12-2001

Effects of Binary Combinations of Herbicides on Freshwater Algae

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EFFECTS OF BINARY COMBINATIONS OF HERBICIDES ON FRESHWATER ALGAE

by

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A THESIS

Presented to the Faculty of
The Graduate College at the University of Nebraska
In Partial Fulfillment of Requirements
For the Degree of Master of Science

Major: Natural Resource Sciences

Under the Supervision of Professors
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Lincoln, Nebraska

December, 2001

ACKNOWLEDGEMENTS

I would like to thank the U. S. Geological Survey (section 104) for the funding of this project, as well as the University of Nebraska-Lincoln and the School of Natural Resource Sciences for providing the facilities and resources necessary to make this project a success.

I would also like to thank Drs. Kyle Hoagland and Blair Siegfried for serving as my major advisors and for their direction and helpful advice throughout the course of this research. I thank Dr. Pat Shea for serving as the third member on my committee and for providing additional advice and ideas that added to the overall quality of this research project. A special thank-you goes to Lindsay Rogers for her help with research that added greatly to the initial findings and enabled me to pursue another avenue of research that proved extremely meaningful. Thank-you to Hillary Hansen, who always kept the lab running smoothly and was helpful in solving any problems that came up. A special thank-you also goes to Christine Lockert, who helped me tremendously when I first started this project and continued to provide me with good advice whenever needed.

In addition, I would like to say thank-you to my friends and family who gave needed moral support and encouragement to me throughout this time period. And finally, thanks to my husband, Brent, for your love and encouragement these last few years.

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Introduction

Pesticides are of vital importance in agriculture and are applied in great amounts in the midwestern United States. For example, it is estimated that 300 million kilograms of pesticide active ingredient are applied to agricultural fields each year (Carder and Hoagland 1998). A survey of several midwestern states in 1994 showed that more than 97% of corn and soybean acreage was treated with herbicides (USDA 1995). Such practices have resulted in herbicide contamination of surface waters as a result of non-point source runoff from agricultural fields, particularly during spring storm events.

It is important to understand how pesticides affect the biota and overall health of aquatic ecosystems. Because herbicides inhibit metabolic processes in plants, non-target algae, cyanobacteria, and aquatic vascular plants may be affected by herbicides in the aquatic environment. Algae are primary producers at the base of the aquatic food chain, serving as a food source for other organisms in aquatic ecosystems. Algae also function in the uptake, storage, release and deposition of nutrients (Fairchild *et al.* 1998). Herbicides can change the ecophysiology and population dynamics of aquatic communities, causing more sensitive species to decline, while providing a competitive advantage to more tolerant species (Seguin *et al.* 2001). Thus, it is possible for herbicides like atrazine to indirectly influence entire food webs through damage caused to phytoplankton, periphyton and macrophytes, reducing the ability of aquatic habitats to sustain higher trophic levels (Solomon *et al.* 1996). For example, changes in the composition of phytoplankton species can impact the rate of growth of zooplankton grazers (Ahlgren *et al.* 1990). Due to the possibility of such harmful ecological effects, numerous studies have recently been conducted to determine the effect of pesticides on freshwater algae.

Thus far, most aquatic research has been conducted on algal responses to atrazine. Some tests on other pesticides, such as alachlor, have also been reported (Weisshaar *et al.* 1988, Couderchet and Böger 1993, Spawn *et al.* 1997, Carder and Hoagland 1998). Atrazine and alachlor are the

most heavily applied pesticides in the U.S., with application rates of 36 million and 39 million kilograms per year, respectively (Carder and Hoagland, 1998). However, little research has been done on the effects of various combinations of pesticides. Such research is critical, especially given the high potential for mixtures of pesticides to enter aquatic systems in agricultural runoff (Faust *et al.* 1994, Fairchild *et al.* 1997). The number of pesticide combinations and interactions are potentially enormous. Not only are pesticides present, but as pesticides break down due to the action of bacteria, sunlight, etc., their degradation products are also included in these mixtures, with some degradation products exhibiting a greater toxicity than the parent compound. Studies on alachlor have found at least 14 different degradation products (Chiron *et al.* 1995). This is an important area requiring further study.

Pesticide Interactions

Three interactions are possible when organisms are exposed to mixtures of pollutants: additive, synergistic and antagonistic. An additive effect is one in which the combined effect of two chemicals applied in combination causes a response that is the same as that obtained when one chemical is substituted for the other. A synergistic effect is a response that is greater than the sum of the toxicities of the individual chemical components, and an antagonistic effect is a response less than the sum of the toxicities of the individual chemical components (Akobundu *et al.* 1975, Preston *et al.* 2000).

There is now widespread agreement within the scientific community that mixtures of substances with a similar mode of action obey the concept of concentration addition. Experimental evidence (Boedeker *et al.* 1993) supports the idea that concentration addition might also be a reliable tool for the ecotoxicological hazard assessment of mixtures of dissimilar acting substances (Faust *et al.* 1993). Faust *et al.* (1993) concluded that whenever experimental data on the toxicity of a specific mixture are not available, concentration addition may be a reasonable assumption in hazard assessment. Carder and Hoagland (1998) indicated that atrazine and

alachlor affect stream algal communities in an additive manner. However, the potential for synergistic effects of pesticide combinations remains of great concern (Steevens and Benson 1997, Tripathi and Agarwal 1997).

Herbicide characteristics and modes of action

The herbicides chosen for the current study are all commonly used throughout the midwestern U.S. Atrazine, alachlor, metolachlor, simazine and isoxaflutole are used primarily to control broadleaf weeds in corn and soybeans. Metolachlor, a chloroacetamide, is very similar in structure to alachlor and shares a similar mode of action. The same is true for simazine and atrazine; both are triazine herbicides with similar modes of action. Isoxaflutole is a relatively new herbicide that is likely to increase in importance and in acres treated (Figure 1).

Atrazine and Simazine

Triazine herbicides disrupt photosynthesis through reversibly binding to the plastoquinone B (QB) protein binding site on the D1 protein in the Photosystem II complex (Velthuys 1981, Fuerst and Norman 1991, Weed Society of America 1994, Wilson *et al.* 2000). Binding of these herbicides effectively halts photosynthetic electron transport from Plastoquinone B (QB) to Plastoquinone A (QA), CO_2 fixation, and the production of ATP and $NADPH_2$ (Wilson *et al.* 2000). This causes excessive radiative excitation in the blocked photosynthetic pigment system. Consequences of this blockage include maximum fluorescence emission, energy spillover to oxygen and other molecules, photoxidation, and eventually phytotoxicity at the organelle, cell, and tissue levels (Devine *et al.* 1993a). Plant death is most commonly caused by the resulting process of lipid peroxidation, which results from the formation of triplet state chlorophyll and singlet state oxygen. Triplet chlorophyll and singlet oxygen are common products that result from the inability to reoxidize QA, because of binding of the herbicide to QB (Ahrens 1994).

Atrazine is widely used for controlling certain annual broadleaf weeds and grasses and is moderately soluble in water (33 mg/L at 22 °C) (Weed Science Society of America 1994). The properties of atrazine enable this pesticide to move from the point of application into ground and/or surface waters (Solomon *et al.* 1996). Atrazine is decomposed by UV light and is hydrolytically stable at pH 5, 7, and 9 in buffered water, with hydrolysis rates increasing at lower or higher pH. The half-life of atrazine has been reported as 335 days in water at 12-45°C (Weed Science Society of America 1994), however, this will vary with relative water chemistry. Stratton (1984) reported atrazine persistence in the aqueous phase from 3 to 12 days and in sediments from 15-20 days. Jones *et al.* (1982) reported that atrazine can persist in soils for up to one year, and can leach into surface water long after the date of initial application. Degradation products of atrazine, such as hydroxyatrazine and deethylatrazine, tend to persist longer and are more mobile in water than the parent compound (DeLorenzo *et al.* 2001).

Deethylatrazine provides an excellent example of the potential for increased persistence, solubility, and toxicity of degradation products. It is the most commonly detected transformation product of atrazine in aquatic ecosystems (Adams and Randtke 1992, Thurman *et al.* 1994) and has lower K_d (sorption distribution coefficient) and K_{oc} (organic carbon sorption distribution coefficient) values than atrazine, causing it to be more readily transported in water. It is also persistent in surface waters (DeLorenzo *et al.* 2001). Pereira and Hostettler (1993) found no evidence of deethylatrazine degradation during its transit time of 46-65 days in the Mississippi River, USA. A laboratory study found deethylatrazine to be almost as toxic as atrazine to estuarine microbial communities (DeLorenzo *et al.* 1999a). Studies have shown degradation products of atrazine to be less toxic than atrazine alone [Stratton 1984, Eisler 1989, Hoagland *et al.* (in press)], although DeLorenzo *et al.* (1999a) has provided some evidence suggesting that these compounds are still potentially toxic.

Like atrazine, simazine is used primarily to control broadleaf weeds and grasses and is also moderately soluble in water (6.2 mg/L at 22 °C), which allows it to move off-site and

contaminate surface waters (Ahrens 1994, Weed Society of America 1994). Simazine decomposes when exposed to UV light and is slowly hydrolyzed at neutral pH (Weed Society of America 1994).

Effects of atrazine and simazine on freshwater algae

Because of the widespread use of atrazine and its relative water solubility and persistence in aqueous environments, it has been identified as a principle contaminant of surface waters (Solomon *et al.* 1996). This has resulted in numerous studies on the effects of atrazine on aquatic organisms. Studies indicate that freshwater algae tend to be the most susceptible aquatic organisms to atrazine (Solomon *et al.* 1996) and that green algae (Chlorophyta) tend to be the most susceptible group within the algae (Tang *et al.* 1998a, Nelson *et al.* 1999). Many algal studies have indicated a wide range of sensitivities to atrazine (DeNoyelles *et al.* 1982, Kasai and Hatakeyama 1993; Tang *et al.* 1997, Hughes *et al.* 1998). Differential toxicity among the algae can have serious implications to aquatic communities, leading to drastic changes in community structure and function (Hersh and Crumpton 1987). Glutathione conjugation appears to be the detoxification pathway responsible for plant tolerance to atrazine, however, it does not appear that the small differences among algal species and divisions in GST activity are responsible for the relatively large differences in atrazine sensitivity (Tang *et al.* 1998b).

Several studies on various single-celled algae indicated some signs of growth inhibition or effects on chlorophyll *a* content at atrazine concentrations as low as 1.0 µg/L (Butler *et al.* 1975, Torres and O'Flaherty 1976, O'Kelly and Deason 1976). However, concentrations at this level are well under the range that will be environmentally relevant during a storm runoff event. For example, a water sample taken from Loeske Creek near Columbus, NE after a storm runoff event had an atrazine concentration of 691 µg/L (Langan *et al.* unpublished data). However, concentrations near 100 µg/L are more commonly found. These higher atrazine concentrations stand in stark contrast to the 1µg/L concentration reported to inhibit algal growth.

Few studies have been conducted to determine the effects of simazine on algal growth. Because simazine's mode of action is the same as that of atrazine, the effects are likely to be similar. According to O'Neal and Lembi (1993), photosynthesis was inhibited by 50% in filamentous algae at 222-949 μg/L and at 949 μg/L in nonfilamentous algae. Other studies report 1.0 mg/L simazine significantly reduced chlorophyll production in three filamentous algal species after 7 days (Torres and O'Flaherty 1976) and inhibited carbon uptake by 50% in all algae at a concentration of 2.67 mg/L (Peterson *et al.* 1994).

Alachlor and Metolachlor

Alachlor and metolachlor belong to the class of herbicides known as the acetanilides, also called the chloroacetamides. The mode of action for this herbicide class is still uncertain. Acetanilides are an established class of preemergent herbicides (Weisshaar *et al.* 1988). Known activity of these compounds include inhibition of biosyntheses of fatty acids and lipids, proteins, isoprenoids and flavonoids. Most susceptible grass and broadleaf weeds fail to emerge. Susceptible monocots that do emerge appear twisted and malformed due to leaves not emerging properly from the coleoptile (Weed Society of America 1994). The basis for selective phytotoxicity of alachlor and metolachlor is the lack of metabolic deactivation in susceptible plants. The herbicides are detoxified rapidly by conjugation with glutathione (GSH) and/or homoglutathione (hGSH) (Lamoureaux *et al.* 1971, Breaux *et al.* 1986, 1987). About 37 million kilograms of alachlor and 22 million kilograms of metolachlor are used annually in the United States. Over 98% of alachlor and metolachlor manufactured are used on field corn, soybeans, sorghum, and peanuts, with 60 and 30% being used on corn and soybeans, respectively (Chesters *et al.* 1989). Although alachlor use is almost twice that of metolachlor for corn and soybeans, metolachlor use has increased dramatically since 1980 (Hogue 1986).

The half-life of alachlor is approximately 80 days in soil and less than 239 days in water, and it generally persists in the field for up to 21 days. For metolachlor the half-life is 70 days in water

from 8-45 °C and it can persist in the field for 30-50 days in northern areas and 15-20 days in southern areas. Alachlor is low to moderately mobile in soil and losses due to volatilization are low. Water solubility for alachlor is 200 mg/L at 20 °C. Field experiments have shown that metolachlor has low mobility in soil and leaching is generally insignificant when soil organic matter is above 2% (Weed Society of America 1994).

Effects of alachlor and metolachlor on freshwater algae

Few studies have specifically looked for effects of alachlor on freshwater algae, even though this herbicide is widely used (Chesters *et al.* 1989). Even fewer studies have been done on the effects of metolachlor. As in higher plants, the modes of action of alachlor and metolachlor for algae are not well understood. Recent studies have indicated that these herbicides interfere in fatty acid synthesis, which results in the inhibition of cell division and growth (Deal and Hess 1980, Fuerst 1987, LaBaron *et al.* 1988, Weisshaar and Böger 1991, Couderchet and Böger 1993). Alachlor has been found to inhibit algal growth and photosynthesis at concentrations as low as 0.1 µg/L and concentrations as high as 105 µg/L have been found in midwestern streams (Baker *et al.* 1981, Spawn *et al.* 1997, Carder and Hoagland 1998). Alachlor concentrations as high as 635 µg/L were measured after a storm runoff event in a Nebraska stream (Langan *et al.* unpublished data), and therefore, pulses of alachlor can be extremely high, as well.

Isoxaflutole

Isoxaflutole is a relatively new herbicide marketed for broadleaf and grass weed control in corn. Isoxaflutole is a proherbicide, which requires hydrolytic activation to form the diketonitrile derivative. This diketonitrile derivative is formed through hydrolysis and is, therefore, activated by rainfall events. In this way, isoxaflutole provides control of small weeds that emerge after herbicide application has occurred (Taylor-Lovell *et al.* 2000). Environmental benefits of isoxaflutole include rapid degradation, low rates of application, and a favorable toxicology in

comparison to conventional herbicides such as atrazine (Luscombe and Pallett 1996). Because isoxaflutole is the only registered herbicide exhibiting this mode of action, there is a high potential for a rapid increase in use (Taylor-Lovell *et al.* 2000).

The diketonitrile derivative is known to inhibit the 4-hydroxyphenylpyruvate dioxygenase (HPPD), which blocks an essential plastoquinone biosynthetic pathway. The toxicity is due to the *in vivo* inhibition of phytoene desaturase resulting from a depletion of the plastoquinone, which is an essential cofactor in carotenoid biosynthesis (Pallett *et al.* 1998). Carotenoids function to rid plants of excess excitation energy that occurs naturally during photosynthesis. When there are no carotenoids due to inhibition of the biosynthetic pathway, there is no way for plants to get rid of this excess excitation energy. This causes a chain reaction of free radical formation and lipid peroxidation, leading to loss of functional integrity of cells, and eventually, cell and plant death (Devine *et al.* 1993). Plants tolerant of isoxaflutole, such as corn, are able to metabolize the active diketonitrile derivative very quickly, thus avoiding its toxicity (Pallett *et al.* 1998). Toxicity of isoxaflutole to freshwater algae is unknown, however due to the likely increased use of this herbicide in the future, investigation into possible toxic effects is warranted.

Objectives

The purpose of this study was to investigate the potential synergistic effects among various herbicides important in agriculture. There is a general lack of knowledge concerning the impacts of pesticides on midwestern aquatic communities, and therefore, a general lack of knowledge of the potential effects of multiple contaminants on aquatic biota. Although a number of pesticides have been shown to be relatively harmless at environmentally relevant concentrations when tested individually, little is known about the potential interaction effects when they occur together in the same system. Given the importance of freshwater algae to the overall functional integrity of

aquatic ecosystems, a greater knowledge of the potential interaction of pesticides is necessary and will be of importance to assessing pesticide impacts on aquatic communities.

Materials and Methods

Algal culturing

Chlorella vulgaris, *Cyclotella meneghiniana*, and *Ankistrodesmus falcatus* were obtained from a culture collection maintained in our laboratory; all three represent taxa commonly associated with aquatic systems in the midwestern U.S. Algae from culture collections were chosen for use because of their lack of previous exposure to herbicides. Algal taxa represented two divisions, including the green algae (Division Chlorophyta) and the diatoms (Division Chrysophyta). Algae were grown in WC freshwater medium (Guillard 1975) under unialgal, axenic conditions in 25 x 150 mm glass culture tubes, and were transferred to fresh growth medium every two weeks to provide optimal growth. Cultures were incubated at 20 °C with an alternate 12:12 light:dark cycle under cool white fluorescent lamps (100 μmol $m^{-2}s^{-1}$). Experimental conditions for algal bioassays are summarized in Table 1.

Pesticide exposures

Technical grade herbicides varying in percent purity (Table 2) were purchased from Chem Service (West Chester, PA, USA), including atrazine, simazine, alachlor and metolachlor. An isoxaflutole metabolite (diketonitrile) was purchased from Rhône Poulenc Ag Company (Research Triangle Park, NC). Because isoxaflutole in the form of the parent compound is not actively toxic (Taylor-Lovell *et al.* 2000), only the active metabolite was tested. Herbicide chemical formulations and modes of action are listed in Tables 2 and 3. Serial dilutions of a 1 mg/mL stock solution of each herbicide were prepared in reagent grade acetone. Acetone bioassays were initially performed to ensure that the solvent would not cause a toxic response to

algal species tested. Solutions were added at a volume of 100 µL to autoclaved growth medium at various concentrations based on their relative algal toxicities. A volume of 100 µL acetone was also added to control treatments. Treated growth medium was dispensed into autoclaved culture tubes (40 mL) and subsequently inoculated with a suspension of algal cells (6-9 days old). Each tube was replicated five times for each herbicide concentration, providing five replications for each species at each treatment level. All algal transfer procedures were performed aseptically under a laminar flow hood. *In vivo* chlorophyll *a* fluorescence was non-destructively measured on day 0, 1, 3, 5, and 7 with a Turner Designs fluorometer (Sunnyvale, CA, USA, model 10-AU) using a 436-nm excitation filter and a 680-nm emission filter. Fluorescence measurements were taken after vortexing the tube and directly inserting it into the fluorometer chamber (i.e. with no sub-sampling or extraction). Growth rates in cell doublings per day (dpd) were calculated based on the fluorometer readings using the formula: $\ln ft_2 - \ln ft_1 / (t_2-t_1) \ln 2$; where ft_1 = fluorescence at time 1 and ft_2 = fluorescence at time 2 (Guillard 1975). Cultures were transferred into fresh herbicide medium on day 7 to avoid possible nutrient limitation, which can be a significant factor in declining growth rates.

Single species bioassays were initially performed for each herbicide individually. Combination bioassays were then performed using a single concentration of simazine, alachlor, metolachlor, and isoxaflutole at 100 µg/L in combination with a range of atrazine concentrations that corresponded to the EC_{35}, EC_{50}, EC_{75}, and EC_{95} levels calculated for *Chlorella vulgaris* and *Cyclotella meneghiniana* (Table 4). *Ankistrodesmus falcatus* was assayed with alachlor and metolachlor in combination with atrazine. Atrazine concentrations ranged from 10 µg/L to 100 µg/L in combination with 50 and 100 µg/L alachlor or 25 and 50 µg/L metolachlor. Individual effects of isoxaflutole on *Ankistodesmus* were also measured. No experiments were conducted for this species using atrazine or simazine individually.

Data analysis

All analyses were conducted using data collected after three days of exposure because growth rates were highest on that day, indicating a non-limiting nutrient supply in culture. Mean growth rates (dpd) for each concentration were compared to control means and the percent inhibition of algal growth on day 3 was calculated using 100*(1-T/C); where T = mean growth rate of treated cultures based on fluorescence or cell density and C = mean growth rate of control cultures. Statistically significant effects of individual and combination herbicide exposure were determined using an ANOVA in conjunction with non-linear regression analysis (SAS Institute Inc. 1999).

Results

Algal responses to individual herbicides

The three algal species tested were differentially susceptible to atrazine, simazine, alachlor, and metolachlor, and all exhibited a high tolerance for isoxaflutole with no inhibition at concentrations up to 1000 µg/L (Figures 2-4).

All species were significantly inhibited by atrazine alone ($P < 0.0001$), with *C. vulgaris* (Figure 2) and *A. falcatus* (Figure 7) most susceptible and *C. meneghiniana* most tolerant (Figure 3). *C. vulgaris* and *C. meneghiniana* were less inhibited by simazine than atrazine, with *C. vulgaris* more inhibited than *C. meneghiniana* (Figure 2-3).

Alachlor and metolachlor significantly inhibited growth of *C. meneghiniana*, but only at 1000 µg/L (Figure 3), while *C. vulgaris* was not significantly inhibited at that concentration. The inhibition curves for alachlor and metolachlor were not significant for either *C. vulgaris* or *C. meneghiniana.* In contrast, *A. falcatus* was highly susceptible to both alachlor and metolachlor, with significant inhibition at concentrations as low as 10 µg/L metolachlor (Figure 4). The inhibition curves for alachlor and metolachlor with this species were highly significant ($P < 0.0001$).

Algal responses to binary herbicide mixtures

The effects of 100 µg/L alachlor in combination with atrazine did not differ from those of the atrazine alone in *C. vulgaris*. Similar results were obtained for *C. meneghiniana,* except at the highest concentration combination, where toxicity was significantly enhanced ($P = 0.0179$) by the presence of alachlor (Figure 5-6). However, for *A. falcatus* the presence of alachlor significantly enhanced the atrazine toxicity. There was a significant difference between atrazine inhibition alone and combined inhibitions at all concentrations levels, however the effects of the two levels of alachlor (50 and 100 µg/L) in combination with atrazine did not differ significantly from one another (Figure 7).

Atrazine in combination with metolachlor yielded results similar to atrazine/alachlor combinations for all species. The combination of 100 µg/L metolachlor with atrazine was not significantly different from inhibition by atrazine alone for *C. vulgaris* (Figure 5), except at the lowest concentration combination level, where toxicity was significantly enhanced ($P = 0.0188$). The combination increased atrazine toxicity to *C. meneghiniana* at two concentrations (atrazine EC_{50} and EC_{95} + metolachlor 100 µg/L), where growth inhibition was slightly greater in combination than with atrazine alone (Figure 6). Results of metolachlor and atrazine combinations for *A. falcatus* were also similar to those for alachlor, although lower concentrations of metolachlor were tested (25 and 50 µg/L). Atrazine combinations ranged from 10–100 µg/L in one series of experiments and 100–1000 µg/L in another. Greater differences in inhibition were observed at lower atrazine concentration combinations. Growth inhibition increased dramatically when 10 µg/L atrazine was combined with 50 µg/L alachlor and 25 µg/L metolachlor. Combined effects were significantly different between metolachlor concentrations (25 and 50 µg/L) at the lowest atrazine concentration of 10 µg/L. Therefore, inhibition may increase even more in a combination with 50 µg/L metolachlor (Figure 7 and Appendix Table 6).

Simazine in combination with atrazine did not cause significant increases in inhibition for *C. vulgaris* or *C. meneghiniana,* with one exception: *C. vulgaris* exposed to the lowest concentration combination (EC_{35} atrazine + 100 µg/L simazine; $P<0.0001$) exhibited significant inhibition from atrazine alone.

The combination of isoxaflutole with atrazine was not significantly different for growth inhibition in *C. vulgaris* and had little effect on growth in *C. meneghiniana.* For *C. meneghiniana* a relatively small, yet significant ($P = 0.0099$) decrease in growth inhibition at the lowest combined concentration occurred consistently throughout the experiment.

In *C. vulgaris*, inhibition curves for alachlor, metolachlor, isoxaflutole and simazine in combination with atrazine were not significantly different from atrazine alone (Figure 5). Differences in inhibition curves in *C. meneghiniana* were not significant for atrazine in combination with the herbicides alachlor and simazine, while combinations with metolachlor and isoxaflutole were significant (Figure 6). Inhibition curves for atrazine in combination with alachlor and metolachlor in *A. falcatus* were significant from inhibition curves obtained for atrazine alone (Figure 7).

Discussion

Differential toxicity and individual atrazine effects

The results of atrazine bioassays were consistent with previous studies, which have shown that atrazine is highly toxic to all three algal species (Tang *et al.* 1997, King 2000). Previous reports indicated a wide range of sensitivity to atrazine among algal species and divisions, with green algae being 4 to 10 times more sensitive than diatoms (Tang *et al.* 1998a).

A microcosm study of periphyton communities found that atrazine concentrations of 10 µg/L or less had a stimulatory effect on protein biomass, chlorophyll *a* biomass, and species richness, whereas higher concentrations led to a significant loss of benthic algal species and biomass, as

well as lower net oxygen production (Pratt *et al.* 1988). It has also been reported that atrazine concentrations of 0.8 to 1.56 µg/L reduced the productivity of attached lake algae by 21 to 82%, depending on the species (Hamilton *et al.* 1988). Still other studies have shown that periphyton communities do not necessarily become resistant to atrazine after previous exposures (Kosinski 1984a, Brockway *et al.* 1984, Kosinski and Merkle 1984b, Hamala and Kollig 1985, Nystrom et al. 2000), but rather show a greater sensitivity to atrazine in acute exposures following a chronic exposure and period of recovery (Hamala and Kollig 1985, Pennington 1996, Nelson *et al.* 1999).

Changes in algal community composition due to atrazine exposure have been described in many studies; however the relative sensitivity of different taxa have been inconsistent. Planktonic green algae and flagellates were reduced while cryptophytes (especially *Cryptomonas*) and chrysophytes (especially *Mallomonas*) increased in abundance in pond mesocosm studies (deNoyelles *et al.* 1982). Similarly, Hamilton *et al.* (1988) found that green algae, diatoms, and dinoflagellates in enclosed phytoplankton communities exposed to 100 µg/L atrazine were reduced, whereas chrysophytes were unchanged and cryptophytes increased slightly in relative abundance. Using single species bioassays, King (2000) observed that green algae were most susceptible to atrazine, while cyanobacteria were less sensitive, followed by the diatoms *Cyclotella meneghiniana* and *Nitzschia palea*, and the cryptophyte *Cryptomonas ovata*. *Euglena gracilis* was unaffected or even slightly stimulated at 100 µg/L atrazine. Hamala and Kollig (1985) found a reduction in chrysophytes and diatoms in laboratory streams exposed to 100 µg/L atrazine, whereas cyanobacteria increased in relative abundance. In another study, cyanobacteria were eliminated when exposed to 100 µg/L atrazine, while diatoms were almost unaffected in a DeLorenzo *et al.* (1999b) study.

Results of the present study support the conclusion that the effects of atrazine contamination on aquatic communities will vary depending on the species present and their relative sensitivity (King 2000, Belden and Lydy 2000). Algal communities dominated by green algae will likely shift to more tolerant species, whereas those communities already dominated by tolerant species

may not show adverse effects. Any changes in algal community structure and composition may affect organisms at higher trophic levels in the aquatic food web (Pratt *et al.* 1988).

Individual simazine effects and comparisons with atrazine

In general, simazine appeared to be less toxic than atrazine to the species tested. These results were somewhat unexpected, because the two compounds share identical modes of action (Ahrens 1994, Weed Society of America 1994). It is possible that simazine binds more loosely or with less affinity to the plastoquinone binding site in Photosystem II than atrazine. There could also be differences in the uptake of the two herbicides, with atrazine being transported to the site of action more efficiently than simazine. There are differences in K_{ow} (octanol/water partition coefficient) values for the two herbicides, with a K_{ow} of 481 (at 25 °C) and 122 for atrazine and simazine respectively (Weed Society of America 1994). The greater the K_{ow} value, the more lipophilic a molecule is, which is pertinent because the cell membranes of algae are composed primarily of lipids (Stewart 1974). This may indicate that atrazine is better able to penetrate algal cell membranes than simazine, and is therefore more toxic at lower concentrations. Algal metabolism may also play an important role in the relative differences of atrazine and simazine toxicity.

Individual alachlor effects

The results of the alachlor experiments indicated that *C. vulgaris* was completely unaffected up to 5,000 µg/L, while *C. meneghiniana* was only slightly inhibited at higher alachlor concentrations. In contrast, alachlor inhibited *A. falcatus* growth even at relatively low concentrations. Spawn *et al.* (1997) found that alachlor concentrations greater than 10 µg/L reduced chlorophyll *a* levels and altered algal taxonomic composition in microcosm experiments, while concentrations greater than 30 µg/L significantly reduced algal cell densities.

It is clear that alachlor, like atrazine, is differentially toxic to algae. The difference in sensitivities among the three species tested in this investigation was dramatic, especially between *C. vulgaris*. and *A. falcatus*, both green algae. Although *C. meneghiniana* was significantly inhibited, such effects were noted only at very high concentrations (1000 µg/L), which are not environmentally relevant. In contrast, *A. falcatus* was greatly inhibited even at 25 µg/L, a level found in midwestern streams (Spawn *et al.* 1997, Carder and Hoagland 1998, Nelson *et al.* 1999).

A previous microcosm study reported that *A. falcatus* was not significantly affected by alachlor at concentrations of 1.0 to 10 µg/L, but was significantly reduced in relative abundance at concentrations of 30, 100 and 1000 µg/L (Spawn *et al.* 1997). Interestingly, algal species that showed no significant negative effects included centric diatoms and unicellular green algae.

Mechanisms of fatty acid synthesis and differential toxicity

Weisshaar *et al.* (1988) reported that a 40-hour exposure to 5 µM alachlor led to a strong decrease in linolenic acid (18:3) content in the green alga *Scenedesmus acutus*. Their data suggested that alachlor inhibited fatty acid biosynthesis by preventing the C-elongation step of palmitic acid (16:0) and the desaturation of oleic acid (18:1) to form linolenic acid (18:3). In *Scenedesmus acutus*, inhibition of acyl-lipid formation was the most rapid effect, and after 40 hours, linolenic acid (18:3) content was greatly reduced, while palmitic acid and oleic acid content was greatly increased.

These results indicate that alachlor may block the desaturation step from palmitic to linolenic acid. In general, freshwater green algae contain few fatty acids with more than three double bonds or 18 carbon atoms, and just as in higher plants, linolenic acid is the primary 18:3 acid. Some algae can progressively desaturate monoenoic acids, yielding di- and polyenoic acids, yet algae fall into two groups in their ability to desaturate exogenously supplied saturated fatty acids. Some species can undergo direct desaturation, while others resemble higher plants in being unable to desaturate exogenously supplied stearic (18:0) and palmitic acids (16:0). According to

Stewart (1974), *Chlorella* species can directly desaturate stearic acid that is exogenously supplied. This observation may explain why *C. vulgaris* showed no signs of inhibition in the current study. In contrast, *A. falcatus* may not be able to desaturate exogenously supplied palmitic and stearic acids, and is therefore highly susceptible to alachlor.

Diatoms contain an odd, but consistent distribution pattern of fatty acids. There is a considerable quantity of acids of the palmitic (C16) series. The very low level of linolenic acids is noteworthy (Stewart 1974), because it may explain why *C. meneghiniana* was only slightly inhibited by alachlor. Diatoms do not need fatty acids of the stearic (C18) series, and unlike chlorophytes, do not require the desaturation steps necessary to synthesize linolenic acids. However, in the microcosm study by Spawn *et al.* (1997) several species of diatoms were significantly inhibited, including *Melosira varians* and *Nitzschia* spp. Therefore, this explanation may not sufficiently explain differential toxicity in diatoms. However, knowledge of fatty acid content for individual species has not been extensively studied. Thus, it may be possible that diatom species with few or no fatty acid carbon chains exceeding C16 may be better able to cope with alachlor exposure than species dependent upon longer carbon chain fatty acids.

Individual metolachlor effects

Metolachlor and alachlor caused similar effects in the three study species. These results were not surprising, due to the similar properties of the two compounds. Metolachlor likely affects fatty acid synthesis in a manner similar to alachlor, and therefore, the basis for alachlor differential toxicity should apply to metolachlor as well.

Individual isoxaflutole effects

The isoxaflutole metabolite had no significant effects on the algal species in this study. These results were somewhat surprising because this herbicide affects carotenoid synthesis. These pigments play an important role in quenching harmful excitation energy in photosynthesis. Crops

such as corn survive the toxic effects of this herbicide through efficient metabolism (Pallett *et al.* 1998). It may be possible that the algal species tested were able to metabolize the toxic product quickly before it could accumulate within cells and cause inhibition. Another possible tolerance mechanism could be that the algae tested utilize different biosynthetic pathways than vascular plants in their production of carotenoids, thereby avoiding the inhibitory effects of the herbicide.

Combination effects

The results of the herbicide combinations were consistent with results from individual herbicide exposure bioassays. Herbicides that cause little or no significant inhibition individually did not significantly affect the toxicity of atrazine. This included atrazine combinations with all herbicides for *C. vulgaris* and combinations with simazine and alachlor for *C. meneghiniana.* The only combinations that significantly enhanced toxicity included atrazine in combination with alachlor and metolachlor for *A. falcatus*, which is also consistent with results from the single herbicide bioassays. For this species, it appears that alachlor and metolachlor interacted with atrazine in an additive manner, in that both alachlor and metolachlor added to the toxicity of atrazine (Carder and Hoagland 1998). These results may have environmental implications due to the relatively low concentrations of both herbicides (e.g. 10 µg/L atrazine and 25 µg/L metolachlor), which in combination caused significant growth inhibitions.

Small but significant increases in inhibition occurred in *C. meneghiniana* in response to alachlor and metolachlor mixtures. The effects of metolachlor in combination with atrazine were significant at EC_{50} (P=0.0191) and EC_{95} (P=0.0074) concentrations. For both of these combinations, toxicity increased due to the effects of metolachlor as compared to the effects of atrazine alone. In contrast, alachlor mixed with atrazine exhibited only one significant combination level for *C. meneghiniana* (EC_{95} level for atrazine). Growth inhibition was highest at this atrazine concentration, even without the effects of another herbicide. The underlying basis

for this difference in sensitivity of *C. meneghiniana* to alachlor and metolachlor in combination with atrazine remains unclear.

Isoxaflutole appeared to cause some antagonism of atrazine toxicity in *C. meneghiniana.* Combinations at the lowest levels for atrazine (EC_{35}) and isoxaflutole (100 µg/L) consistently caused a significant decrease in toxicity in comparison to bioassays with atrazine alone. However, this antagonism did not occur at higher atrazine concentrations. Overall, there was no significant difference between the toxicities of atrazine alone and isoxaflutole at 100 µg/L for atrazine at EC_{50} and EC_{75} levels. From the results of the single herbicide bioassays, it would be expected that the isoxaflutole would have no significant effect on atrazine toxicity. If there were a significant effect, it would be to enhance toxicity, since atrazine is a photosynthetic inhibitor and blocks electron flow, while isoxaflutole reduces carotenoids which act to rid cells of excess excitation energy caused by singlet oxygen formation resulting from atrazine toxicity (Devine *et al.* 1993b). The combination of atrazine with isoxaflutole should cause additive toxicity or synergism to occur, thus the antagonism found in this study is problematic.

Summary and Conclusions

Contamination of surface waters by herbicide mixtures has been documented (Christensen and Zielski 1980, De March 1987, Boedeker *et al.* 1993, Faust *et al.* 1994, Lozano and Pratt 1994, Day and Hodge 1996, Backhaus *et al.* 2000). In most cases, concentrations of individual chemicals will remain quite low (< 1µg/L) in first order streams, but even at low concentrations some algal species will likely be growth inhibited (Hawxby *et al.* 1977, Hamilton *et al.* 1988). For short periods, herbicides can reach relatively high concentrations due to runoff during spring rains. Herbicide concentrations during such events can be extremely high and may disrupt aquatic ecosystems receiving non-point source inputs.

At the same time, algal species may respond differently to various herbicides. This differential toxicity can cause shifts in species composition within algal communities that may

then resonate throughout the aquatic food web. With herbicide mixtures, there may be many instances where combined effects may be additive. Although synergism was not shown in any of the interactions in this study, it remains an environmental concern (Steevens and Benson 1997, Tripathi and Agarwal 1997, Belden and Lydy 2000).

Concentration addition appears to apply in most cases of herbicide mixture toxicity (Boedeker *et al.* 1993, Faust *et al.* 1993, Carder and Hoagland 1998). The present study has shown that atrazine, in combination with alachlor and metolachlor at relatively low concentrations elicited an additive response in the common green alga *A. falcatus,* and when in combination, these herbicides can cause extremely high growth inhibition in this alga. Other algal species may be affected in a similar manner, however relatively little is known about many other algal responses to herbicide combinations. There are far too many herbicides and algal species to describe all potential interactions, thus future research should focus on mixture toxicity to other representative taxa and classes of herbicides in order to learn more about potential interactions. These mixtures are extremely prevalent in aquatic ecosystems and the knowledge gained through mixture toxicity studies should be useful for adapting environmental and water quality standards to account for their potential additive effects.

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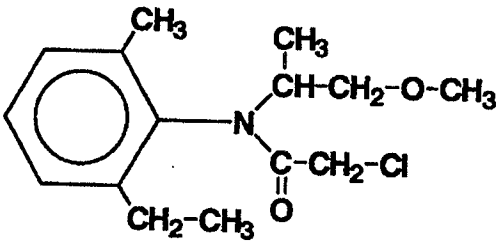
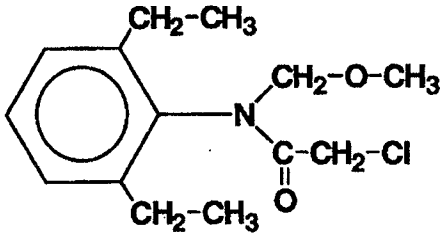
FIGURES

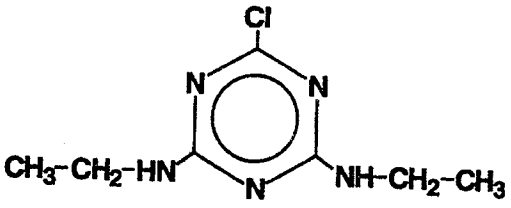
Figure 1. Chemical structures for metolachlor, alachlor, simazine, atrazine, isoxaflutole and diketonitrile metabolite (Weed Society of America 1994 and 1998).



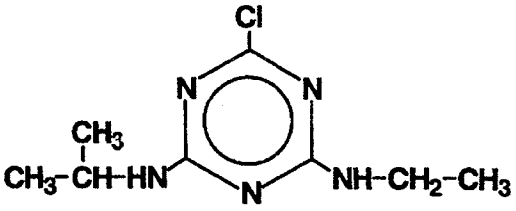
Metolachlor



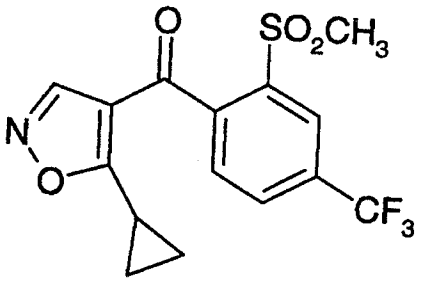
Alachlor



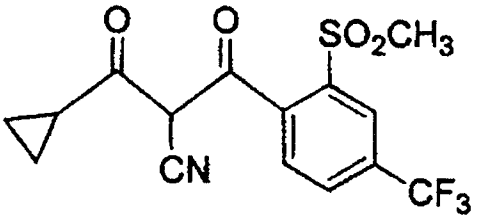
Simazine



Atrazine

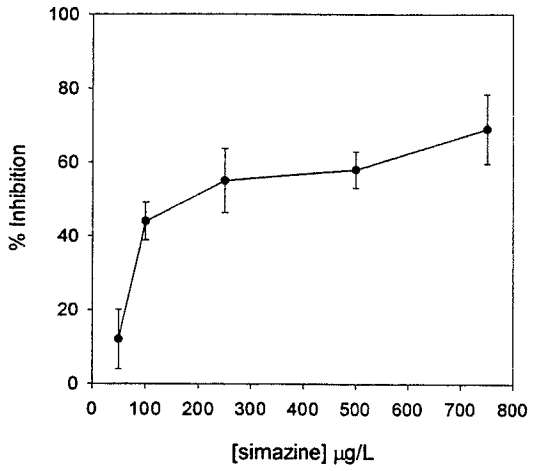


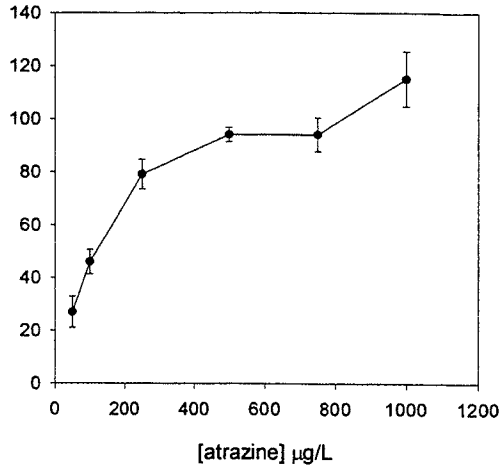
Isoxaflutole

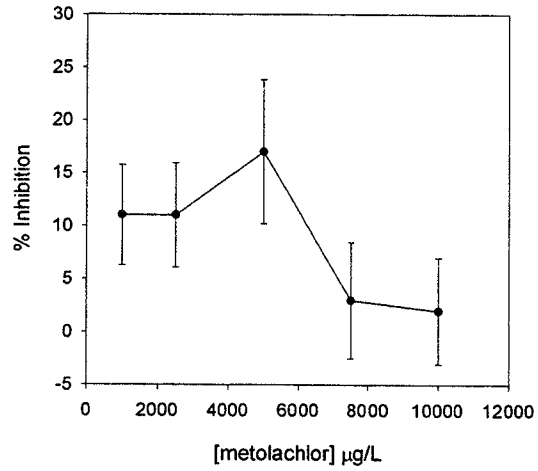


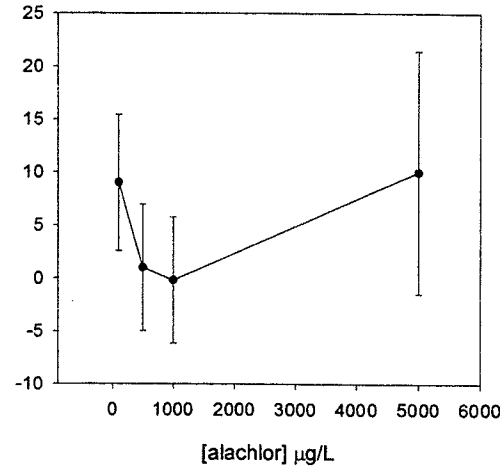
Diketonitrile

Figure 2. Response of *Chlorella vulgaris* to simazine, atrazine, metolachlor, alachlor and isoxaflutole as determined by fluorescence measurements (± SE).


% Inhibition
100
80
60
40
20
0
0 100 200 300 400 500 600 700 800
[simazine] µg/L


140
120
100
80
60
40
20
0
0 200 400 600 800 1000 1200
[atrazine] µg/L


% Inhibition
30
25
20
15
10
5
0
-5
0 2000 4000 6000 8000 10000 12000
[metolachlor] µg/L


25
20
15
10
5
0
-5
-10
0 1000 2000 3000 4000 5000 6000
[alachlor] µg/L

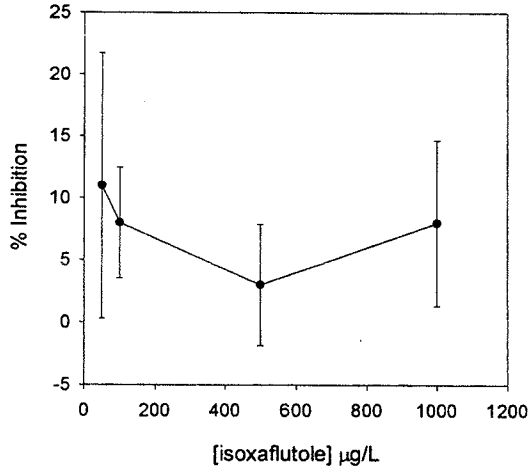
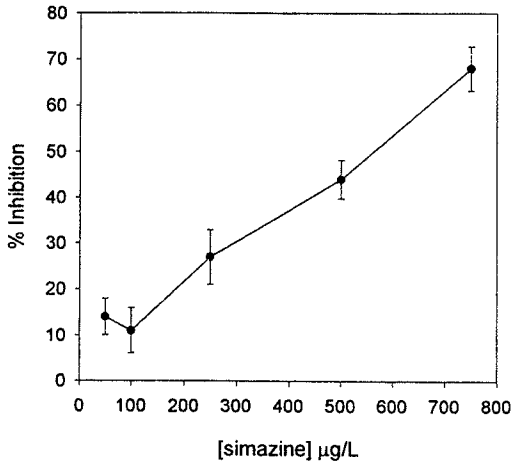
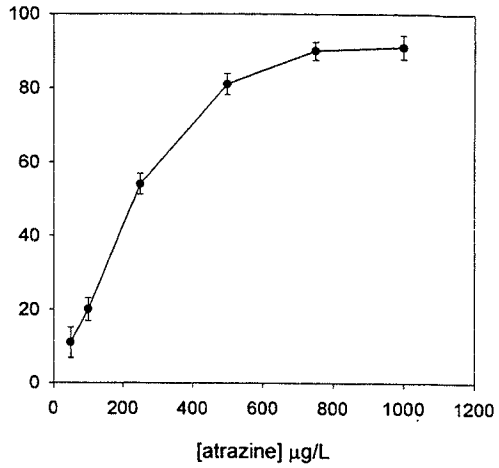
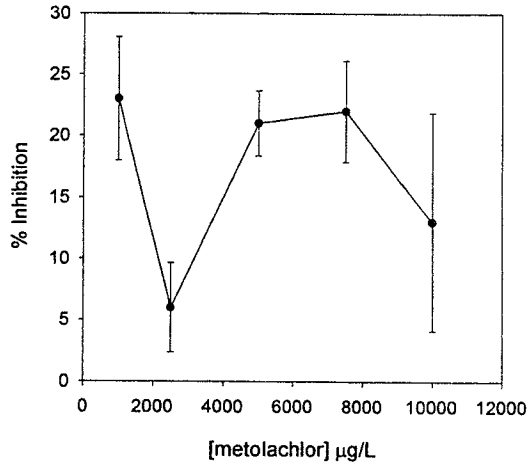
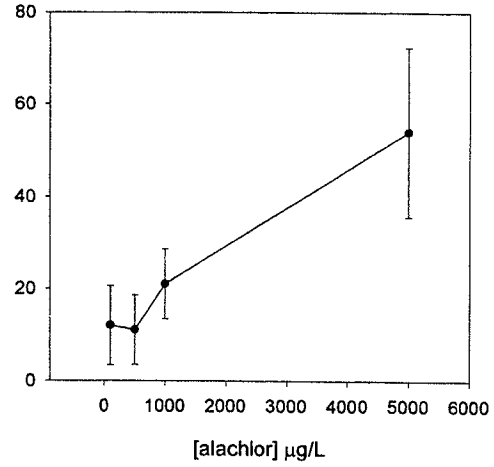

% Inhibition
25
20
15
10
5
0
-5
0 200 400 600 800 1000 1200
[isoxaflutole] µg/L

Figure 3. Response of *Cyclotella meneghiniana* to simazine, atrazine, metolachlor, alachlor and isoxaflutole as determined by fluorescence measurements (± SE).


% Inhibition
[simazine] µg/L


[atrazine] µg/L


% Inhibition
[metolachlor] µg/L


[alachlor] µg/L

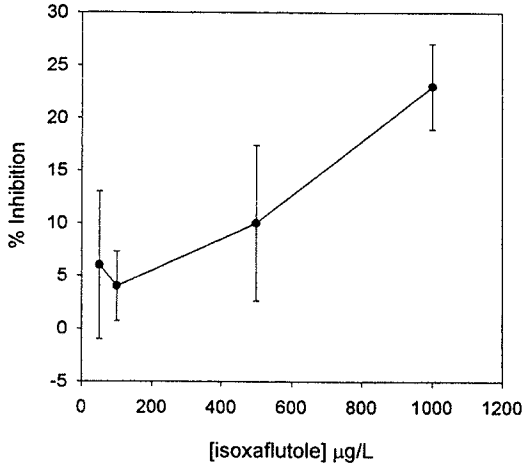
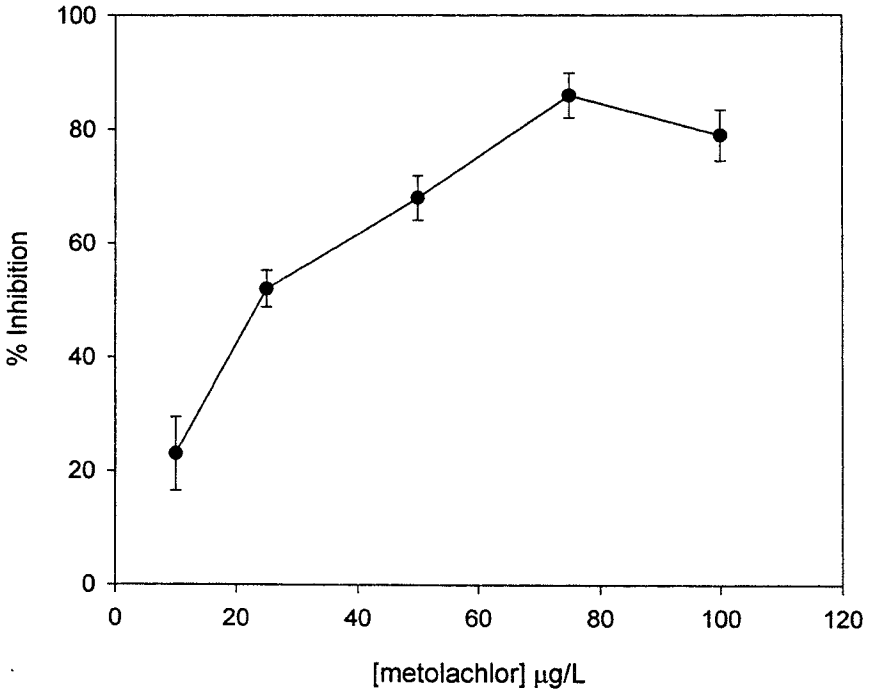

% Inhibition
[isoxaflutole] µg/L

Figure 4. Response of *Ankistrodesmus falcatus* to metolachlor and alachlor as determined by fluorescence measurements (± SE).


100
80
60
40
20
0
% Inhibition
0
20
40
60
80
100
120
[metolachlor] µg/L

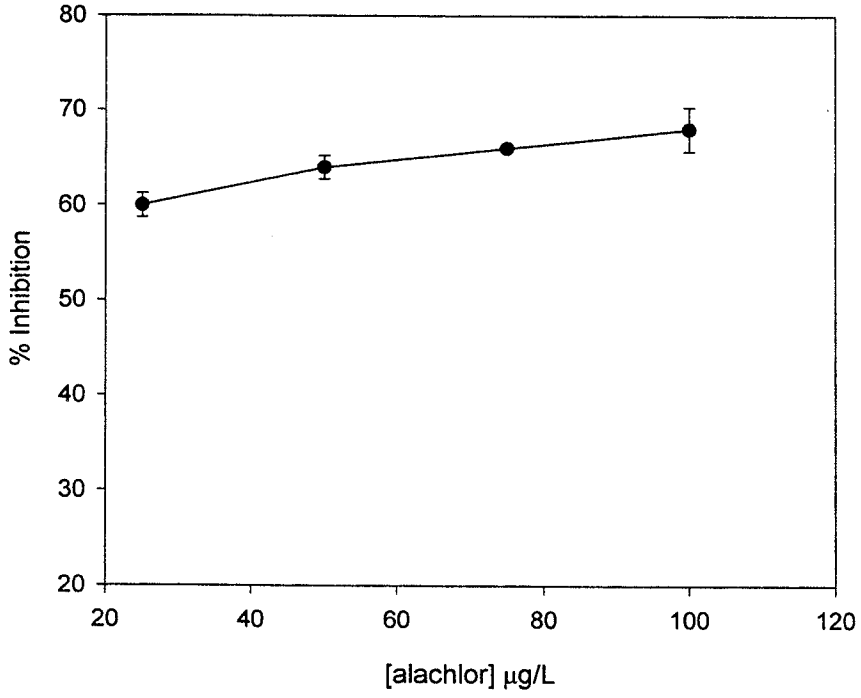
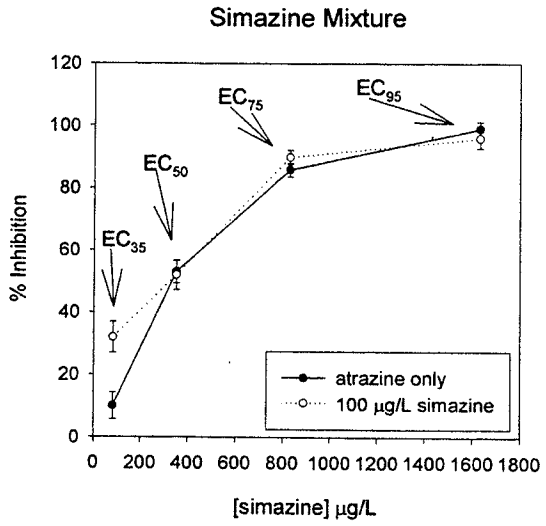
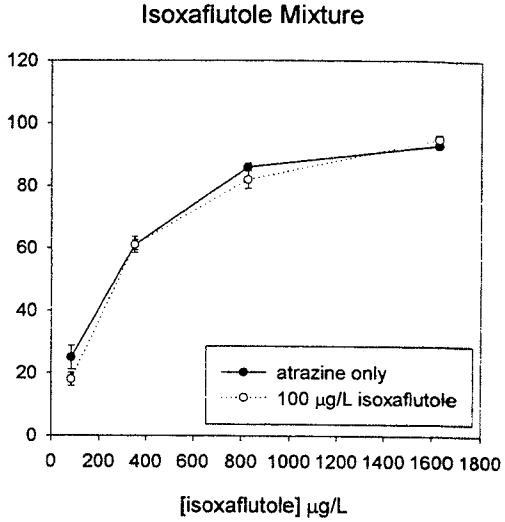
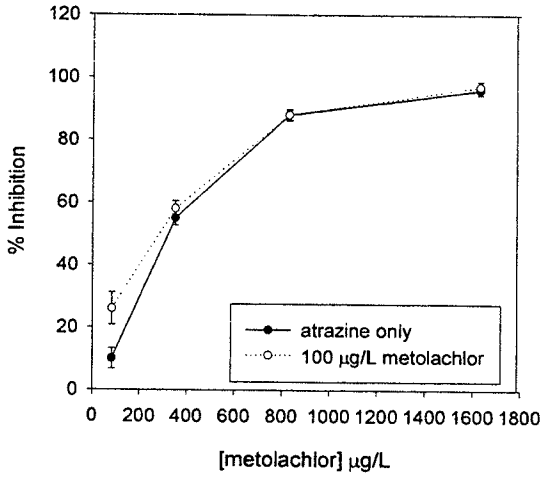

80
70
60
50
40
30
20
% Inhibition
20
40
60
80
100
120
[alachlor] µg/L

Figure 5. Toxicity of atrazine in combination with 100 µg/L simazine, isoxaflutole, metolachlor and alachlor for *Chlorella vulgaris* (± SE).


Simazine Mixture
% Inhibition
EC35
EC50
EC75
EC95
atrazine only
100 µg/L simazine
[simazine] µg/L


Isoxaflutole Mixture
atrazine only
100 µg/L isoxaflutole
[isoxaflutole] µg/L


Metolachlor Mixture
% Inhibition
atrazine only
100 µg/L metolachlor
[metolachlor] µg/L

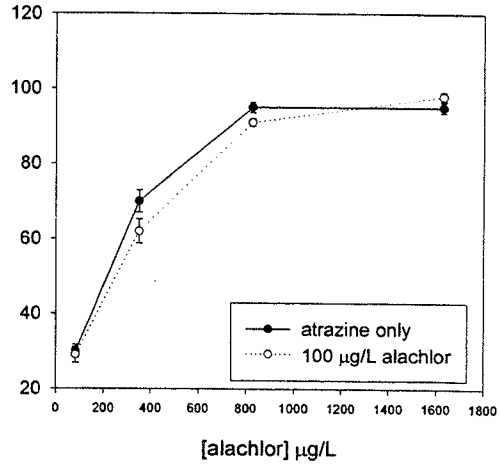
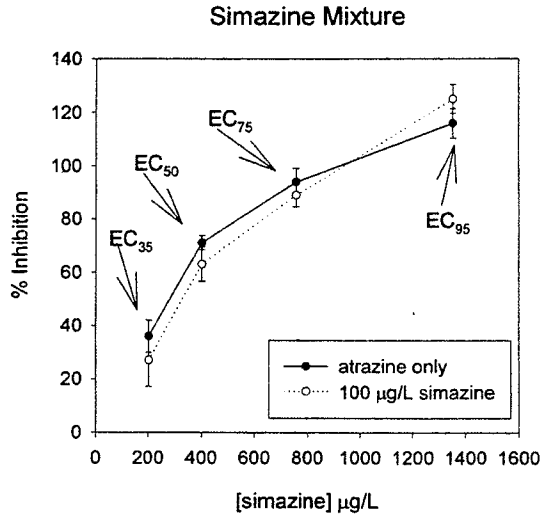
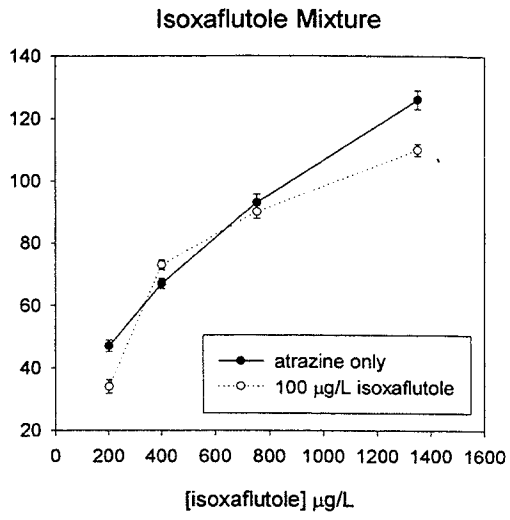
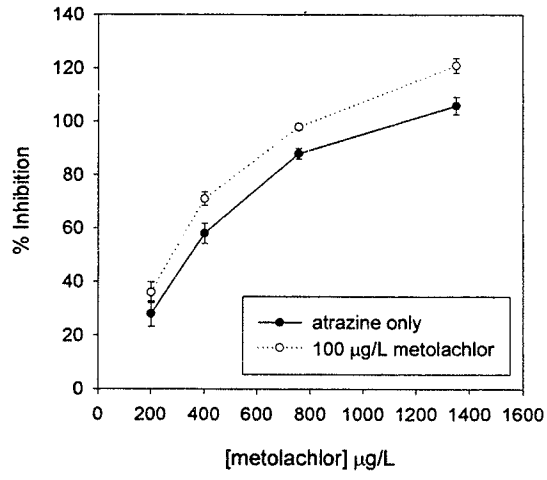

Alachlor Mixture
atrazine only
100 µg/L alachlor
[alachlor] µg/L

Figure 6. Toxicity of atrazine in combination with 100 µg/L simazine, isoxaflutole, metolachlor and alachlor for *Cyclotella meneghiniana* (± SE).


Simazine Mixture
% Inhibition
[simazine] µg/L
EC35
EC50
EC75
EC95
atrazine only
100 µg/L simazine


Isoxaflutole Mixture
[isoxaflutole] µg/L
atrazine only
100 µg/L isoxaflutole


Metolachlor Mixture
% Inhibition
[metolachlor] µg/L
atrazine only
100 µg/L metolachlor

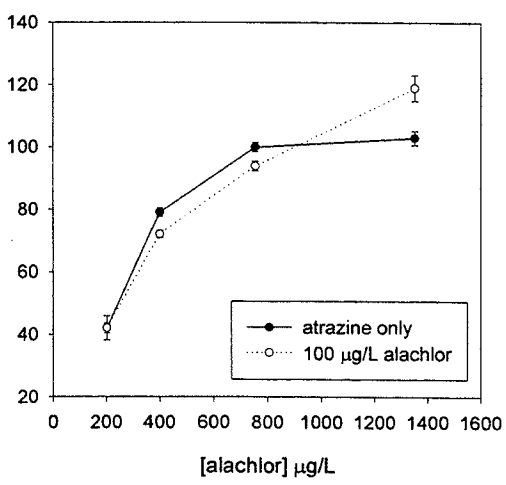
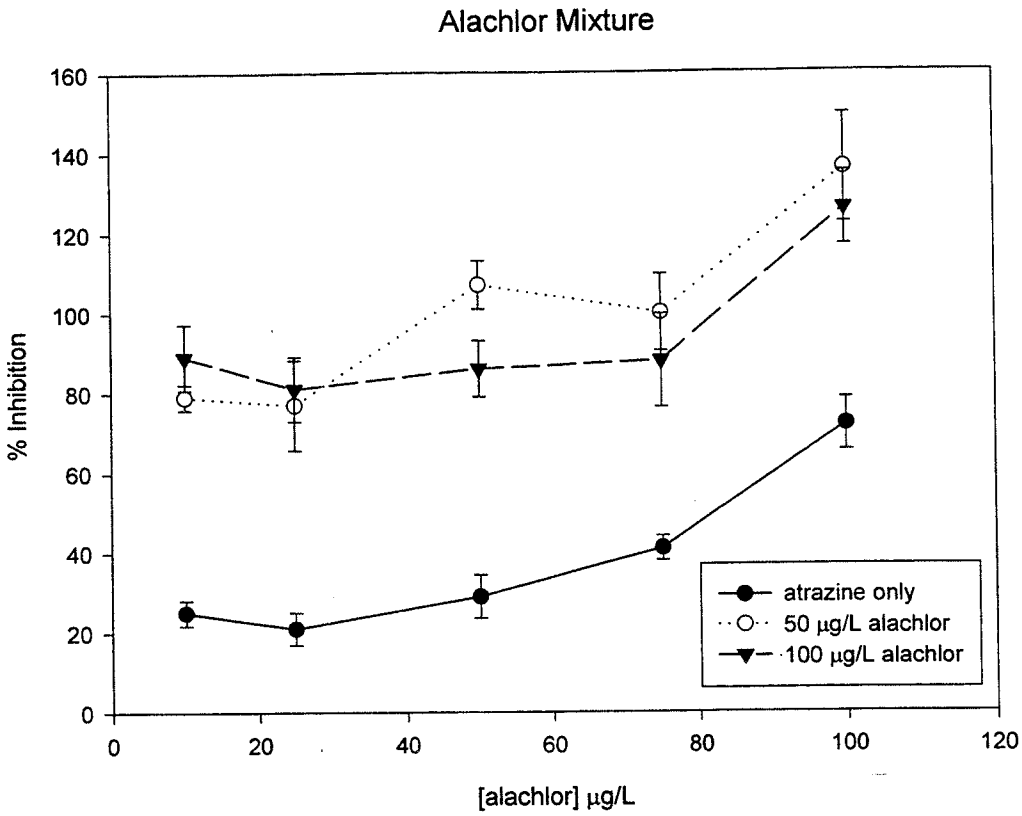

Alachlor Mixture
[alachlor] µg/L
atrazine only
100 µg/L alachlor

Figure 7. Toxicity of atrazine in combination with 50 and 100 µg/L alachlor (from single experiment only) and 25 and 50 µg/L metolachlor for *Ankistrodesmus falcatus* (± SE).


Alachlor Mixture
% Inhibition
[alachlor] µg/L
atrazine only
50 µg/L alachlor
100 µg/L alachlor

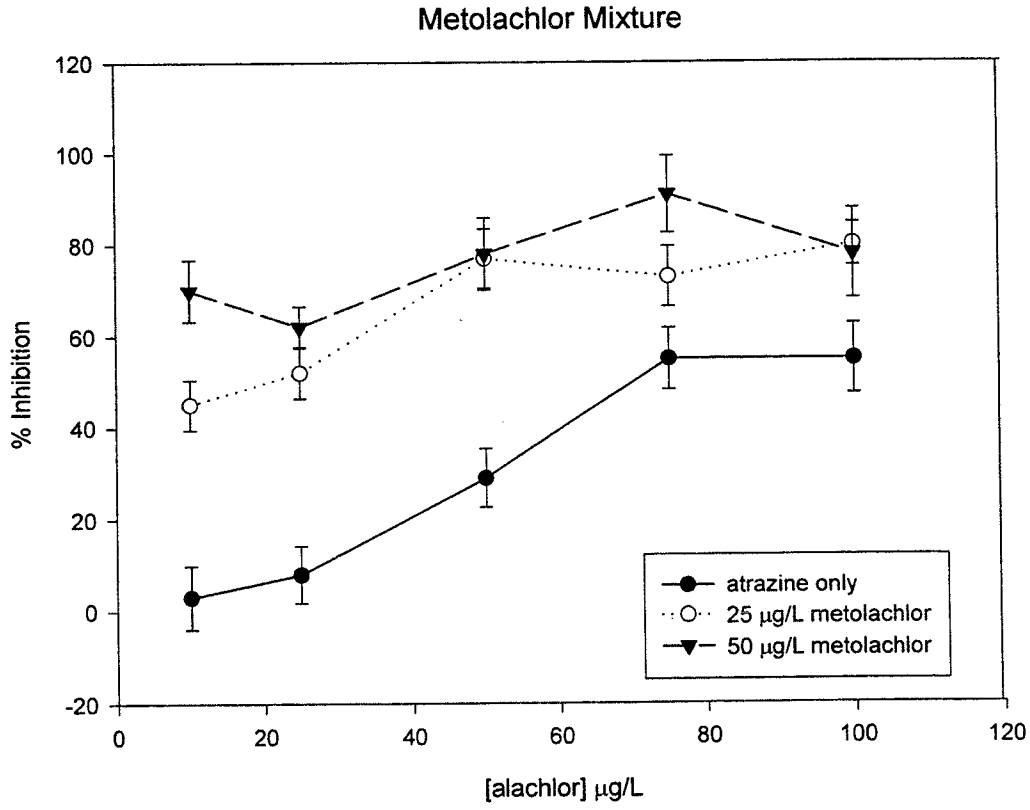
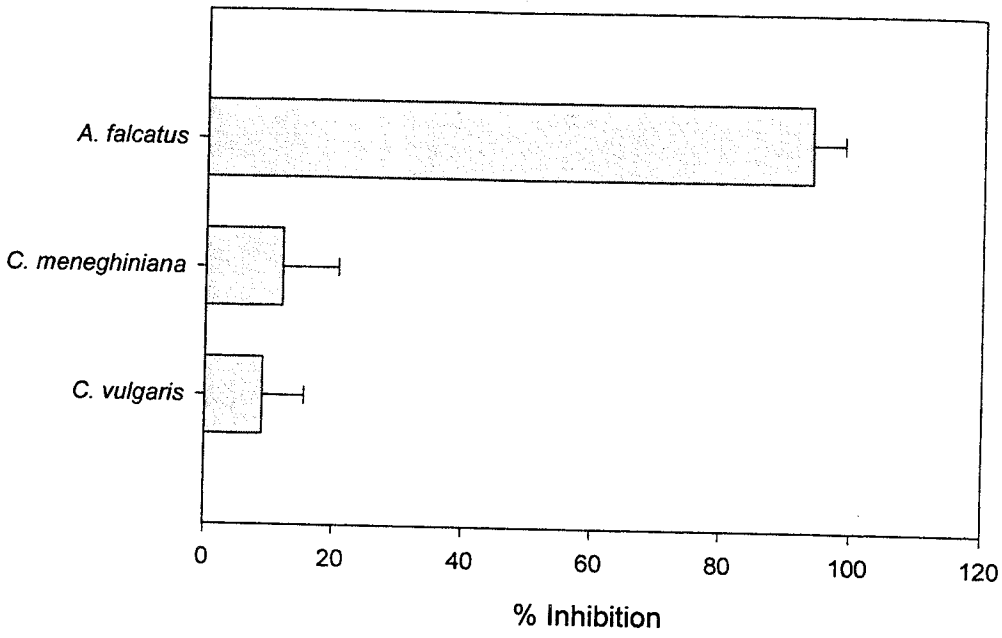
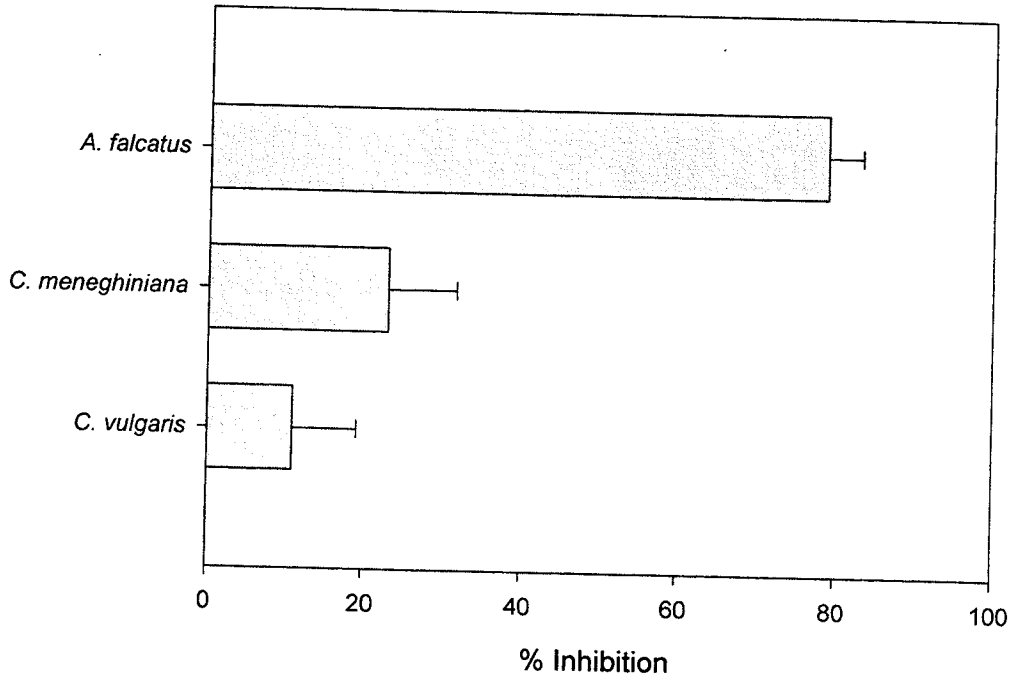

Metolachlor Mixture
% Inhibition
[alachlor] µg/L
atrazine only
25 µg/L metolachlor
50 µg/L metolachlor

Figure 8. Differential toxicity of *Ankistrodesmus falcatus*, *Chlorella vulgaris*, and *Cyclotella meneghiniana* to 100 µg/L metolachlor and alachlor (± SE).

Metolachlor Selective Toxicity



Alachlor Selective Toxicity



TABLES

Table 1. Experimental conditions for algal bioassays.

Test Parameter	Test Conditions
Temperature (°C)	20
Light source	cool-white fluorescent
PFD (μmol $m^{-2}s^{-1}$)	100
Photoperiod	12:12
Test chamber	50 mL test tube
Test volume (mL)	40
Replicates	5
Medium	WC*
Assessment endpoint	biomass
Measurement endpoint	fluorescence

*From Guillard (1975)

Table 2. Percent purity of herbicide formulations and herbicide modes of action.

Herbicide	Mode of Action	% Purity
atrazine	inhibits photosynthesis	99.0
alachlor	inhibits multiple biosyntheses	99.3
simizine	inhibits photosynthesis	99.0
metolachlor	inhibits multiple biosyntheses	96.1
isoxaflutole	inhibits HPPD synthesis	99.9

Table 3. Chemical classification and formulations of herbicides.

Chemical	Chemical Class	Chemical Name and Number
Alachlor	Acetanilide	2-chloro-N-(2,6-diethylphenyl)-N-(methoxymethyl)acetamide; cas #15972-60-8
Atrazine	Triazine	6-chloro-N-ethyl-N′-(1-methylethyl)-1,3,5-triazine-2,4-diamine; cas #1912-24-9
Isoxaflutole	Isoxazole	5-cyclopropyl-4-(2-methylsulphonyl-4-trifluoromethyl-benzoyl)isoxazole cas #141112-29-0
Metolachlor	Acetanilide	2-chloro-N-(2-ethyl-6-methylphenyl)-N -2-(methoxy-1-methylethyl) acetamide; cas #51218-45-2
Simazine	Triazine	6-chloro-N,N′-diethyl-1,3,5-triazine-2,4-diamine; cas #122-34-9

From *Herbicide Handbook* (1994 and 1998)

Table 4. EC values for atrazine (µg/L).

Species	EC35	EC50	EC75	EC95
Chlorella vulgaris	83	352	829	1632
Cyclotella meneghiniana	202	402	756	1352

APPENDIX

Appendix Table 1. Percent inhibition values (µg/L) for *Chlorella vulgaris* of all herbicides individually.

Simazine

Concentration	% Inhibition
50	**12** (± 8.112)
100	**44** (± 5.075)
250	**55** (± 8.638)
500	**58** (± 4.912)
750	**69** (± 9.368)

Atrazine

Concentration	% Inhibition
50	**27** (± 5.859)
100	**46** (± 4.587)
250	**79** (± 5.609)
500	**94** (± 2.712)
750	**94** (± 6.343)
1000	**115** (± 10.371)

Metolachlor

Concentration	% Inhibition
1000	**11** (± 4.742)
2500	**11** (± 4.911)
5000	**17** (± 6.804)
7500	**3** (± 5.485)
10000	**2** (± 5.007)

Alachlor

Concentration	% Inhibition
50	**5** (± 7.068)
100	**9** (± 6.430)
500	**1** (± 5.946)
1000	**-0.2** (± 5.946)
5000	**10** (± 11.455)

Isoxaflutole

Concentration	% Inhibition
50	**11** (± 10.709)
100	**8** (± 4.460)
500	**3** (± 4.875)
1000	**8** (± 6.678)

Appendix Table 2. Percent inhibition values (µg/L) for *Cyclotella meneghiniana* of all herbicides individually.

Simazine

Concentration	% Inhibition
50	**14** (± 3.953)
100	**11** (± 4.923)
250	**27** (± 5.979)
500	**44** (± 4.224)
750	**68** (± 4.784)

Atrazine

Concentration	% Inhibition
50	**11** (± 4.222)
100	**20** (± 3.029)
250	**54** (± 2.870)
500	**81** (± 2.805)
750	**90** (± 2.427)
1000	**91** (± 3.172)

Metolachlor

Concentration	% Inhibition
1000	**23** (± 5.034)
2500	**6** (± 3.653)
5000	**21** (± 2.645)
7500	**22** (± 4.113)
10000	**13** (± 8.893)

Alachlor

Concentration	% Inhibition
50	**12** (± 10.230)
100	**12** (± 8.646)
500	**11** (± 7.572)
1000	**21** (± 7.572)
5000	**54** (± 18.334)

Isoxaflutole

Concentration	% Inhibition
50	**6** (± 6.980)
100	**4** (± 3.300)
500	**10** (± 7.372)
1000	**23** (± 4.048)

Appendix Table 3. Percent inhibition values (µg/L) for *Ankistrodesmus falcatus* of alachlor and metolachlor individually.

Metolachlor

Concentration	% Inhibition
10	**23** (± 6.454)
25	**52** (± 3.224)
50	**68** (± 3.923)
75	**86** (± 3.907)
100	**79** (± 4.474)

Alachlor

Concentration	% Inhibition
25	**60** (± 1.257)
50	**64** (± 1.222)
75	**66** (± 0.207)
100	**68** (± 2.303)

Appendix Table 4. Percent inhibition values (µg/L) for *Chlorella vulgaris* of all herbicide mixture combinations.

Simazine (100 µg/L) Mixture

Concentration	Atrazine	Mixture
EC_{35}	**10** (± 4.260)	**32** (± 4.959)
EC_{50}	**53** (± 3.641)	**52** (± 4.749)
EC_{75}	**86** (± 2.257)	**90** (± 2.290)
EC_{95}	**99** (± 2.246)	**96** (± 3.163)

Isoxaflutole (100 µg/L) Mixture

Concentration	Atrazine	Mixture
EC_{35}	**25** (± 3.805)	**18** (± 2.091)
EC_{50}	**61** (± 1.665)	**61** (± 2.598)
EC_{75}	**86** (± 1.202)	**82** (± 2.890)
EC_{95}	**93** (± 1.191)	**95** (± 1.356)

Metolachlor (100 µg/L) Mixture

Concentration	Atrazine	Mixture
EC_{35}	**10** (± 3.275)	**26** (± 5.220)
EC_{50}	**55** (± 2.239)	**58** (± 2.408)
EC_{75}	**88** (± 1.639)	**88** (± 1.764)
EC_{95}	**96** (± 1.495)	**97** (± 1.760)

Alachlor (100 µg/L) Mixture

Concentration	Atrazine	Mixture
EC_{35}	**30** (± 1.696)	**29** (± 2.163)
EC_{50}	**70** (± 2.944)	**62** (± 3.261)
EC_{75}	**95** (± 1.365)	**91** (± 1.036)
EC_{95}	**95** (± 1.384)	**98** (± 1.215)

Appendix Table 5. Percent inhibition values (µg/L) for *Cyclotella meneghiniana* of all herbicide combinations.

Simazine (100 µg/L) Mixture

Concentration	Atrazine	Mixture
EC_{35}	**36** (± 6.010)	**27** (± 9.828)
EC_{50}	**71** (± 2.650)	**63** (± 6.407)
EC_{75}	**94** (± 5.101)	**89** (± 4.355)
EC_{95}	**116** (± 5.500)	**125** (± 5.393)

Isoxaflutole (100 µg/L) Mixture

Concentration	Atrazine	Mixture
EC_{35}	**47** (± 1.837)	**34** (± 2.195)
EC_{50}	**67** (± 1.610)	**73** (± 1.603)
EC_{75}	**93** (± 2.686)	**90** (± 2.106)
EC_{95}	**126** (± 2.978)	**110** (± 1.899)

Metolachlor (100 µg/L) mixture

Concentration	Atrazine	Mixture
EC_{35}	**28** (± 4.719)	**36** (± 3.832)
EC_{50}	**58** (± 3.715)	**71** (± 2.580)
EC_{75}	**88** (± 1.951)	**98** (± 1.120)
EC_{95}	**106** (± 3.276)	**121** (± 2.750)

Alachlor (100 µg/L) mixture

Concentration	Atrazine	Mixture
EC_{35}	**42** (± 3.821)	**42** (± 1.583)
EC_{50}	**79** (± 1.382)	**72** (± 1.182)
EC_{75}	**100** (± 1.552)	**94** (± 1.524)
EC_{95}	**103** (± 2.275)	**119** (± 4.128)

Appendix Table 6. Percent inhibition values (µg/L) for *Ankistrodesmus falcatus* of alachlor and metolachlor combinations.

Alachlor (50 and 100 µg/L) Mixture

Concentration	Atrazine	Mixture at 50	Mixture at 100
10	**25** (± 3.200)	**79** (± 3.187)	**89** (± 8.227)
25	**21** (± 4.074)	**77** (± 11.205)	**81** (± 8.013)
50	**29** (±5.401)	**107** (± 6.035)	**86** (± 6.996)
75	**41** (± 3.083)	**100** (± 9.614)	**88** (± 11.622)
100	**72** (± 6.517)	**136** (± 13.520)	**126** (± 9.164)

Metolachlor (25 and 50 µg/L) Mixture

Concentration	Atrazine	Mixture at 25	Mixture at 50
10	**3** (± 6.905)	**45** (± 5.500)	**70** (± 6.758)
25	**8** (± 6.246)	**52** (± 5.638)	**62** (± 4.558)
50	**29** (± 6.346)	**77** (± 6.473)	**78** (± 7.889)
75	**55** (± 6.712)	**73** (± 6.589)	**91** (± 8.424)
100	**55** (± 7.592)	**80** (± 4.689)	**78** (± 9.772)