94

Once all the parameters have been estimated from the experimental data, and the model equations have been developed, the last step in the modelling process involves validation of the model. In anticipation of this step, two sets of competition experiments were run at different times, the first in 1993-94 and the second in 1994-95. The two separate experimental runs serve as validations for each other.

The validation process involved running a series of simulations to generate a data set which was then compared to the data set from the other experimental run. These simulations were run using, as closely as was possible, the same inputs as those used in the actual experiments. Since the actual experiments were terminated on day 11, and the models were set to run until day 40, final outputs were not entirely comparable. Therefore for the purposes of model evaluation, day 11 output values of the simulations were compared to the day 11 cell density values of the actual competition experiments.

The comparison of the experimental results, and the simulation outputs, was done by comparing the ratio of (Species 1 divided by Species 2) day 5 to 11 average cell density produced by the actual competition experiments with the ratio of (Species 1 divided by Species 2) day 11 cell density values produced by the simulations. Recall that the day 5 to day 11 average cell density was the parameter, steady state cell density, that was used in the determination of the outcome of the continuous culture competition experiments described in chapter 5. Therefore, in this analysis the outcomes of the actual continuous culture experiments were defined by the the ratio of (Species 1 divided by Species 2) day 5 to 11 average cell density.

These ratios were calculated for each species pair at each herbicide concentration tested. Tables 5.5a, 5.5b, and 5.5c present these ratios. These ratios represent the degree of competitive success of species 1 compared to species 2. If the ratio equals 1.0 the cell density of the two competing species was equal. If the ratio was less than 1.0, it indicates that species 2 has the higher cell density. Alternately if the ratio was greater than 1.0, then species 1 has the numerical advantage. The difference in the magnitude of the experimental ratio compared to the simulation ratio indicates the degree of difference between the actual and simulation results. For example an experimental ratio of 0.1 indicates that species 2 achieved a cell density ten times greater then species 1. Comparing that ratio with a simulation ratio of 0.2 indicates that species 2 was twice as large in the simulation as it was in the actual experiment.

Table 5.5a Comparison of the ratios of the experimental, continuous simulation and threshold simulation cell densities of *Chlorella vulgaris* divided by *Scenedesmus quadricauda*. (-) indicates the simulation could not function with the inputs of the actual experiment.

[Herbicide] (mol/L)	Experimental		Continuous Simulation		Threshold Simulation	
	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
0	18.8	9.9	7.2	8.0	6.3	6.3
$1.0X10^{-8}$	66.0	58.7	6.8	0.9	5.9	10.4
$5.0X10^{-8}$	11.9	20.0	4.9	5.0	4.4	150.0
$1.0X10^{-7}$	19.8	22.1	3.1	2.3	2.5	122580.6
$5.0X10^{-7}$	7.6	27.2	1.8	-	90.4	5483.9
$8.2X10^{-7}$	36.7	41.2	-	-	19.7	0.4
$1.0X10^{-6}$	16.1	21.4	-	-	180.6	12.4

Table 5.5b Comparison of the ratios of the experimental, continuous simulation and threshold simulation cell densities of *Chlamydomonas musicola* divided by *Scenedesmus quadricauda*. (-) indicates the simulation could not function with the inputs of the actual experiment.

[Herbicide] (mol/L)	Experimental		Continuous Simulation		Threshold Simulation	
	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
0	0.5	0.5	0.9	1.0	8.8	1.0
$1.0X10^{-8}$	1.5	1.5	1.0	1.0	9.5	1.0
$5.0X10^{-8}$	0.4	0.4	0.9	0.9	0.9	0.9
$1.0X10^{-7}$	0.5	1.1	0.9	1.0	1.0	1.0
$5.0X10^{-7}$	1.5	1.3	-	-	1.0	0.3
$8.2X10^{-7}$	1.8	1.2	-	-	1.6	0.3
$1.0X10^{-6}$	1.1	0.6	-	-	1.8	0.7

Table 5.5c Comparison of the ratios of the experimental, continuous simulation and threshold simulation, cell densities of *Chlorella vulgaris* divided by *Chlamydomonas musicola*. (-) indicates the simulation could not function with the inputs of the actual experiment.

[Herbicide] (mol/L)	Experimental		Continuous Simulation		Threshold Simulation	
	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
0	24.4	24.4	8.2	13.8	10.2	12.2
$1.0X10^{-8}$	20.3	20.3	9.2	13.1	9.8	11.5
$5.0X10^{-8}$	87.8	43.0	8.5	10.9	8.1	10.0
$1.0X10^{-7}$	35.7	65.0	8.1	8.7	6.2	11.2
$5.0X10^{-7}$	22.4	62.5	6.0	1.1	0.4	1.8
$8.2X10^{-7}$	29.3	59.6	1.4	0.4	1.3	0.6
$1.0X10^{-6}$	53.2	39.6	14.8	2.5	0.0	1.1

These analyses show that the threshold model appears to give the best prediction as to the outcome of competition under the influences of atrazine. As a result the data from both experimental runs were combined and a series of equations fitted to these pooled data.

One further question that was of interest was, did the simulation output patterns resemble the output patterns from the competition experiments? That is, were the trends in the changes in the ratios the same in the simulation models as they were in the actual experiments? It was also evident from the ratio analysis the results of actual experiments run 1 and run 2 were slightly different from each other due to the inherent variation of such complex experiments. These differences could impact the validation comparisons of the simulation and experimental results. Therefore a correlation analysis was performed in order to determine if the results of the simulations and experimental results were behaving in a similar fashion. In this correlation analysis, if the two data sets being compared are perfectly positively correlated then the analysis returns a value of +1.0. If a perfect negative correlation exists between the two data sets then the correlation is -1.0. If the analysis returns a 0.0 value, the two data sets have no correlation. Table 5.6 presents the results of this correlation analysis.

Table 5.6 Correlation analysis of simulation outputs of continuous and threshold models compared with the actual results of the competition experiments from each of experimental run 1 and 2. (-) indicates comparisons of no interest.

Scenedesmus vs Chlorella	**Ratio (Run 1 actual)**	**Ratio (Run 2 actual)**	**Ratio (Run 1 sim)**	**Ratio (Run 2 sim)**
Continuous				
Ratio (Run 1 actual)	1			
Ratio (Run 2 actual)	0.88	1		
Ratio (Run 1 sim)	-	0.0039	1	
Ratio (Run 2 sim)	-0.250	-	-	1
Threshold				
Ratio (Run 1 actual)	1			
Ratio (Run 2 actual)	0.88	1		
Ratio (Run 1 sim)	-	-0.181	1	
Ratio (Run 2 sim)	-0.1383	-	-	1
Scenedesmus vs Chlamydomonas				
Continuous				
Ratio (Run 1 actual)	1			
Ratio (Run 2 actual)	0.712	1		
Ratio (Run 1 sim)	-	0.298	1	
Ratio (Run 2 sim)	0.800	-	-	1
Threshold				
Ratio (Run 1 actual)	1			
Ratio (Run 2 actual)	0.712	1		
Ratio (Run 1 sim)	-	0.087	1	
Ratio (Run 2 sim)	-0.729	-	-	1
Chlamydomonas vs Chlorella				
Continuous				
Ratio (Run 1 actual)	1			
Ratio (Run 2 actual)	0.034	1		
Ratio (Run 1 sim)	-	-0.461	1	
Ratio (Run 2 sim)	0.084	-	-	1
Threshold				
Ratio (Run 1 actual)	1			
Ratio (Run 2 actual)	0.034	1		
Ratio (Run 1 sim)	-	-0.644	1	
Ratio (Run 2 sim)	0.048	-	-	1

The correlation between the two runs of the actual continuous culture experiments was relatively close to +1.0, for the *Scenedesmus quadricauda* vs. *Chlorella vulgaris*, and the *Scenedesmus quadricauda* vs. *Chlamydomonas musicola* pairings. However the patterns of the species 1/species 2 ratios with respect to atrazine concentration for the two experimental runs for the *Chlamydomonas musicola* vs. *Chlorella vulgaris* were quite different. I have no reason to suspect that the culture conditions under which these two runs were conducted were different enough to produce such different outcome patterns. The correlations for almost all the simulation runs compared to the actual experiments were very poor. Since the model equations are not perfect representations of these competitive systems under the influences of atrazine, I would not have expected perfect correlations. One potential source for the lack of numerical correlation could have been the problems the model had with initial cell inoculum levels that exceeded the carrying capacity of the culture at high herbicide concentrations. The model equations behaved very erratically under these circumstances, whereas the actual competition cultures showed no such dramatic fluctuations in population levels. There must be some dampening mechanism inherent within the algae which allows the cells to withstand an initial exposure to high atarzine levels so that the populations do not fluctuate so dramatically.

The principal conclusion that can be derived from Table 5.6 is that the model simulations do not reproduce the actual numerical results that were seen in the competition experiments. However, the utility of the model does not rest on the prediction of the precise numerical outcomes. The model would still be of value if one

could predict which species would dominate the system even if one could not predict the exact degree of that dominance.

Because of the poor correlation analysis results, a more qualitative comparison of the results of the simulations and experimental results was made. In this assessment the competitive outcome of the simulation (on day 11) was compared to the outcome of the actual experiment. That is, if species 1 was predicted by the simulation to outnumber species 2 on day 11, did the actual experiment agree with this prediction? This qualitative assessment showed that the simulations based on the threshold models correctly predicted the day 11 outcomes in every case. The simulations based on the continuous models broke down and incorrectly predicted the day 11 outcomes at the three highest herbicide concentrations.

While the inability of these models to correctly predict the actual cell densities of any given experiment was disappointing, the threshold models did correctly predict the day 11 outcome of the competition. They always underestimated the cell density values of the competing species. As well one can see from Table 5.6 that there was little agreement in simulation and experimental runs in the pattern of the change in numerical difference between the competing species, with changes in the herbicide concentration. This fact could be due to the inability of the models to function under the input conditions of the actual experiments. During the different experiments, little effort was made to inoculate the cultures with the same initial cell densities. The competition literature is full of references to the lack of importance of initial cell density in the outcome of resource competition. One should not take this for granted. As it turns out when a xenobiotic is

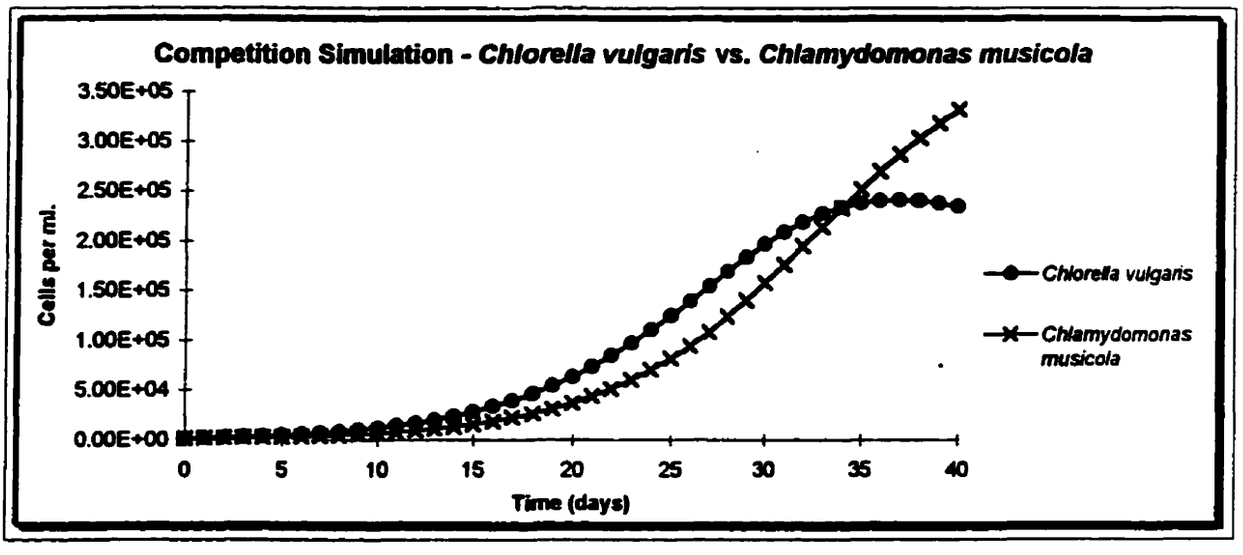
influencing the competitive abilities of the algae, the initial cell densities do play an important part in the final outcome of the competition. All further experiments in this area should pay very close attention to the initial inoculum levels.

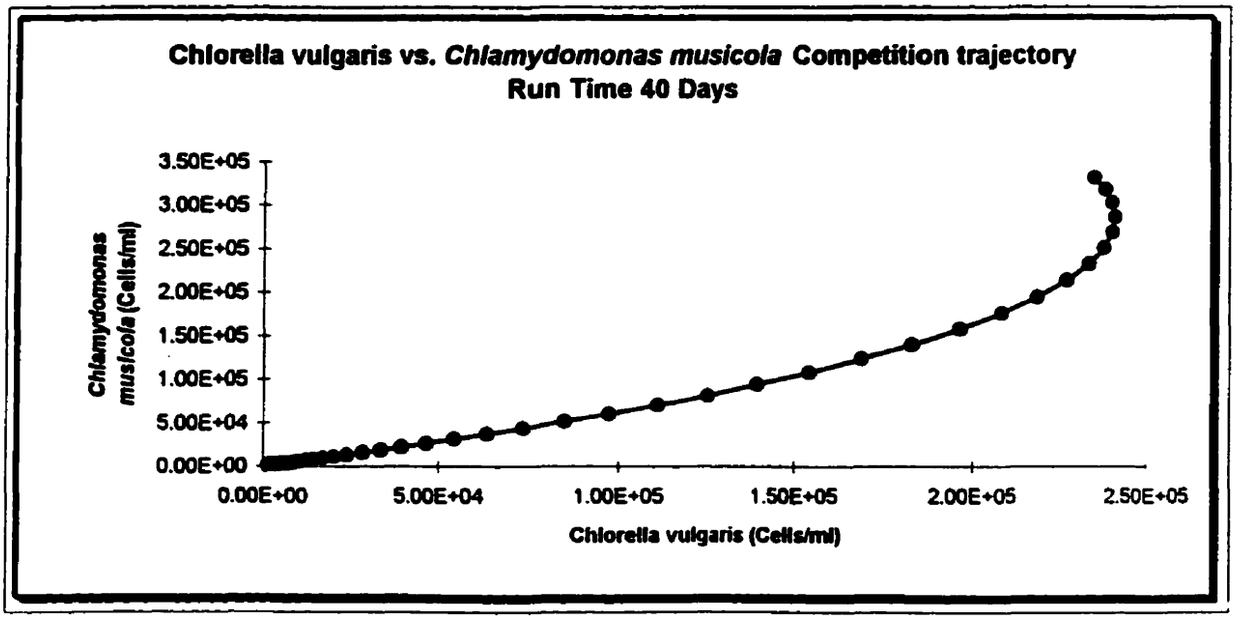
This model allows one to simulate the population growth of any of these species pairs by inputting any initial population levels, and any atrazine concentration. Figure 5.10 shows an example of the output of these models. In order to evaluate the range of behaviour of these models, several simulations were run inputting midrange as well as extreme values for the input variables. The models behave erratically when the initial population densities of either species exceeded the carrying capacity calculated for the desired herbicide level by two or more orders of magnitude. The only situation where this characteristic of the model might prove to be a problem could be when a culture were grown to steady state with no herbicide present, and then a large dose of atrazine were applied in a single pulse. This situation would cause the carrying capacity to be suddenly reduced far below the existing population densities of the two species. It is not surprising that our model equations do not behave properly under these circumstances since all experiments, from which the equations were derived, began with both test species inoculated at very low cell densities. It might be interesting to repeat some of these competition experiments with the herbicide added as a pulse to a culture already in steady state. Such a set of circumstances would closely resemble a spill of a herbicide into an uncontaminated aquatic ecosystem.

Figure 5.10 Typical output of competition simulation models.

ALGAL COMPETITION SIMULATION
EFFECTS OF ATRAZINE ON COMPETITION
THRESHOLD MODEL EQUATIONS FROM RUN 2 LOW HERBICIDE ZERO TO $1.0x10^{-7}$
SPECIES 1 - Cholrella vulgaris SPECIES 2 - Chlamydomonas musicola

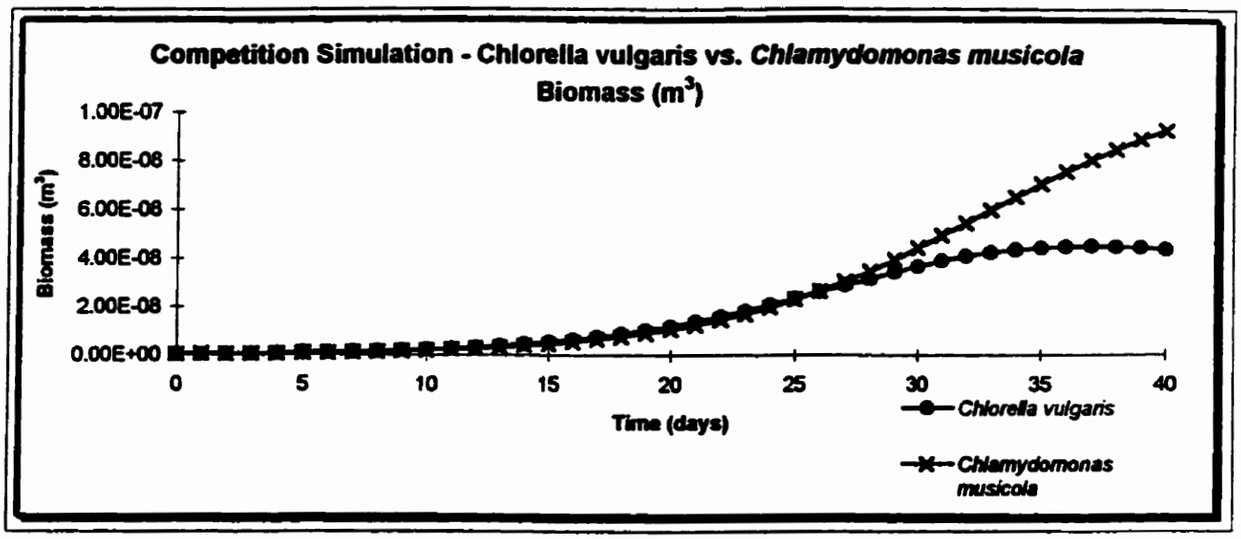
	Speceis 1	Species 2
Steady State Growth constant (r) =	0.2	0.2
Starting cells/ml (N_0) =	1.00E+03	1.00E+03
Steady State cells/ml (K) =	4.60E+05	4.62E+05
Atrazine concentration =	1.00E-07	

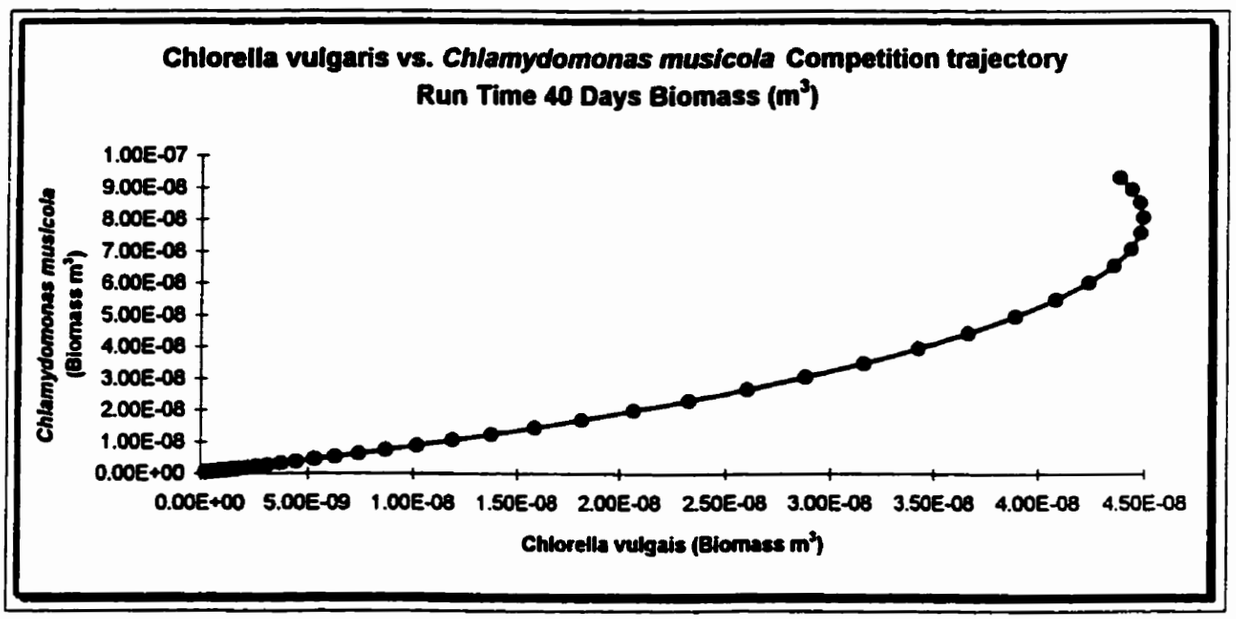




	Equilibrium point Cells/ml
Chlorella vulgaris	2.35E+05
Chlamydomonas musicola	3.32E+05

Figure 5.10 (cont.)





	Equilibrium point Biomass (m^3)
Scenedesmus quadracauda	**4.38E-08**
Chlamydomonas musicola	**9.31E-08**

Inoculum simulation experiments.

From the validation process, I am satisfied that the threshold model equations fitted to the combined data sets from experimental runs 1 and 2, would adequately predict the qualitative outcomes of competition under varying levels of atrazine stress. These simulations can now be used to explore the behaviour of these two species systems under various initial population levels and various herbicide stresses. To this end, numerous simulation experiments were run for each species pair using the threshold model. The first set of experiments simulated the competitive behaviour of *Scenedesmus quadricauda* and *Chlorella vulgaris* with the initial inoculum levels for each species set at $1.0X10^{-3}$ cells per ml. The next two experiments alternately set the initial inoculums at $1.0X10^{-3}$ and $1.0X10^{-4}$ cells per ml for these same two species. The hypothesis being tested in these simulation experiments was, that the initial population density has no effect on the outcome of the competitions under various levels of herbicide stress. Tables 5.7a, 5.7b and 5.7c present the results of these simulation experiments.

Table 5.7a Results of the threshold simulation initial inoculum experiments for *Scenedesmus quadricauda* vs. *Chlorella vulgaris*, (=) means both species achieved a more or less equal population density by day 40 of the simulation. If both genus names appear, this indicates that on day 40 both species were still present with the first species name indicating the dominant population. If only one genus name occurs this indicates that by day 40 of the simulation the other species had disappeared.

Initial inoculum levels equal at $1.0X10^{-3}$ cells per ml.

[Herbicide] (mol/L)	Winner in terms of cell density	Winner in terms of biomass
0	(=)	*Scenedesmus, Chlorella*
$1.0X10^{-8}$	(=)	*Scenedesmus, Chlorella*
$5.0X10^{-8}$	(=)	*Scenedesmus, Chlorella*
$1.0X10^{-7}$	*Scenedesmus, Chlorella*	*Scenedesmus, Chlorella*
$5.0X10^{-7}$	*Scenedesmus*	*Scenedesmus*
$8.2X10^{-7}$	*Scenedesmus*	*Scenedesmus*
$1.0X10^{-6}$	*Scenedesmus*	*Scenedesmus*

Table 5.7b Results of the threshold simulation initial inoculum experiments for *Scenedesmus quadricauda* vs. *Chlorella vulgaris*, (=) means both species achieved a more or less equal population density by day 40 of the simulation. If both genus names appear, this indicates that on day 40 both species were still present with the first species name indicating the dominant population. If only one genus name occurs this indicates that by day 40 of the simulation the other species had disappeared.

Initial inoculum levels - *Scenedesmus* =$1.0X10^{-4}$, *Chlorella* =$1.0X10^{-3}$ cells per ml.

[Herbicide] (mol/L)	Winner in terms of cell density	Winner in terms of biomass
0	*Scenedesmus, Chlorella*	*Scenedesmus, Chlorella*
$1.0X10^{-8}$	*Scenedesmus, Chlorella*	*Scenedesmus, Chlorella*
$5.0X10^{-8}$	*Scenedesmus, Chlorella*	*Scenedesmus, Chlorella*
$1.0X10^{-7}$	*Scenedesmus, Chlorella*	*Scenedesmus, Chlorella*
$5.0X10^{-7}$	*Scenedesmus*	*Scenedesmus*
$8.2X10^{-7}$	*Scenedesmus*	*Scenedesmus*
$1.0X10^{-6}$	*Scenedesmus*	*Scenedesmus*

Table 5.7c Results of the threshold simulation initial inoculum experiments for *Scenedesmus quadricauda* vs. *Chlorella vulgaris*, (=) means both species achieved a more or less equal population density by day 40 of the simulation. If both genus names appear, this indicates that on day 40 both species were still present with the first species name indicating the dominant population. If only one genus name occurs this indicates that by day 40 of the simulation the other species had disappeared.

Initial inoculum levels - *Scenedesmus* =$1.0X10^{-3}$, *Chlorella* =$1.0X10^{-4}$ cells per ml.

[Herbicide] (mol/L)	Winner in terms of cell density	Winner in terms of biomass
0	*Chlorella*	*Scenedesmus, Chlorella*
$1.0X10^{-8}$	*Chlorella*	*Scenedesmus, Chlorella*
$5.0X10^{-8}$	*Chlorella*	*Scenedesmus, Chlorella*
$1.0X10^{-7}$	*Chlorella, Scenedesmus*	*Scenedesmus, Chlorella*
$5.0X10^{-7}$	*Scenedesmus*	*Scenedesmus*
$8.2X10^{-7}$	*Scenedesmus*	*Scenedesmus*
$1.0X10^{-6}$	*Scenedesmus*	*Scenedesmus*

These results show that in terms of cell density, these two species would maintain a stable equilibrium at low atrazine stress, if both were inoculated at equal levels. However if either species were given an initial advantage, at low herbicide levels the

species with that advantage would maintain the higher population level. In terms of cell density *Scenedesmus quadricauda* appears to be the superior competitor at high atrazine stress. One interesting result of these experiments was that in terms of biomass, *Scenedesmus quadricauda* always dominated the cultures, even if *Chlorella vulgaris* occurred in greater numbers.

A similar set of simulation experiments were performed for *Scenedesmus quadricauda* in competition with *Chlamydomonas musicola*. Tables 5.8a 5.8b, and 5.8c present the results of these simulation experiments.

Table 5.8a Results of the threshold simulation initial inoculum experiments for *Scenedesmus quadricauda* vs. *Chlamydomonas musicola*, (=) means both species achieved a more or less equal population density by day 40 of the simulation. If both genus names appear, this indicates that on day 40 both species were still present with the first species name indicating the dominant population. If only one genus name occurs this indicates that by day 40 of the simulation the other species had disappeared.

Initial inoculum levels, *Scenedesmus* $=1.0X10^{-3}$, *Chlamydomonas* $=1.0X10^{-3}$ cells per ml.

[Herbicide] (mol/L)	Winner in terms of cell density	Winner in terms of biomass
0	*Scenedesmus, Chlamydomonas*	*Scenedesmus, Chlamydomonas*
$1.0X10^{-8}$	*(=)*	*Scenedesmus, Chlamydomonas*
$5.0X10^{-8}$	*Scenedesmus, Chlamydomonas*	*Scenedesmus, Chlamydomonas*
$1.0X10^{-7}$	*(=)*	*Scenedesmus, Chlamydomonas*
$5.0X10^{-7}$	*Scenedesmus, Chlamydomonas*	*Scenedesmus, Chlamydomonas*
$8.2X10^{-7}$	*Chlamydomonas, Scenedesmus*	*Scenedesmus, Chlamydomonas*
$1.0X10^{-6}$	*Chlamydomonas, Scenedesmus*	*Scenedesmus, Chlamydomonas*

Table 5.8b Results of the threshold simulation initial inoculum experiments for *Scenedesmus quadricauda* vs. *Chlamydomonas musicola*, (=) means both species achieved a more or less equal population density by day 40 of the simulation. If both genus names appear, this indicates that on day 40 both species were still present with the first species name indicating the dominant population. If only one genus name occurs this indicates that by day 40 of the simulation the other species had disappeared.

Initial inoculum levels, *Scenedesmus* $=1.0X10^{-4}$, *Chlamydomonas* $=1.0X10^{-3}$ cells per ml.

[Herbicide] (mol/L)	Winner in terms of cell density	Winner in terms of biomass
0	*Scenedesmus, Chlamydomonas*	*Scenedesmus, Chlamydomonas*
$1.0X10^{-8}$	*Scenedesmus, Chlamydomonas*	*Scenedesmus, Chlamydomonas*
$5.0X10^{-8}$	*Scenedesmus, Chlamydomonas*	*Scenedesmus, Chlamydomonas*
$1.0X10^{-7}$	*Scenedesmus, Chlamydomonas*	*Scenedesmus, Chlamydomonas*
$5.0X10^{-7}$	*Scenedesmus, Chlamydomonas*	*Scenedesmus, Chlamydomonas*
$8.2X10^{-7}$	*(=)*	*Scenedesmus, Chlamydomonas*
$1.0X10^{-6}$	*(=)*	*Scenedesmus, Chlamydomonas*

Table 5.8c Results of the threshold simulation initial inoculum experiments for *Scenedesmus quadricauda* vs. *Chlamydomonas musicola*, (=) means both species achieved a more or less equal population density by day 40 of the simulation. If both genus names appear, this indicates that on day 40 both species were still present with the first species name indicating the dominant population. If only one genus name occurs this indicates that by day 40 of the simulation the other species had disappeared.

Initial inoculum levels, *Scenedesmus* $=1.0X10^{-3}$, *Chlamydomonas* $=1.0X10^{-4}$ cells per ml.

[Herbicide] (mol/L)	Winner in terms of cell density	Winner in terms of biomass
0	*Chlamydomonas, Scenedesmus*	*Scenedesmus, Chlamydomonas*
$1.0X10^{-8}$	*Chlamydomonas, Scenedesmus*	*Scenedesmus, Chlamydomonas*
$5.0X10^{-8}$	*Chlamydomonas, Scenedesmus*	*Scenedesmus, Chlamydomonas*
$1.0X10^{-7}$	*Chlamydomonas, Scenedesmus*	*Scenedesmus, Chlamydomonas*
$5.0X10^{-7}$	*Chlamydomonas, Scenedesmus*	*Scenedesmus, Chlamydomonas*
$8.2X10^{-7}$	*Chlamydomonas, Scenedesmus*	*(=)*
$1.0X10^{-6}$	*Chlamydomonas, Scenedesmus*	*Chlamydomonas, Scenedesmus*

In terms of cell densities, the results of the *Scenedesmus quadricauda* vs. *Chlamydomonas musicola* simulation experiment indicate that at low atrazine concentrations, *Scenedesmus quadricauda* has a marginal competitive advantage over *Chlamydomonas musicola* when both are inoculated at equal densities. However,

Chlamydomonas musicola does appear to have the advantage at high atrazine levels. *Scenedesmus quadricauda* can hold its own at the higher atrazine levels only if it is inoculated at a ten fold higher cell density, whereas *Chlamydomonas musicola* will dominate the cultures at all atrazine levels if it is given a ten fold initial advantage. As in the competitive simulation of *Scenedesmus quadricauda* and *Chlorella vulgaris*, *Scenedesmus quadricauda* dominates these experiments in terms of biomass. Only at the $8.2X10^{-7}$ and $1.0X10^{-6}$ mol/L atrazine levels, when *Chlamydomonas musicola* was given a ten fold initial inoculum advantage did the biomass of *Chlamydomonas musicola* exceed that of *Scenedesmus quadricauda* by the end of the simulation run.

The last competition simulation experiment completed the three way species pairing by pitting *Chlamydomonas musicola* against *Chlorella vulgaris*. Tables 5.9a, 5.9b, and 5.9c present the results of this set of competition simulations.

Table 5.9a Results of the threshold simulation initial inoculum experiments for *Chlorella vulgaris* vs. *Chlamydomonas musicola*, (=) means both species achieved a more or less equal population density by day 40 of the simulation. If both genus names appear, this indicates that on day 40 both species were still present with the first species name indicating the dominant population. If only one genus name occurs this indicates that by day 40 of the simulation the other species had disappeared.

Initial inoculum levels, *Chlorella* $=1.0X10^{-3}$, *Chlamydomonas* $=1.0X10^{-3}$ cells per ml.

[Herbicide] (mol/L)	Winner in terms of cell density	Winner in terms of biomass
0	*Chlorella, Chlamydomonas*	*Chlorella, Chlamydomonas*
$1.0X10^{-8}$	*Chlorella, Chlamydomonas*	*Chlorella, Chlamydomonas*
$5.0X10^{-8}$	(=)	(=)
$1.0X10^{-7}$	*Chlamydomonas*	*Chlamydomonas*
$5.0X10^{-7}$	*Chlamydomonas*	*Chlamydomonas*
$8.2X10^{-7}$	*Chlamydomonas*	*Chlamydomonas*
$1.0X10^{-6}$	*Chlamydomonas*	*Chlamydomonas*

Table 5.9b Results of the threshold simulation initial inoculum experiments for *Chlorella vulgaris* vs. *Chlamydomonas musicola*, (=) means both species achieved a more or less equal population density by day 40 of the simulation. If both genus names appear, this indicates that on day 40 both species were still present with the first species name indicating the dominant population. If only one genus name occurs this indicates that by day 40 of the simulation the other species had disappeared.

Initial inoculum levels, *Chlorella* $=1.0X10^{-3}$, *Chlamydomonas* $=1.0X10^{-4}$ cells per ml.

[Herbicide] (mol/L)	Winner in terms of cell density	Winner in terms of biomass
0	(=)	*Chlamydomonas, Chlorella*
$1.0X10^{-8}$	*Chlamydomonas, Chlorella*	*Chlamydomonas, Chlorella*
$5.0X10^{-8}$	*Chlamydomonas, Chlorella*	*Chlamydomonas, Chlorella*
$1.0X10^{-7}$	*Chlamydomonas, Chlorella*	*Chlamydomonas, Chlorella*
$5.0X10^{-7}$	*Chlamydomonas*	*Chlamydomonas*
$8.2X10^{-7}$	*Chlamydomonas*	*Chlamydomonas*
$1.0X10^{-6}$	*Chlamydomonas*	*Chlamydomonas*

Table 5.9c Results of the threshold simulation initial inoculum experiments for *Chlorella vulgaris* vs. *Chlamydomonas musicola*, (=) means both species achieved a more or less equal population density by day 40 of the simulation. If both genus names appear, this indicates that on day 40 both species were still present with the first species name indicating the dominant population. If only one genus name occurs this indicates that by day 40 of the simulation the other species had disappeared.

Initial inoculum levels, *Chlorella vulgaris* =$1.0X10^{-4}$, *Chlamydomonas* =$1.0X10^{-3}$ cells per ml.

[Herbicide] (mol/L)	Winner in terms of cell density	Winner in terms of biomass
0	*Chlorella, Chlamydomonas*	*Chlorella, Chlamydomonas*
$1.0X10^{-8}$	*Chlorella, Chlamydomonas*	*Chlorella, Chlamydomonas*
$5.0X10^{-8}$	*Chlorella, Chlamydomonas*	*Chlorella, Chlamydomonas*
$1.0X10^{-7}$	(=)	*Chlamydomonas, Chlorella*
$5.0X10^{-7}$	*Chlamydomonas*	*Chlamydomonas*
$8.2X10^{-7}$	*Chlamydomonas*	*Chlamydomonas*
$1.0X10^{-6}$	*Chlamydomonas*	*Chlamydomonas*

From these simulations, in terms of cell densities, it would appear that *Chlorella vulgaris* has the competitive advantage at lower atrazine concentrations, when these two species are inoculated in equal amounts. However with equal initial inoculum levels, at $1.0X10^{-7}$ mol/L atrazine, *Chlamydomonas musicola* gains the advantage. As the atrazine level continues to rise to $1.0X10^{-6}$ mol/L, *Chlorella vulgaris* is competitivly displaced by *Chlamydomonas musicola*. *Chlamydomonas musicola* dominated at all but the zero atrazine levels, when it was given a ten fold initial numerical advantage. *Chlorella vulgaris* on the other hand dominated at the zero, $1.0X10^{-8}$, and $5.0X10^{-8}$mol/L atrazine concentrations when it was given a ten fold initial advantage. In these simulations it would appear that both cell density and biomass were equally accurate as predictors of the final outcome. When a large species dominates numerically over a smaller species logically it should also dominate in terms of biomass.

These results would tend to indicate that at least in these species, the initial cell density does have an impact on the final outcome of the competition under low levels of herbicide stress. If atrazine were released into a lake or stream, the shift in the population levels to a new equilibrium of these species would be different, depending on the initial population densities at the time of the release. If this type of trend is more universal across other phytoplankton taxa, then given the natural seasonal shifts in population densities an accidental herbicide spill, or a change in the seasonal use of a herbicide, could cause a greater change in community structure at one time of the year then at others. Therefore from a management point of view, it would be important to know when a herbicide release would be most damaging to an aquatic ecosystem, so as to minimize the chances of a release occurring at that time of year.

Clearly this is just a preliminary model. More experiments need to be carried out on phytoplankton from other dominant taxa, such as diatoms and the cyanobacteria. If such competition trends are present within one taxon, it is quite possible that such trends may be even more prevalent across more anatomically and physiologically diverse groups. The process of creating models for such ecotoxicological interactions is in itself a very useful activity which only helps to enhance our understanding of these intricate processes.

Chapter 6

Conclusions

Two major results are evident from this research. First, atrazine does cause changes the interspecific interactions between the phytoplankton examined in this study; and secondly, there appears to be a threshold pattern to the changes these interactions. Neither of these results would be inferred from the standard herbicide evaluation protocols. This research focused on a very narrow ecological process, the effects of atrazine on the competitive abilities of three phytoplankton species, and undoubtedly such xenobiotics also impact on other ecological processes that influence community structure. It is evident from this research, that new toxicological evaluation protocols that evaluate the impacts of these toxins on population level processes would vastly improve our abilities to protect aquatic ecosystems.

To this end I would propose the following recommendations;

Recommendation 1. ***That for the purposes of toxicological assessment, batch culture methods be supplemented with continuous culture methods.***

Rational: The continuous culture methods provide a stable steady state culture system which can allow for the evaluation of long term effects.

Recommendation 2. ***That the test species for bioassays be isolated from the ecosystem to be evaluated.***

Rational: In order to evaluate the population level effects of a toxin the test species must naturally occur together. This will allow any past coevolutionary adaptations

to be part of the analysis. Even if a single species test is to be performed that species should still be isolated from the ecosystem to be evaluated. Local water chemistry should be mimicked as closely as is possible, so as to account for any local effects of water chemistry on the toxicant.

Recommendation 3. ***Multi-species tests should be used along with other physiological tests in order to give a broader picture of the effects of the toxins on population processes.***

Rational: Multi-species test systems could include species of the same ecological level such as two phytoplankton species. These test would evaluate the effects of the toxicant on competition or other allopathic type processes. Multi-species tests could also include predator prey species in order to test trophic level processes.

The type of bioassay used in this research gives a new perspective to the environment and its inhabitants. Such methods have been advocated for more than a decade (Munawar *et. al* 1989). Calow (1989) posed a series of questions that raise a wide range of fundamental issues for both ecotoxicology in particular and ecology in general. Some of these issues have been addressed in this research. One such idea is that of an "indicator species". Many researchers have suggested that certain "keystone" species could play an important role in community structure. A competitive bioassay survey of some of the dominant species using synthetic microcosms, could be used to help identify these species. Of course, the concept of "indicator species" assumes some generality in the sensitivities of different species to the xenobiotic toxins being introduced into the

ecosystem. What we have seen from this research is that there is considerable variation in the changes in competitive responses due to atrazine stress even in these green algal genera. The competitive interactions of the three species of phytoplankton used in this study are even different at different concentrations of the same herbicide.

One of the most important results of this research is a demonstration of the validity of the assertion made by Calow (1989) that researchers need to discover the relationships between changes at one biological level (e.g. physiological impacts of a pollutant) and those at another (e.g. population dynamics). This research has showed that responses at one level do not adequately predict responses at another. Of course in order to understand such relationships it will be necessary to establish, experimentally, the mechanistic links between these ecological levels. The determination of the mechanisms by which atrazine causes the observed changes in the competition coefficients was beyond the scope of this study. However establishing these mechanistic links should be a high priority in future research.

While the principles of multispecies, continuous culture, synthetic microcosms bioassays are well accepted these assays do have their drawbacks. One of the major problems with this method is the requirement for the accurate enumeration of each species used in the assay. Many researchers have used the estimation of culture optical density to reduce the labor involved in the determination of cell densities of unialgal cultures. However such methods do not work when one needs to enumerate separate species in a mixed culture. In order to overcome this problem, for the past two years, I and the students in my lab, have been investigating the use of computer assisted image analysis to

automate the cell counting process. Images of the mixed culture are captured and digitized as computer image files using a video camera mounted on a microscope. The video image is fed through an analogue to digital converter, and the resulting digital signal is captured using special software on a Pentium® computer. The digital images can be altered or data collected using commercially available image analysis software. Most recently I have been investigating the use of artificial intelligence software for pattern recognition. This technology holds the promise of being able to distinguish between different phytoplankton species based on their morphologies, without the need to write complex algorithms. I am confident that it is only a matter of time before such technical problems are overcome.

For ecologists, a more daunting task will be to convince the decision makers in the environmental agencies of government and industry that these multi-species bioassays should be incorporated into the mainstream evaluation process. Finally, much more basic research needs to be done in the development of a solid body of ecological theory upon which to base the development of a more ecologically based evaluation of toxic substances.

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Appendix 1

WC' Algal Growth Medium for phosphate limited algal cultures.

Modified from Guillard and Lorenzen (1972).

Major Elements

Compound	Molecular Wt.	Stock (100mM) gm/100ml	[Final] mg/L	[Final] µM	Add ml/L
$NaNO_3$	85.0	0.850	17.0	500	5.0
KH_2PO_4	236.5	1.361	1.4	10	0.1
KCl	74.6	0.746	3.0	40	0.4
$MgSO_4.7H_2O$	246.5	2.465	37.0	150	1.5
$CaCl_2.2H_2O$	147.0	1.471	36.8	250	2.5
$NaHCO_3$	84.0	0.840	12.6	150	1.5
$Na_2SiO_3.9H_2O$	284.2	2.842	56.8	200	2.0

Trace Elements

Combine the following compounds, in the order listed, to one liter of glass distilled water. Add 2.5 ml of this stock to one liter of medium.

Na_2EDTA	874.0 mg
$FeCl_3.6H_2O$	630.0 mg
H_3Bo_3	200.0 mg
$MnCl_2.4H_2O$	36.0 mg
$Na_2MoO_4.2H_2O$	1.2 mg
$ZnSO_4.7H_2O$	4.4 mg
$CoCl_2.6H_2O$	2.0 mg
$CuSO_4.5H_2O$	2.0 mg

Buffer

Prepare stock bicine buffer by adding 16.32 g of bicine (M.W. 163.2) to 1 liter of distilled H_2O. Adjust the pH of the stock to 7.0 Add 10ml/L to medium. Adjust the pH of the medium to 7.6. using HCl or NaOH.

Vitamins

Perpare three separate solutions

A: cyanocobolamine (B_{12}) 10.0 mg/100ml H_2O

B: biotin 10.0 mg/100ml H_2O

C: thiamine 10.0 mg + 0.5ml solution A + 0.5 ml solution B + 99.0 ml H_2O.

Add 1.0ml of solution C to one liter of media.

Appendix 2

Isolation of test species

The test species used in this study were isolated from the University of Manitoba Field Station at Delta Marsh Manitoba in the spring of 1988. On May 28, 1988 a phytoplankton sample was collected using a 153 micron mesh plankton net. The sample was placed on ice and transported back to the lab at the University of Winnipeg. Less than 4 hours after collection the plankton sample was divided into three sub-samples, and one of which was preserved with Lugols iodine for future reference. The other two sub-samples were inoculated into two 500 ml Erlenmeyer flasks, containing 350 ml of WC' medium. These cultures were maintained in a growth chamber at 20 C°, on a rotary shaker table at approximately 60 RPM under a continuous illumination of 75 $\mu Em^{-2} s^{-1}$. Illumination was provided by four GE cool white fluorescent bulbs. These cultures were allowed to grow for three days. On day 4 the cultures were removed from the growth chamber and the cells which had adhered to the glass walls of the flasks were resuspended with a small test tube cleaning brush. Twenty samples of these nutrient enrichment cultures were inoculated onto WC' agar plates using an sterile inoculating loop. The WC' agar plates were prepared by adding 15 g of bacteriological agar to 1 L of WC' medium. then autoclaving for 30 minutes at 123 C°. The plates were poured in a laminar flow hood, and stored in sealed plastic bags in the dark at 5 C° until needed. Once the plates had been streaked, they were placed back in the growth chamber at 20 C° under 75 $\mu Em^{-2} s^{-1}$ and allowed to grow four several days. These plates were checked daily for the appearance of algal growth. When a individual colony that was clearly separated from the

others was detected that colony was remove from the plate using a micropipet and a micromanipulator viewed under a compound microscope. These pure colonies were transferred to a screw top test tube containing 15 ml of WC' medium. These culture tube were then placed back into the growth chamber under the same light and temperature conditions as described above, and allowed to grow for several more days. When growth in these test tube cultures was detected they were checked for purity under the microscope. If the cultures were determined to be pure unialgal cultures the test tube culture was transferred to a 250 ml Erlenmeyer flask containing 150 ml of WC' medium. These unialgal cultures were subcultured every 7 days, and served as the stock cultures for all batch and continuous culture experiments.

Appendix 3

BASIC program for Approximate Randomization Test

```
CLS
RANDOMIZE TIMER
PRINT "*************************************************************"
PRINT "This program performs a two-tailed test"
PRINT "*************************************************************"
PRINT "Do you want to display the intermediate calculations?"
INPUT "If your answer is yes, then type '1'"; question
PRINT "*************************************************************"
PRINT "*************************************************************"
INPUT "Sample size for sample 1"; m1
INPUT "Sample size for sample 2"; m2
PRINT "*************************************************************"
PRINT "*************************************************************"
mT = m1 + m2
DIM Y(mT), x(mT), v(100): REM DIMENSION ARRAYS FOR SHUFFLING
kk = 0: ll = 0
FOR i = 1 TO mT
IF i <= m1 THEN ll = ll + 1
IF i > m1 THEN kk = kk + 1
IF i <= m1 THEN PRINT "datum number"; ll, " for sample 1 "
IF i > m1 THEN PRINT "datum number"; kk; " for sample 2 "
INPUT " Please enter datum"; Y(i)
IF i = m1 THEN PRINT "*************************************************************"
IF i <= m1 THEN x(i) = 0: REM A ZERO IN THE ARRAY X(i) DENOTES "sample1" DATA
IF i > m1 THEN x(i) = 1: REM A ONE IN THE ARRAY X(i) DENOTES "sample 2" DATA
NEXT i
GOSUB 1000: REM COMPUTE TEST STATISTIC
actualstat = statistic
mean1 = sum0 / count0
mean2 = sum1 / count1
PRINT "*************************************************************"
PRINT "*************************************************************"
PRINT "mean of sample 1 = "; mean1; " n1 = ", m1
PRINT "mean of sample 2 = "; mean2; " n2 = ", m2
PRINT "*************************************************************"
PRINT "*************************************************************"
PRINT "observed difference ( the test statistic) = "; actualstat
PRINT "*************************************************************"
PRINT "*************************************************************"
INPUT "Desired number of shuffles = "; NS
PRINT "Please be patient, this may take some time.. "
PRINT "*************************************************************"
PRINT "*************************************************************"
PRINT
NGE = 0: NLT = 0
FOR shuffle = 1 TO NS
GOSUB 2000 REM SHUFFLE SUBROUTINE
GOSUB 1000
pseudostat = statistic
```

```
REM NGE = NUMBER GREATER THAN OR EQUAL TO
REM THIS WILL BE USED TO EVALUATE THE PROBABILITY OF THE RESULT
IF ABS(pscudostat) >= ABS(actualstat) THEN NGE = NGE + 1 ELSE NLT = NLT + 1
IF question = 1 AND ABS(pscudostat) >= ABS(actualstat) THEN PRINT shuffle: " *** ". " Pscudo-stat = ": pscudostat.
" >= ": actualstat
IF question = 1 AND ABS(pscudostat) < ABS(actualstat) THEN PRINT shuffle: "    ". " Pscudo-stat = ": pscudostat. " <
": actualstat
NEXT shuffle
PRINT
PRINT "*************************************************************"
PRINT
PRINT "Probability of the observed difference = ": (NGE) / (NGE + NLT)
PRINT "Number of shuffles = ": NGE + NLT
PRINT
PRINT "*************************************************************"
PRINT "*************************************************************"
END
1000 REM COMPUTE TEST STATISTIC
sum1 = 0: sum0 = 0: count1 = 0: count0 = 0
FOR i = 1 TO mT
IF x(i) > 0 THEN
sum1 = sum1 + Y(i): count1 = count1 + 1
ELSE sum0 = sum0 + Y(i): count0 = count0 + 1
END IF
NEXT i
statistic = (sum1 / count1) - (sum0 / count0)
REM STATISTIC = ABS((SUM1/COUNT1)-(SUM0/COUNT0))
RETURN
2000 REM: SHUFFLE SUBROUTINE
FOR j = 1 TO mT - 1
U = RND
K = j + INT(U * (mT - j + 1))
TEMP = Y(K)
Y(K) = Y(j): Y(j) = TEMP
NEXT j
RETURN
```

Appendix 4

Combined Experimental competition data

Scenedesmus quadracauda(2) vs. Chlorella vulgaris(1)

HERBICIDE CONCENTRATION

0 mol			$1.0x10^{-8}$ mol.			5.0x10-8 mol	
$\alpha_{2,1}$	$\alpha_{1,2}$		$\alpha_{2,1}$	$\alpha_{1,2}$		$\alpha_{2,1}$	$\alpha_{1,2}$
0.036671	0.507442		0.858266	0.28764		0.54349	0.565472
0.035041	0.484889		0.813873	0.240899		0.480332	0.737459
0.040746	0.563825		0.828671	0.240899		0.486981	0.52899
0.03879	0.536761		0.756532	0.298427		0.373961	0.698371
0.043517	0.602165		0.858266	0.28764		0.652355	0.488599
0.040094	0.554804		0.813873	0.240899		0.67036	0.466884
0.051046	0.70636		0.828671	0.240899		0.761773	0.542888
0.071973	0.99594		0.756532	0.298427		0.515235	0.51683
0.039931	0.552548	mean	0.814335	0.266966	mean	0.560561	0.568187
0.045146	0.624718	sd	0.039558	0.028164	sd	0.125348	0.097848
0.13821	1.912494						
0.129898	1.797474						
0.080351	1.111863		1.0x10-7 mol.			5.0x10-7 mol.	
0.096975	1.341903		$\alpha_{2,1}$	$\alpha_{1,2}$		$\alpha_{2,1}$	$\alpha_{1,2}$
0.042702	0.590889		0.690903	0.237844		0.330539	
0.031619	0.437528		0.295877	0.294221		0.234731	4.369475
0.03879	0.536761		1.369216	0.798097		0.258683	
0.042702	0.590889		0.82153	0.546159		0.244311	5.954679
0.036671	0.507442		1.137866	0.588443		0.287425	3.739457
0.035041	0.484889		0.758577	0.500352		0.182036	4.084951
0.040746	0.563825		1.175637	0.30303		0.277844	3.719134
0.03879	0.536761		0.45011	0.396406		0.182036	2.78427
0.043517	0.602165	mean	0.837465	0.458069	mean	0.249701	4.108661
0.040094	0.554804	sd	0.370073	0.187216	sd	0.051047	1.050564
0.051046	0.70636						
0.071973	0.99594						
0.039931	0.552548		8.2x10-7 mol			1.0x10-6 mol.	
0.045146	0.624718		$\alpha_{2,1}$	$\alpha_{1,2}$		$\alpha_{2,1}$	$\alpha_{1,2}$
0.13821	1.912494		2.404215	1.212766		2.209189	2.471104
0.129898	1.797474		1.637931	2.911348		2.013685	3.42766
0.080351	1.111863		1.427203	1.148936		1.251222	3.228378
0.096975	1.341903		1.312261	1.549645		3.264907	2.152252
0.042702	0.590889		1.90613	1.787234		3.851417	2.949382
0.031619	0.437528		1.877395	1.241135		2.346041	1.71383
0.03879	0.536761		1.886973	1.677305		2.580645	3.507373
0.042702	0.590889		1.369732	1.255319		1.857283	1.913113
Mean 0.058011	0.802736	mean	1.72773	1.597961	mean	2.421799	2.670387
sd 0.032133	0.444641	sd	0.365743	0.581152	sd	0.818212	0.703039

Combined Experimental competition data
Scenedesmus quadracauda(2) vs. Chlamydomonas musicola(1)

HERBICIDE CONCENTRATION

0 mol

	$\alpha_{2,1}$	$\alpha_{1,2}$
	1.183391	3.755074
	1.100346	1.488498
	1.183391	2.151556
	1.076125	2.266576
	1.183391	3.755074
	1.100346	1.488498
	1.183391	2.151556
	1.076125	2.266576
Mean	1.135813	2.415426
sd	0.05168	0.885702

$1.0x10^{-9}$ mol.

	$\alpha_{2,1}$	$\alpha_{1,2}$
	0.515614	0.561798
	0.635439	0.575281
	0.63907	0.489888
	0.628177	0.377528
	0.515614	0.561798
	0.635439	0.575281
	0.63907	0.489888
	0.628177	0.377528
Mean	0.604575	0.501124
sd	0.055068	0.083807

5.0X10-8 mol

	$\alpha_{2,1}$	$\alpha_{1,2}$
	1.227102	2.18241
	0.764115	2.084691
	1.035132	1.758958
	1.023839	1.824104
	0.176161	0.416938
	0.209787	0.492942
	0.21606	0.412595
	0.28005	0.521173
Mean	0.616531	1.211726
sd	0.442181	0.814386

1.0X10-7 mol

	$\alpha_{2,1}$	$\alpha_{1,2}$
	0.174167	0.312896
	0.201389	0.291755
	0.196111	0.255814
	0.253611	0.317125
	0.661111	0.350951
	0.563889	0.395349
	0.502778	0.560254
	0.863889	0.414376
Mean	0.427118	0.362315
sd	0.258667	0.095556

5.0X10-7 mol.

	$\alpha_{2,1}$	$\alpha_{1,2}$
	1.24427	1.412458
	0.86444	0.988721
	0.687623	0.567016
	1.093648	1.245808
	0.75442	0.865766
	0.555599	0.721878
	0.628684	0.746266
	0.472299	0.609694
Mean	0.787623	0.894701
sd	0.266648	0.302734

8.2X10-7 mol.

	$\alpha_{2,1}$	$\alpha_{1,2}$
	1.452482	0.761135
	1.165957	0.723404
	1.202837	0.582411
	1.129078	0.684539
	1.211348	0.791489
	1.605674	1.177305
	1.004255	0.913475
	1.296454	1.370213
Mean	1.258511	0.875496
sd	0.190816	0.268037

1.0X10-6 mol

	$\alpha_{2,1}$	$\alpha_{1,2}$
	0.816869	1.059386
	1.111162	1.078517
	0.794866	0.860901
	1.760257	0.538063
	0.948888	0.542846
	1.116204	0.514149
	1.074948	0.324831
	0.857208	0.48625
Mean	1.06005	0.675618
sd	0.311503	0.284221

Combined Experimental competition data
Chlorella vulgaris(2) vs. Chlamydomonas musicola(1)

HERBICIDE CONCENTRATION

0 mol

	$\alpha_{2,1}$	$\alpha_{1,2}$
	0.394464	0.821436
	0.539792	0.572071
	0.460208	0.96812
	0.460208	0.872775
	0.394464	0.821436
	0.539792	0.572071
	0.460208	0.96812
	0.460208	0.872775
mean	0.463668	0.808601
sd	0.055054	0.156457

1.0×10^{-8} mol.

	$\alpha_{2,1}$	$\alpha_{1,2}$
	0.377633	0.665896
	0.522876	0.300578
	0.374001	0.60578
	0.424837	0.610405
mean	0.424837	0.545665
sd	0.06934	0.165658

5.0X10-8 mol

	$\alpha_{2,1}$	$\alpha_{1,2}$
	0.126223	0.616343
	0.127478	0.556787
	0.126725	0.873961
	0.163363	0.573407
	0.40276	0.845568
	0.376412	0.808172
	0.304893	0.777008
	0.331242	0.889197
mean	0.244887	0.742555
sd	0.120567	0.138319

1.0X10-7 mol

	$\alpha_{2,1}$	$\alpha_{1,2}$
	0.427778	0.961284
	0.35	0.432483
	0.291667	1.042493
	0.488889	1.359773
	0.233333	1.29745
	0.211111	0.457035
	0.193056	0.970727
	0.1875	0.710104
mean	0.297917	0.903919
sd	0.114005	0.348291

5.0X10-7 mol.

	$\alpha_{2,1}$	$\alpha_{1,2}$
	1.225933	0.004196
	0.423576	0.064096
	0.44165	0.086802
	0.609037	0.062084
	1.19188	0.311377
	1.087099	0.196407
	0.949574	0.180838
	0.543549	0.186826
mean	0.809037	0.136578
sd	0.340478	0.099588

8.2X10-7 mol.

	$\alpha_{2,1}$	$\alpha_{1,2}$
	0.788652	0.735632
	0.788652	0.704981
	0.947518	0.628352
	0.75461	0.499617
	0.703546	1.057471
	0.797163	1.509579
	0.646809	0.911877
	0.507801	0.854406
mean	0.741844	0.862739
sd	0.128275	0.313197

1.0X10-6 mol

	$\alpha_{2,1}$	$\alpha_{1,2}$
	0.709603	1.853372
	0.536328	1.571848
	0.539079	2.27566
	0.772863	0.235777
	0.694476	1.094819
	0.69906	0.659824
	0.84804	1.446725
	0.405684	1.241447
mean	0.650642	1.297434
sd	0.144995	0.648232

Sumary Table of mean and standard deviations of Delta alpha values
Combined run1 and run 2
Scenedesmus quadracauda(2) vs. Chlorella vulgaris(1)

Herb con = 1.0X10-8

	delta $a_{1,2}$	delta $a_{2,1}$
	0.515096	-0.80025
	0.561838	-0.75586
	0.561838	-0.77066
	0.504309	-0.69852
	0.515096	-0.80025
	0.561838	-0.75586
	0.561838	-0.77066
	0.504309	-0.69852
mean $\Delta\alpha$	0.53577	-0.75632
sd $\Delta\alpha$	0.028164	0.039558

Herb con = 5.0X10-8

	delta $a_{1,2}$	delta $a_{2,1}$
	0.237264	-0.48548
	0.065277	-0.42232
	0.273746	-0.42897
	0.104365	-0.31595
	0.314137	-0.59434
	0.335853	-0.61235
	0.259848	-0.70376
	0.285907	-0.45722
mean $\Delta\alpha$	0.23455	-0.50255
sd $\Delta\alpha$	0.097848	0.125348

Herb con = 1.0X10-7

	delta $a_{1,2}$	delta $a_{2,1}$
	0.564893	-0.63289
	0.508515	-0.23787
	0.004639	-1.31121
	0.256577	-0.76352
	0.214294	-1.07985
	0.302384	-0.70057
	0.499706	-1.11763
	0.406331	-0.3921
mean $\Delta\alpha$	0.344667	-0.77945
sd $\Delta\alpha$	0.187216	0.370073

Herb con = 5.0X10-7

	delta $a_{1,2}$	delta $a_{2,1}$
		-0.27253
	-3.56674	-0.17672
		-0.20067
	-5.15194	-0.1863
	-2.93672	-0.22941
	-3.28221	-0.12402
	-2.9164	-0.21983
	-1.98153	-0.12402
mean $\Delta\alpha$	-3.30592	-0.19169
sd $\Delta\alpha$	1.050564	0.051047

Herb con = 8.2X10-7

	delta $a_{1,2}$	delta $a_{2,1}$
	-0.41003	-2.3462
	-2.10861	-1.57992
	-0.3462	-1.36919
	-0.74691	-1.25425
	-0.9845	-1.84812
	-0.4384	-1.81938
	-0.87457	-1.82896
	-0.45258	-1.31172
mean $\Delta\alpha$	-0.79522	-1.66972
sd $\Delta\alpha$	0.581152	0.365743

Herb con = 1.0X10-6

	delta $a_{1,2}$	delta $a_{2,1}$
	-1.66837	-2.15118
	-2.62492	-1.95567
	-2.42564	-1.19321
	-1.34952	-3.2069
	-2.14665	-3.79341
	-0.91109	-2.28803
	-2.70464	-2.52263
	-1.11038	-1.79927
mean $\Delta\alpha$	-1.86765	-2.36379
sd $\Delta\alpha$	0.703039	0.818212

Sumary Table of mean and standard deviations of Delta alpha values
Combined run1 and run 2
Scenedesmus quadracauda(2) vs. Chlamydomonas musicola(1)

Herb con = 1.0X10-8

	delta $a_{1,2}$	delta $a_{2,1}$
	1.853628	0.620199
	1.840145	0.500374
	1.925539	0.496743
	2.037898	0.507636
	1.853628	0.620199
	1.840145	0.500374
	1.925539	0.496743
	2.037898	0.507636
mean $\Delta\alpha$	1.914303	0.531238
sd $\Delta\alpha$	0.083807	0.055068

Herb con = 5.0X10-8

	delta $a_{1,2}$	delta $a_{2,1}$
	0.233016	-0.09129
	0.330736	0.371698
	0.656469	0.100681
	0.591322	0.111974
	1.998488	0.959653
	1.922484	0.926026
	2.002831	0.919753
	1.894254	0.855763
mean $\Delta\alpha$	1.2037	0.519282
sd $\Delta\alpha$	0.814386	0.442181

Herb con = 1.0X10-7

	delta $a_{1,2}$	delta $a_{2,1}$
	2.10253	0.961646
	2.123671	0.934424
	2.159612	0.939702
	2.098302	0.882202
	2.064475	0.474702
	2.020077	0.571924
	1.855173	0.633035
	2.00105	0.271924
mean $\Delta\alpha$	2.053111	0.708695
sd $\Delta\alpha$	0.095556	0.258667

Herb con = 5.0X10-7

	delta $a_{1,2}$	delta $a_{2,1}$
	1.002968	-0.10846
	1.426706	0.271373
	1.848411	0.44819
	1.169618	0.042165
	1.549661	0.381393
	1.693548	0.580214
	1.669161	0.507129
	1.805732	0.663515
mean $\Delta\alpha$	1.520726	0.34819
sd $\Delta\alpha$	0.302734	0.266648

Herb con = 8.2X10-7

	delta $a_{1,2}$	delta $a_{2,1}$
	1.654291	-0.31667
	1.692022	-0.03014
	1.833015	-0.06702
	1.730887	0.006735
	1.623937	-0.07553
	1.238121	-0.46986
	1.501951	0.131558
	1.045213	-0.16064
mean $\Delta\alpha$	1.53993	-0.1227
sd $\Delta\alpha$	0.268037	0.190816

Herb con = 1.0X10-6

	delta $a_{1,2}$	delta $a_{2,1}$
	1.35604	0.318944
	1.336909	0.024651
	1.554525	0.340947
	1.877363	-0.62444
	1.87258	0.186925
	1.901277	0.019609
	2.090596	0.060865
	1.929177	0.278605
mean $\Delta\alpha$	1.739808	0.075763
sd $\Delta\alpha$	0.284221	0.311503

Sumary Table of mean and standard deviations of Delta alpha values
Combined run1 and run 2
Chlamydomonas musicola(1) vs. Chlorella vulgaris(2)

Herb con = 1.0X10-8

	delta $a_{1,2}$	delta $a_{2,1}$
	0.142705	0.086035
	0.508023	-0.05921
	0.20282	0.089666
	0.198196	0.038831
mean $\Delta\alpha$	0.262936	0.038831
sd $\Delta\alpha$	0.165658	0.06934

Herb con = 5.0X10-8

	delta $a_{1,2}$	delta $a_{2,1}$
	0.192257	0.337444
	0.251814	0.33619
	-0.06536	0.336943
	0.235193	0.300305
	-0.03697	0.060907
	0.000429	0.087256
	0.031592	0.158774
	-0.0806	0.132426
mean $\Delta\alpha$	0.066045	0.218781
sd $\Delta\alpha$	0.138319	0.120567

Herb con = 1.0X10-7

	delta $a_{1,2}$	delta $a_{2,1}$
	-0.15268	0.03589
	0.376117	0.113668
	-0.23389	0.172001
	-0.55117	-0.02522
	-0.48885	0.230334
	0.351566	0.252557
	-0.16213	0.270612
	0.098497	0.276168
mean $\Delta\alpha$	-0.09532	0.165751
sd $\Delta\alpha$	0.348291	0.114005

Herb con = 5.0X10-7

	delta $a_{1,2}$	delta $a_{2,1}$
	0.804404	-0.76227
	0.744505	0.040092
	0.721798	0.022018
	0.746517	-0.14537
	0.497223	-0.72821
	0.612193	-0.62343
	0.627762	-0.48591
	0.621774	-0.07988
mean $\Delta\alpha$	0.672022	-0.34537
sd $\Delta\alpha$	0.099588	0.340478

Herb con = 8.2X10-7

	delta $a_{1,2}$	delta $a_{2,1}$
	0.072968	-0.32498
	0.10362	-0.32498
	0.180248	-0.48385
	0.308984	-0.29094
	-0.24887	-0.23988
	-0.70098	-0.3335
	-0.10328	-0.18314
	-0.04581	-0.04413
mean $\Delta\alpha$	-0.05414	-0.27818
sd $\Delta\alpha$	0.313197	0.128275

Herb con = 1.0X10-6

	delta $a_{1,2}$	delta $a_{2,1}$
	-1.04477	-0.24594
	-0.76325	-0.07266
	-1.46706	-0.07541
	0.572823	-0.30919
	-0.28622	-0.23081
	0.148777	-0.23539
	-0.63812	-0.38437
	-0.43285	0.057984
mean $\Delta\alpha$	-0.48883	-0.18697
sd $\Delta\alpha$	0.648232	0.144995