INVESTIGATIONS OF THE EFFECTS OF TWO TRIAZINE HERBICIDES ON PERIPHYTIC ALGAL COMMUNITIES IN THE DELTA MARSH, MANITOBA

by

Sharon Elizabeth Gurney

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INTRODUCTION

Triazine herbicides are used in aquatic management primarily to control macrophytes and undesirable phytoplankton populations. Triazines used for terrestrial weed control also enter aquatic systems through runoff, aerial drift or from spillage. This study assesses the impact of two of these triazines, simazine and terbutryn, on the structure, function and biomass of periphytic algae within in-situ littoral enclosures. Secondary effects of treatment on water chemistry were also monitored during an 87 day experimental period.

The second half of this study describes an automated quantitative grain density microautoradiography procedure. The effects of Lugol's iodine on isotope leakage from cells preserved for autoradiography, was demonstrated using three haptobenthic diatom species. The applicabilty of using this technique to evaluate intra and interspecific variation in algal response to triazine exposure in-situ was also discussed.

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CHAPTER 1

LITERATURE REVIEW

1.1 TRIAZINES: MODE OF ACTION

Although atrazine is the most commonly used triazine herbicide, a number of other triazines have been used for aquatic and terrestrial weed control (Fedtke, 1982). All s-triazines consist of a central 6 membered triazine ring (alternating nitrogen and carbon) with alkylamino side chains originating from the 4 and 6 position of the ring. Triazines are further defined by differences in the alkylamino side chains and the substituted constituent on the remaining ring carbon at position 2. The chloro-triazines, simazine, cyanazine, procyazine, trietazine and atrazine, include a chlorine atom at this position. A substituted methylthio group in terbutryn, prometryn, cyanatryn, simetryn and ametryn, classifies these compounds as methylthio-triazines. The third class of s-triazines is referred to as methoxy-triazines. Prometon, atraton and simeton all contain substituted methoxy groups on the triazine ring. The physical and chemical properties of these herbicides are further described in WSSA (1983).

The primary mode of action of all triazines is inhibition of photosysnthesis. Triazines are one of several

classes of herbicides which are considered electron transport inhibitors (Moreland, 1980). These herbicides bind noncovalently to a special high-affinity site on PS II, inhibit electron transfer from Q- to B, and effectively prevent electron transfer through PS II (Shipman, 1982). The binding site is located on the thylakoid surface and is proteinaceous in nature (Fedtke, 1982). The binding is believed to lead to a change in the redox potential or an equilibrium shift in the plastoquinone B (Moreland, 1980) which prevents its participation in electron transport. The relationship between triazine structure and level of inhibition has been investigated by Radosevich et al. (1979). This research suggests that the inhibition is favored by asymmetric alkylamino substitution at the 4 and 6 position of the triazine ring, and the orientation of the triazine molecule at the active site may be controlled by these alkylamino groups. Of the three groups of s-triazines, Radosevich et al. (1979) has found methylthio-s-triazines to be the strongest Hill reaction inhibitors. Thylakoid suspensions were least affected by the methoxy-s-triazines, and chloro-s-triazines were intermediate in effect. This finding was substantiated by Goldsborough and Robinson (1983) as the methylthio-triazine terbutryn was found to be 55x more toxic to periphyton than the chloro-triazine simazine, at a concentration of 0.1 $mg \cdot L^{-1}$. Competitive binding studies have suggested that many of the s-triazines,

triazinones, pyridazinones, biscarbamates, phenylureas, N-phenylcarbamates, uracils, acylanilides, cyclic ureas and thiadiazoles interfere at the same electron carrier in the electron transport chain through a similar mechanism (Tischer and Strotmann, 1977; Pallett and Dodge, 1979; Moreland, 1980; Van Assche and Carles, 1982). Pfister et al. (1981) and Gardner (1981) identify a 32 kD protein present in PS II which is associated with herbicide binding, however Gressel (1982) suggests that this 32 kD protein may only be adjacent to the thylakoid binding site. Shipman (1982) states that each herbicide molecule must have a flat polar component with hydrophobic substituents to be active. These hydrophobic components serve to partition the molecule into lipid regions of the cell and to fit the hydrophobic region of the binding site. The flat polar region of the herbicide is used for electostatic binding to the polar region of the herbicide binding site.

Although the primary influence of triazine toxicity is the inhibition of electron transport, evidence of secondary effects, have been documented (Fedtke, 1982).

1.2 TRIAZINE RESISTANCE

Triazine resistance and tolerance in higher plants have been well documented. The chloro-s-triazines can be degraded in higher plants by three major metabolic pathways:

hydrolysis at the 2 position of the triazine ring; N-dealkylation of the side chains; or conjugation with glutathione (Jensen, 1982). Corn which is extremely tolerant to atrazine and simazine, utilizes all three degradation pathways. Sorghum, however which is also tolerant to atrazine, primarily utilizes conjugation and to a lesser extent N-dealkylation in detoxification. Sorghum is not able to hydrolyze atrazine since it is deficient in benzoxazinone, the cyclic hydroxamic acid that catalyzes the hyrdolysis of chloro-s-triazines nonenzymatically (Jensen, 1982). Plants which are extremely or moderately suceptible to atrazine (eg. oats, wheat and peas) are devoid of the enzyme glutathione-s-transferase, which catalyzes the conjugation of chloro-s-triazines with glutathione. Less is known about the metabolism of methoxy or methylthio-s-triazines. Plants can degrade terbutryn and prometryn by oxidation of the methylthio group to hydroxy-metabolites and by dealkylation of the side chains, however degradation is slow compared to atrazine in corn (WSSA, 1983).

Many of the triazine resistant weeds do not have the enhanced rate of herbicide degradation such as that found in corn. Instead, these species are resistant because the triazines do not bind to the thylakoids. Triazine tolerance and resistance have evolved differently even within the same

species. Gressel (1986) cites an example where one population of Senecio vulgaris slowly increased tolerance to sublethal triazine doses, while another population suddenly evolved plastid resistance to high levels of atrazine. Biotypes which have evolved triazine resistance via a chloroplast membrane alteration, contain a thylakoid component that has a subtle alteration responsible for reduced triazine binding affinity (Arntzen et al., 1982). Nucleotide sequence analysis done by Hirschberg and McIntosh (1983), has demonstrated that the 32 kD protein in a triazine resistant biotype of Amaranthus hybridus differs from the protein of a herbicide susceptible biotype by only one amino acid. A number of studies have focused on the characterization of the chloroplast membranes between triazine-resistant and triazine-susceptible biotypes (Pfister et al., 1979; Holt et al., 1981; Pillai and St. John, 1981; Burke et al., 1982; Ducruet and DePrado, 1982; Oettmeier et al., 1982; Gasquez and Darmency, 1983; Ort et al., 1983; Turcsányi and Faludi-Dániel, 1983; Galloway and Mets, 1984). Pfister and Arntzen (1979) have found that the PS II complex of chloroplasts from several different triazine-resistant weed biotypes share common traits. These include an alteration in the thylakoid membrane which results in very strong resistance to all symmetrical triazines and asymmetrical triazinones, but only slight resistance to ureas or amides. Chloroplasts of both

triazine-susceptible and triazine-resistant biotypes of Amaranthus hybridus synthesize a 34 kD herbicide binding protein, but the resistant biotypes do not bind the herbicide (Steinback et al., 1981). Mattoo et al. (1984) found that atrazine adaptation in Spirodela oligorrhiza coincided with high proportions of thylakoid polyunsaturated fatty acid constituents which increase membrane fluidity. They suggest that alterations in the lipid environment may mediate conformational, orientational or functional changes of the 32 kD binding protein which could hinder herbicide binding.

A few studies have reported triazine resistance in certain algal species. Using visual observations, Vance and Smith (1969) report simazine to be non-toxic to Scenedesmus quadricauda and Chlamydomonas eugametos. Four Chlamydomonas reinhardii mutants were found to have reduced herbicide binding to thylakoid membranes (Galloway and Mets, 1984). A reduced inhibition of carbon assimilation by phytoplankton from atrazine treated ponds to further atrazine additions, has been considered to be an indication of induced resistance (deNoyelles et al., 1982). Goldsborough and Robinson (1987) also found evidence of triazine resistance in periphyton exposed to simazine concentrations over 0.8 $mg \cdot L^{-1}$

1.3 TRIAZINE USE IN AQUATIC MANAGEMENT

Triazines have been used in a number of countries for the management of aquatic macrophytes and algae. Simazine has been used to control phytoplankton (Tucker and Boyd, 1978a), filamentous algae and Chara sp. (Tucker and Boyd, 1978b; Tucker et al., 1983) in catfish ponds in the southern United States, and in the control of undesirable blue-green algal populations in Moriane Lake, New York (Harman, 1978). Simazine, atrazine, propazine and prometone have been used to control aquatic vegetation in fish habitats in Missouri (Walker, 1964). In this study, control of filamentous algae and Chara sp. required a higher simazine concentrations than was required to control other aquatic vegetation. Mauck et al. (1976) and Crawford (1981) both report simazine as being an effective herbicide in the management of aquatic vegetation in ponds. Simazine and terbutryn have been used to control vegetation growing in irrigation canals in California, Colorado and Washington (Anderson et al., 1978; Bowmer et al., 1979). Preliminary investigations into the effectiveness of cyanatryn in controlling aquatic macrophytes and algae in small ponds, suggest that excellent control can be obtained with herbicide concentrations ranging from 50-150 $\mu g \cdot L^{-1}$ (MacKenzie et al., 1985). Murphy et al. (1981) has evaluated the use of terbutryn and cyanatryn for aquatic weed control in navigable canals in Britain UK, and terbutryn has been applied to Lake

Peterborough, Britain UK, in an attempt to control filamentous algal growth (Robson et al., 1976). The effects of cyanatryn, on the aquatic vegetation in a drainage channel, has been evaluated by Scorgie (1980). Terbutryn has been applied to Ontario farm ponds and lakes to assess its effectiveness in controlling filamentous algae, submerged macrophytes and emergent aquatics (Mackenzie et al., 1983). During this study, best control occurred under static water conditions, and filamentous algae were found to be most sensitive to terbutryn treatment.

1.4 TRIAZINE CONTAMINATION IN NON-TARGET AQUATIC SYSTEMS

Although some of the triazine herbicides have been used in aquatic management, many used for terrestrial weed control incidentally contaminate aquatic systems through aerial drift or runoff. Hall (1974) found that with an atrazine application rate of 2.2 $kg \cdot ha^{-1}$, a level of 2.3 $ug \cdot L^{-1}$ could be detected in runoff water. With a wide range of atrazine application rates (0.6-9.0 $kg \cdot ha^{-1}$) an average of 2.4% of the herbicide was lost in runoff water from corn fields (Hall et al., 1972). The quantity and the rate of herbicide loss from agricultural fields may depend on the amount of rainfall, and the cultivation techniques employed. Triplett et al. (1978) found that less runoff, containing simazine and atrazine occurred from corn fields planted to no-tillage than to conventionally tilled soil. In that study

the greatest quantity of atrazine and simazine transported in runoff was 64 $g \cdot ha^{-1}$ (5.7% of total applied) and 123 $g \cdot ha^{-1}$ (5.4%), respectively. Ridge planting of corn was also found to greatly reduce atrazine losses when compared to surface-contoured planting (Ritter et al., 1974). A storm occurring seven days after atrazine application resulted in approximately 15% loss from surface contoured watersheds, with concentrations of atrazine in the surface water ranging from 1.17-4.91 $mg \cdot L^{-1}$. Quantities of atrazine and N-deethylated atrazine were monitored in five rivers that drained agricultural areas in Quebec (Muir et al., 1978). Atrazine and N-deethylated atrazine residues ranged in concentration from 0.01-26.9 $\mu g \cdot L^{-1}$ and <0.01-1.34 $\mu g \cdot L^{-1}$, respectively, between April and December of 1974 and 1975. The highest levels were observed in July which coincided with herbicide spraying and occasional heavy rainfall. The total discharge of atrazine into these watersheds was found to range from 0.1-2.9% of that applied. A similar study was conducted for 11 agricultural watersheds in southern Ontario (Frank and Sirons, 1979). In this study atrazine and desethylatrazine were detected in 80% of the stream waters at a mean concentration of 1.4 $\mu g \cdot L^{-1}$. Losses of atrazine were found to be greatest on clay and least on sandy soils. Of the total amount lost, 60% was caused by storm runoff, spills accounted for 20% and base flow associated with drainage accounted for the remaining 20%. The quantity of

atrazine and simazine that entered the Wye River was reported to depend upon the quantity applied in the watershed and the timing of runoff with respect to application rates (Glotfelty et al., 1984). The maximum concentration of atrazine measured in the Wye River estuary was near 15 $\mu g \cdot L^{-1}$, with an average concentration at peak loading of <3 $\mu g \cdot L^{-1}$. Anderson et al. (1978) have determined the amount of simazine present in irrigation water after ditchbank treatment for weed control. At application rates of 2.25-7.43 $kg \cdot ha^{-1}$, simazine levels in flowing canal water immediately after herbicide application did not exceed 60 $\mu g \cdot L^{-1}$. In first-flow samples collected 4 to 6 months after application from sites that were dewatered at application, simazine levels peaked at about 250 $\mu g \cdot L^{-1}$ within the treated section but decreased rapidly to <5 $\mu g \cdot L^{-1}$. The heavy use of atrazine in the South Platte River Valley, Colorado initiated a study which monitored groundwater at gradient points above, directly beneath, and below an atrazine treated site (Wilson et al., 1987). The maximum concentration recorded in a groundwater well during this study was 2.3 $\mu g \cdot L^{-1}$.

1.5 TRIAZINE PERSISTENCE

The persistence of triazine herbicides within water and sediment has been reported to vary considerably. The rate of herbicide disappearance from a water body is likely related

to the flushing rate of the system, the amount of mixing and also rates of biotic and abiotic degradation. In general however, triazine persistence appears to be greater in sediments than in the overlying water. Muir et al. (1981) reported a half-life of terbutryn in water ranging from 21 days in a pond that contained heavy growths of Typha and Lemna, to 30 days in a pond that was free of aquatic macrophytes. With an application rate of 100 $\mu g \cdot L^{-1}$, these two ponds had terbutryn residues in the sediment as high as 1.4 $\mu g \cdot g^{-1}$ (dry wt) and 0.5 $\mu g \cdot g^{-1}$. After 61 weeks, 51% of terbutryn added to the the unvegetated pond, and 35% of that added to the vegetated pond could still be accounted for. MacKenzie et al. (1983) have also found considerable variation in terbutryn persistence in both water and sediment from treated ponds and lakes. In a laboratory study, Muir and Yarechewski (1982) found that the rate of terbutryn degradation was dependent on the redox potential. Under anaerobic conditions degradation rates were considerably slower. In another study monitoring the persistence of herbicides in irrigation ditches, three years following treatment, 50% of the simazine added was still present in the soil of the ditch sides and bottoms (Smith et al., 1975). In this study, atrazine was found to be a less persistent compound, with 30% remaining in the soil after this time period. Atrazine was however more persistent than two non-triazine herbicides, monuron and bromacil, which had

only 15% and negligible amounts remaining, respectively. Absorption constants for simazine on high organic sediments were found to be linear over a 0.01-1.0 $mg \cdot L^{-1}$ concentration range (Glotfelty et al., 1984). Mauck et al. (1976) found the concentration of simazine applied to pond water in Missouri was directly related to the concentration ending up in the sediment. These concentrations were shown to decline after application, however residues of 0.16 $\mu g \cdot g^{-1}$ were measured 456 days after the initial 3.0 $mg \cdot L^{-1}$ application. After strong winds, higher concentrations of simazine were found in the water column, suggesting that the herbicide can quite easily dissociate from the sediment with any physical disturbance. Walker (1978) found simazine to be somewhat more persistent than atrazine, however both herbicides demonstrated considerably shorter half-lives, with increases in temperature and moisture content in the soil. Since triazine herbicides are known to adsorb readily to organic matter (Pillay and Tchan, 1972; Nicholls et al, 1984) the reported variation in persistence may largely be due to differences in the organic content of sediment. The persistence of triazines in water overlying marsh sediments has been shown to increase when sediments are excluded from contact with the overlying water (Goldsborough and Robinson, 1985). Sediment adsorption of pesticides from overlying water has also been documented by Gillott et al. (1975). Murphy (1982) reviews the persistence and degradation of the

methylthio-triazines: ametryn, cyanatryn and terbutryn in freshwater systems.

1.6 TRIAZINE TOXICITY TO AQUATIC MACROPHYTES AND ALGAE

While the physiological effects of triazine herbicides on terrestrial plants have been well documented, comparatively less research has been conducted on aquatic macrophytes and algae. The absorption and translocation of simazine in Myriophyllum brasiliense has been studied by Sutton and Bingham (1969). Root applications greater than 1.0×10^{-7}M inhibited growth and simazine was found to accumulate in the top of the shoot. In a similar study, simazine concentrations of 0.12 -1.0 $mg \cdot L^{-1}$ in cultures of Lemna minor, Elodea canadensis and Myriophyllum brasiliense inhibited oxygen evolution within 24 hr (Sutton et al., 1969). Murphy et al. (1981) found suppression in the growth of aquatic macrophytes for 3-12 months subsequent to terbutryn treatment, followed by blooms of Lemna. A cyanatryn concentration of 0.12 $\mu g \cdot g^{-1}$ was found adequate to eliminate the dominant macrophyte (Myriophyllum spicatum) from a drainage channel (Scorgie, 1980) and cyanatryn levels between 50-150 $\mu g \cdot L^{-1}$ were found effective in controlling growth of vascular macrophytes in shallow ponds in Ontario (MacKenzie et al., 1985). Dabydeen and Leavitt (1981) conducted a study to evaluate the effects of a 3.0 $mg \cdot L^{-1}$ concentration of atrazine and simazine on cultures of Elodea

canadensis. Toxic systems were found to develop faster in the simazine treatment, however absorption of atrazine was actually faster. Although simazine is more toxic to Elodea canadensis, both herbicides caused migration of the chloroplasts to the center of the cell. This was followed by loss of pigment and death.

Laboratory investigations of triazine toxicity in algae have focused mainly on short-term primary effects of triazine inhibition of individual species. Prometryn concentrations between 0.1 and 10 μM were used to evaluate effects on growth, photosynthesis and respiration in four species of algae (Hawxby et al., 1977). Levels of inhibition were found to vary, with Lyngbya being most susceptible. Tubea et al. (1981) report that prometryn concentrations of 10 uM drastically reduced growth of Chlorella pyrenoidosa and Lyngbya birgei. Plumley and Davis (1980) report that atrazine concentrations of 2.2 $mg \cdot L^{-1}$ will reduce photosynthetic rate, chlorophyll content, and cell numbers in unialgal cultures of Nitzschia sigma and Thalassiosira fluviatilis isolated from a salt marsh. The EC_{50} values of atrazine and its degradation products were derived for two species of green algae and three species of blue-green algae (Stratton, 1984). Deethylated atrazine was found to be the most toxic breakdown product. Evidence of synergistic, antagonistic, and additive interaction responses were also

documented in this study. Synergistic inhibition of chlorophyll production by Chlorella vulgaris has been reported for specific concentrations of atrazine and simazine, although other concentrations were found to have stimulatory effects (Torres and O'Flaherty, 1976). Vance and Smith (1969) also report varying degrees of simazine toxicity to three algal species. A study of the effects of simazine on photosynthesis and growth of filamentous algae found that algicidal effects are reduced under low light conditions (O'Neal and Lembi, 1983). Vérber et al. (1981) found atrazine concentrations of 0.25-5.0 $mg \cdot L^{-1}$ to inhibit growth of Chlorella vulgaris. Concentrations of 0.25-12.0 $mg \cdot L^{-1}$ prometryn were evaluated for influence on cell numbers of Chlamydomonas segnis, heterocyst frequency in Anabaena. and generation time of Klebsiella pneumoniae strain M5A1 and Rhizobium japonicum strain 61A76 (Weinberger et al., 1985). A study on the effects of atrazine on salt marsh edaphic algae indicated that a 2.2 $mg \cdot L^{-1}$ concentration will significantly reduce the rate of photosynthesis, chlorophyll content, and cell numbers in cultures treated in the lab (Plumley and Davis, 1980). In their field investigations, a higher level of atrazine was required to produce equivalent inhibition. Robson et al. (1976) have evaluated the effects of various concentrations of terbutryn and cyanatryn on nuisance filamentous algal species. Simazine has been found to induce ultrastructural

changes in the thylakoids of Anacystis nidulans (Mehta and Hawxby, 1979). Kruglov and Mikhailova (1975) have shown that algae can actively absorb and accumulate simazine in an amount exceeding its concentration in the surrounding medium by 100-fold. In another laboratory experiment, Verber et al. (1981) found that Chlorella vulgaris under conditions of atrazine inhibition (2.5 $mg \cdot L^{-1}$), was still capable of removing most of the atrazine present in the medium.

Field experiments with triazine exposure have evaluated physiological and compositional changes in algal communities. Goldsborough and Robinson (1983) used varying concentrations of simazine and terbutryn to report their effect on carbon assimilation rate and chlorophyll a accumulation in marsh periphyton. Effects of these herbicides on carbon assimilation, chlorophyll a, periphyton biovolume and community structure were correlated to increased herbicide concentrations (Goldsborough and Robinson, 1986). Hamilton et al. (1987) have evaluated the impact of atrazine on lake periphyton communities. Consistent with the observations of Goldsborough and Robinson (1986), the community structure shifted from a chlorophyte- to a diatom-dominated community. Herman et al., (1986) have also found the diatom community to be least sensitive to atrazine treatment, and report the major impact on periphyton as a decrease in the Chlorophyta. Density of

diatoms, particularly Achnanthes sp., was found to increase following cyanatryn treatment in a drainage channel (Scorgie, 1980). Plumley and Davis (1980) found that atrazine treatment did not significantly affect species diversity, however there were increases in the numbers of some species. Murphy et al. (1981) found that a major short-term result of terbutryn treatment was an alteration towards filamentous algal dominance at the expense of submerged vascular plants. The effects of atrazine on the community structure (Kosinski, 1984) and productivity (Kosinski and Merkle, 1984) of stream periphyton have been studied in artifical streams. Atrazine concentrations as low as 20 $\mu g \cdot L^{-1}$ were found to inhibit ^{14}C uptake and biomass in pond phytoplankton (deNoyelles et al., 1982). Successional changes towards more resistant species were documented in this study, at higher herbicide concentrations. Three applications of simazine totalling 1.3 $mg \cdot L^{-1}$ were found to effectively depress phytoplankton density throughout most of the summer months. This treatment also reduced the percentage of the phytoplankton community represented by blue-green algae.

The triazine toxicity studies of effects on algal productivity, have assessed whole community or whole population responses, giving little direct information on the differential responses of individual species. It is

possible, however, to monitor species-specific responses using either grain density (GDA) or nuclear track (NTA) microautoradiography. Both of these techniques have been used in aquatic biology primarily in the study of phytoplankton and bacteria. To date, researchers have used autoradiography to evaluate inorganic carbon assimilation rates of phytoplankton (Maguire and Neill, 1971; Watt, 1971; Stull et al., 1973; Paerl, 1977; Paerl 1978a; Descolas-Gros, 1980; Ogawa and Ichimura, 1981), and their heterotrophic potential (Saunders, 1972). Knoechel and DeNoyelles (1980) have used NTA to determine the influence of light and phosphorous enrichment on the carbon fixation rates of hypolimnetic phytoplankton. GDA has been used by Friebele et al. (1978) to investigate the relationship between phytoplankton size and the rate of phosphate uptake. GDA has also been utilized quite extensively to examine bacterial heterotrophic activity (Paerl, 1974; Hoppe, 1976; Paerl, 1978b; Chocair and Albright, 1980; Novitsky, 1983; Marcussen et al., 1984). Paerl (1983) used GDA to determine the partitioning of CO_2 in a colonial cyanobacterium as it relates to the promotion of surface scums. Autoradiography has only recently been utilized for periphyton research. Pip and Robinson (1982a, 1982b) used NTA to analyse the dynamics of individual epiphytic periphyton productivity by quantifying both inorganic and organic carbon assimilation rates. Carbon assimilation rates of epiphytic algae, as they

relate to cell surface area, have been investigated using this same technique (Robinson and Pip, 1983). The species specific productivity rates of epilithic periphyton has also been examined using NTA (Duthie and Hamilton, 1983). To date only two investigators have used autoradiography in toxicological research. Bruno et al. (1982) used GDA to monitor the uptake and biotransformation of benzo(a)pyrene in periphytic algal communities, and Hamilton et al. (1987) have used NTA to assess the impact of atrazine on the productivity rates of lake periphyton.

1.7 USE OF ENCLOSURES AND ARTIFICAL SUBSTRATA IN TRIAZINE TOXICITY STUDIES

Although some triazine toxicological investigations have involved the treatment of entire lakes (Harman, 1978; MacKenzie et al., 1983), ponds (Tucker and Boyd, 1978a, 1978b; Crawford, 1981; deNoyelles, 1982; Tucker et al., 1983; Beckmann et al., 1984; MacKenzie et al., 1985), ditches (Smith et al., 1975; Bowmer et al., 1979 and canals (Murphy et al., 1981), practical limitations plus the need for comparative controls has favored the use of replicable in-situ enclosure systems. Plumley and Davis (1980) used metal cylinder enclosures (1.8 m diameter, 90 cm high) to evaluate the effect of atrazine on salt marsh edaphic algae. A polythene barrier has been used by Scorgie (1980) to partition off an area of a drainage channel treated with

cyanatryn. Hamilton et al. (1987) and Herman et al. (1986) have used enclosures/limnocorrals (5x5x5 m deep) constructed of reinforced polyvinyl-chloride plastic (PVC), to evaluate the impact of atrazine on lake periphyton. Goldsborough and Robinson (1983, 1986) have used cylindrical open-bottom PVC enclosures (78 cm diameter, 120 cm high) to monitor the effects of simazine and terbutryn on marsh periphyton community structure and productivity. A modified sealed bottom enclosure has also been used to evaluate the effect of simazine on sediment nutrient flux in the Delta Marsh, Manitoba (Goldsborough and Robinson, 1985).

The size and design of enclosures will partially influence how closely the enclosed system simulates the surrounding environment. Dense colonization of the enclosure walls with periphyton could significantly affect the nutrient and gas concentrations within the enclosed water. Consequently enclosures with a small wall surface area to enclosed volume ratio, are prefered as they are less likely to produce 'enclosure effects'. It is advisable to use control enclosures as opposed to unenclosed water when evaluating treatment effects, although unenclosed water should also be monitored to identify enclosure effects. Differences between replicate enclosures, and differences between unenclosed and control enclosures have rarely been

addressed (Uehlinger et al., 1984; Goldsborough et al., 1986).

Artifical substrata have often been used to sample periphytic algae due to the difficulties associated with quantitative sampling of natural substrata. The degree to which colonization of artifical substrata represents that on natural substrata is still debated. Goldsborough et al. (1986) discuss types of substrata used, the parameters measured and the variability of replicates. Artifical substrata have been used in studies evaluating the effects of triazines on periphyton. Extruded, clear acrylic rods (ca. 0.6 cm diameter) positioned vertically into the sediment have been used by Goldsborough and Robinson (1983, 1986) to measure periphyton productivity, chlorophyll a, biovolume, density and community composition in control enclosures and enclosures treated with simazine and terbutryn. Herman et al. (1986) has utilized strips of PVC and microscope slides to record periphyton species composition, chlorophyll a, organic matter, biovolume, density and primary productivity in control enclosures and in enclosures treated with atrazine. Hamilton et al. (1987) also utilized microscope slides to monitor periphyton freshweight biomass, ash-free dry weight, chlorophyll a, cell density, species richness, community carbon uptake and species-specific carbon uptake in two control enclosures and

four atrazine treated enclosures. As substrata for periphyton in artifical streams, Kosinski (1984) and Kosinski and Merkle (1984) used microscope slides to assess the impact of atrazine on periphyton productivity, species composition and biovolume.

1.8 TRIAZINE IMPACT ON WATER CHEMISTRY

A number of studies have documented secondary effects resulting from triazine treatment. Included in these are decreases in dissolved oxygen (Tucker and Boyd, 1978a, 1978b; Wingfield and Johnson, 1981; Goldsborough and Robinson, 1983, 1985; MacKenzie et al., 1983; Tucker et al., 1983; Herman et al., 1986), pH (Pruss and Higgins, 1975; Wingfield and Johnson, 1981; Tucker et al., 1983) and redox potential (Wingfield and Johnson, 1981). Other secondary effects include increases in nitrogenous compounds (Murphy et al., 1981; Goldsborough and Robinson, 1983, 1985; Tucker et al., 1983; Herman et al., 1986), total phosphorus (Murphy et al., 1981; Goldsborough and Robinson, 1983, 1985) and silicon (Goldsborough and Robinson, 1983, 1985). Decreases in dissolved carbon dioxide (Tucker et al., 1983) dissolved organic carbon (Hamilton et al., 1987) and increases in dissolved inorganic carbon, sodium and calcium (Hamilton et al., 1987) have also been detected after herbicide treatment. Decreases in dissolved oxygen has usually been attributed to the inhibition of photosynthesis in the

treated site. Murphy (1982) states that when oxygen evolution is halted by photosynthetic inhibition, there seems to be no immediate reduction in respiratory oxygen demand and deoxygenation may be prolonged by enhanced microbial biochemical oxygen demand as plants decay. Although increases in dissolved nutrients after herbicide treatment have most often been attributed to the release of nutrients from decaying vegetation (Peverly and Johnson, 1979; Anderson, 1981; Tucker et al., 1983), deoxygenation of the water may play an equal or greater role. Mortimer (1941) has found that when the oxygen concentration and redox potential fall below approximently 2 $mg \cdot L^{-1}$ and E_{7}=0.4 V., iron, salts, ammonia and silicates are released from the sediments into the overlying water. In an experiment assessing the effect of simazine on nutrient flux from marsh sediments, Goldsborough and Robinson (1985) found that in the absence of decaying macrophytes increases in dissolved nutrients followed treatment. Similar increases were not recorded when treatment excluded sediment contact. Jansson (1980) has found that benthic algae are capable of totally preventing the flux of NH_4-N from the sediment to overlying lake water. These results suggests that chemical interactions and inhibition of the biotic sediment community may both contribute to the secondary effects documented in herbicide studies.

CHAPTER 2

THE INFLUENCE OF TWO TRIAZINE HERBICIDES ON FRESHWATER MARSH PERIPHYTON

2.1 ABSTRACT

This study evaluates some of the primary and secondary effects of simazine and terbutryn treatment on freshwater marsh periphyton. Haptobenthic algae colonizing acrylic substrata and herpobenthic algae living within the sediment were monitored within *in-situ* PVC enclosures. Simazine (2.0 $mg \cdot L^{-1}$) and terbutryn (0.01 $mg \cdot L^{-1}$) treated haptobenthic communities produced photosynthetic inhibition during the first two weeks of exposure, whereas the herpobenthic community remained inhibited throughout the 84 day sampling period. Haptobenthic chlorophyll *a* content also increased after the two week exposure. Although cell density of this community was similar in both treatments to that of the control, biovolume data indicated significant inhibition throughout the experimental period. Nonmetric multidimensional scaling using Euclidean distance indicates that the biomass and composition of the treated communities were distinctly different from the control. Large filamentous Chlorophytes which dominated the biomass of the control community were replaced by smaller diatom species in the treatment communities. The secondary effects of herbicide treatment included increases in dissolved

nutrients and decreases in dissolved oxygen. Increases in phosphorus and ammonia were correlated with accelerated productivity rates of the treated communities.

2.2 INTRODUCTION

Triazine herbicides are commonly used for the control of terrestrial weeds, aquatic macrophytes and nuisance algae. Simazine [2-chloro-4,6-bis(ethylamino)-s-triazine] has been registered for use as an aquatic herbicide to control both algae and macrophytes, and as a terrestrial herbicide primarily for broadleaf weed control in orchards and in corn fields. Terbutryn [2-(tert-butylamino)-4-(ethylamino)-6-(methylthio)-s-triazine], although not registered in Canada, has been tested in a number of countries as an aquatic herbicide for the control of macrophytes (Robson et al., 1976; Bowmer et al., 1979; Murphy et al., 1981; Mackenzie et al., 1983), and has been shown to be more phytotoxic to algal communities than simazine (Goldsborough and Robinson, 1983). Both herbicides are marketed by the Ciba-Geigy Corporation (Basle, Switzerland). The persistence of these herbicides within the water column is often short-lived. The half-life of terbutryn in farm pond water has been reported to vary from 21 to 30 days (Muir et al., 1981), however within irrigation ditch sediments, 50 % of simazine applied to the water remained in the sediment three years later (Smith et al,

1975). The principal mode of action of all triazines is the inhibition of electron transport in photosystem II (Moreland, 1980; van Rensen, 1982). Tolerance or resistance to these herbicides have been reported frequently with higher plants (Burke et al., 1982; Akinyemiju et al., 1983; Turcsányi and Faludi-Dániel, 1983; Gressel, 1986) but to a lesser extent in algal studies (Vance and Smith, 1969; deNoyelles et al., 1982; Galloway and Mets, 1984; Goldsborough and Robinson, 1987).

Although their effects on target species have been well documented (eg. Ellis et al., 1976; Tucker and Boyd, 1978a; MacKenzie et al., 1985), only recently has research begun to investigate their effects on the non-target organisms, such as periphytic algal communities (Goldsborough and Robinson, 1983, 1986; Kosinski, 1984; Kosinski and Merkle, 1984; Herman et al., 1986; Hamilton et al., 1987) which play essential roles in food chains and nutrient cycling in littoral waters. It is expected that the ultimate effect of a herbicide within a community will be related to the interactions that will occur between components of that community, and secondary effects resulting from treatment may prove to be just as influential as the direct effect of treatment. Secondary effects from herbicide exposure include: decreases in pH (Ellis et al., 1976; Wingfield and Johnson, 1981; Tucker et al., 1983), redox potential

(Wingfield and Johnson, 1981) and dissolved oxygen (Tucker and Boyd, 1978a, 1978b; Murphy et al., 1981; Goldsborough and Robinson, 1983), with increases in dissolved nutrients (Walker, 1964; Murphy et al., 1981; Goldsborough and Robinson, 1983, 1985; Tucker et al., 1983). Herbicide mediated changes in any of the physical, chemical or biological conditions would be expected to have some influence on the response of the organisms under investigation, and for this reason, it is dangerous to predict toxicological effects on communities, based on single species laboratory investigations.

Since large scale toxicological studies are not often feasible, the use of in-situ enclosures has become more common (Goldsborough and Robinson, 1983, 1986; Herman et al., 1986; Hamilton et al., 1987). Enclosures used for periphyton research have been designed to prevent leakage of the toxicant while minimizing wall shading (Goldsborough et al., 1986) and the use of artificial substrata for quantitative sampling of periphyton within these enclosures has also been employed (Goldsborough et al., 1986; Herman et al., 1986; Hamilton et al., 1987).

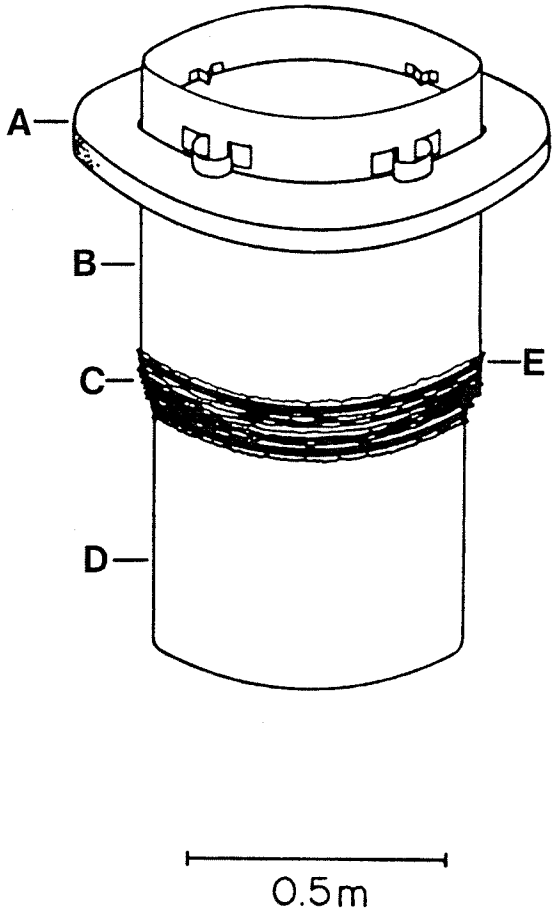
Current research in this field suggests that the response of periphyton to herbicide treatment depends not only on the level of exposure, the duration of exposure, and the species composition at the time of treatment, but also

on secondary effects resulting from the toxicant application. This study evaluates some of the primary and secondary effects of simazine and terbutryn application over an 87 day (single treatment) experiment on two algal communities. Haptobenthic algae (ie. established on solid substrata) colonizing artifical substrata positioned in the water column, and herpobenthic algae (ie. established on soft substrata) living within the first few millimeters of marsh sediment, are monitored within in-situ enclosures.

2.3 METHODS

The experimental site is located in the Blind Channel of the Delta Marsh (20,000 ha) on the southern end of Lake Manitoba, Canada (99° 19'W, 50° 7'N). The proximity of the marsh to the lake results in frequent water level changes within the marsh during high winds. To prevent herbicide loss from the experimental site due to fluctuating water levels, six telescoping enclosures were constructed from cylindrical PVC tubes (height 120 cm, diameter 76 cm). The overlapping telescoping portion was constructed from a PVC cylinder 60 cm high, 78 cm in diameter with a wall thickness of 0.15 cm (Fig. 1). This larger diameter portion was connected to the smaller diameter tube with a water-tight flexible vinyl curtain. The curtain was attached to the two portions by securing it into water-tight Poly Zip (Curry Industries, Winnipeg, Canada) tracking (Cruikshank et al,

Figure 1: Diagram of a PVC telescoping enclosure, illustrating styrofoam float (A), telescoping PVC tube (B), flexible vinyl curtain (C) connecting the telescope to the enclosure (D) and tracking used to secure the curtain to the PVC tube (E).


A
B
C
D
E
0.5m

1983) which was secured to the middle of the fixed tube and to the bottom of the telescoping tube. A circular styrofoam float was attached to the outside of the telescoping portion to maintain the upper edge of the enclosure 15 cm above the water surface. The telescoping modification allowed for a 50 cm fluctuation in water level. All six experimental enclosures were securely placed in a row such that they were embedded to a depth of 30 cm of marsh sediment, with PVC wave breaks positioned at both ends of the row. Although aquatic macrophytes were absent at the time of enclosure placement, occasionally throughout the experiment, removal of vegetation (primarily Potamogeton pectinatus) was necessary in the control enclosures to reduce variability between enclosures. Total enclosed water volume of each enclosure was approximately 320 L, and the wall surface area to volume ratio was approximately 5.2.

Two enclosures were used for simazine application, two for terbutryn application and two were used as controls, receiving no herbicide. On day one of the experiment, 84 extruded acrylic rods (0.6 cm diameter; 90 cm in length), prescored at regular intervals (Goldsborough et al., 1986) were vertically positioned in one of each treatment enclosure. Rods were placed 23 cm into the sediment to provide anchorage. Sampling disturbance to the haptobenthic algae on the rods was minimized by using needle nose pliers

to cleanly break the rod segments at score marks. Enclosures without artificial substata were used to sample the herpobenthic periphyton (epipelon).

On day six of the experiment, following a period during which artifical substrata became colonized by periphytic algae, two enclosures were treated with 2.0 $mg \cdot L^{-1}$ simazine and two with 0.01 $mg \cdot L^{-1}$ terbutryn. These levels of herbicide had previously been demonstrated to significantly inhibit physiological processes in haptobenthic algae (Goldsborough and Robinson, 1983). For each, preweighed unformulated technical grade herbicide was placed in a gauze sac and suspended in the enclosure.

Between 28/05/84 and 21/08/84 haptobenthic primary productivity and pigment content data were collected on nine sampling dates, eight being after herbicide additions. At each sampling period, four randomly selected colonized acrylic rods were removed and subsampled at 20-25 cm below the water surface. Of these four samples, three were used as replicates for the estimation of carbon fixation rates, and one was used to correct for dark assimilation. Each was placed in a culture tube containing 25 mL of twice filtered (Whatman GF/C and Sartorius 0.45 μm cellulose nitrate) enclosure water and transported to the laboratory.

Herpobenthic communities were sampled from within 11 cm diameter PVC coring cylinders which were placed into the sediment within each enclosure. Following sampling, the cylinders were left in position to minimize sediment disturbance. One sediment sample was collected per treatment on each of the nine sampling dates. An aspirator was employed to remove the top few centimeters of sediment delineated by each cylinder. Samples of sediment were returned to the laboratory and settled in darkened beakers (10 cm diameter) for 24 hours. Water overlying the sediment was then carefully removed so as not to disturb the sediment, lens paper tissue traps (4 cm^2) were placed on the sediment surface, and beakers were placed in a light incubation chamber for nine hours (Eaton and Moss, 1966). Three tissue traps containing trapped algae were used as replicates for measuring carbon assimilation and one was used to correct for dark assimilation. The traps were carefully removed from the sediment beakers and singly placed into culture tubes containing 25 mL of prefiltered (as above) enclosure water.

Samples of haptobenthic and herpobenthic communities were inoculated with 1.0 mL of standardized $NaH^{14}CO_3$ and incubated in a growth chamber for two hours at a light intensity of 168 $\mu E \cdot m^{-2} \cdot s^{-1}$ and a temperature of 22°C. These incubation conditions were chosen to represent average

summer marsh conditions. Following incubation, cells were vacuum filtered through 0.45 µm filters, washed with deionized water, fumed for one minute over concentrated HCL to remove residual inorganic ^{14}C and placed in 10.0 mL of Scinti Verse scintillation cocktail (Fisher Scientific). Specific radioactivity was determined by scintillation counting using a Picker Liquimat 220 counter. All samples were corrected for dark assimilation and for colour quenching using the channels ratio method (Wang and Willis, 1965). Inorganic carbon assimilation rates were calculated using the following formula:

$$\mu g\ C\ fixed \cdot cm^{-2} \cdot hr^{-1} = \frac{DPM(S) \times C \times 1.05}{DPM(T) \times A \times T}$$

where: DPM(S) is the specific activity of the algal sample, DPM(T) is the total specific activity of the isotope added, C is the dissolved inorganic carbon present in the incubation water determined from alkalinity (APHA, 1980) (µg $C \cdot 25\ mL^{-1}$), 1.05 is the ^{14}C uptake discrimination factor, A is the surface area of the substratum sampled (cm^2), and T is the incubation period (hours).

At each sampling interval, triplicate, 10 cm long acrylic substrata segments were removed from a 25-35 cm water depth for the determination of chlorophyll *a*. Periphyton was detached from the subtrata into distilled

water, collected on GF/C filters, covered with a 1% magnesium carbonate suspension, and then frozen until analysis. Pigments were extracted using a tissue grinder and 90% acetone. Chlorophyll *a* concentrations were measured trichromatically (Strickland and Parsons, 1968), corrected for phaeopigments, and presented as chlorophyll *a*·cm^{-2} of substratum surface area.

Concentrations of dissolved oxygen, silicon, ammonia and total reactive phosphorus (TRP) within all enclosures were measured three times a week. Water samples for nutrient analyses were taken at 10 cm below the water surface. Nutrient concentrations were analyzed spectrophotometrically, following methods of Stainton *et al.*(1968). Dissolved oxygen concentrations were measured 10 cm below the water surface using a YSI model 51B oxygen meter.

Simazine persistence in the enclosed water was measured on a weekly basis using ultraviolet spectrophotometry (Mattson *et al.*, 1970). The detection limit of the ultraviolet method proved to be too high to be used for the terbutryn analysis, therefore GLC analysis (Muir, 1980) was used to determine final terbutryn concentrations 86 days after herbicide addition.

On six of the nine sampling dates, haptobenthic algal samples were collected and preserved for cell enumeration.

On each occasion a 5 cm segment (from 15-20 cm water depth) was collected from each of four rods and placed in 20 mL of filtered distilled water with 0.5 mL of Lugol's iodine. Preserved periphyton was later detached from the substrata using a fine paint brush and a rubber policeman. Uniform algal cell suspensions were prepared by the gentle use of a homogenizer. These were washed by centrifugation and resuspended in a known volume of distilled water (1-5 ml). Diatom identification and quantification slides were prepared by dispensing known quantities (0.1-0.3 mL) of suspensions onto glass coverslips (18x18 mm), which were dried, then ashed at 600°C for six minutes. Naphrax (Northern Biological Supplies) high resolution diatom mounting medium was used to permanently mount coverslips onto glass slides. Diatom identification and cell numbers were recorded along random transects of the coverslips. Diatoms per unit area of colonized substratum was calculated as follows:

$$\text{cells} \cdot \text{cm}^{-2} = \frac{N \times A \times V1}{C \times S \times V2}$$

where: N is the number of cells of each taxon, A is the area covered by the algal suspension on the coverslip (cm^2), V1 is the total volume of the concentrated algal suspension (mL), C is the surface area of the coverslip examined (cm^2), S is the surface area of acrylic rods sampled (cm^2), V2 is

the volume of algal suspension used on the coverslip (mL). Taxonomy was based on Patrick and Reimer (1966, 1975) and Germain (1981).

Cell enumeration for non-diatom taxa was conducted using a 0.10 mL Palmer counting cell at 400X magnification. Taxonomy was based on Prescott (1962). When species level identification was not possible, organisms were grouped (eg. coccoid Chlorophytes).

Algal biovolumes were also tabulated for each species or group. Measurements of a representative number of cells of each species were conducted using a Bioquant II (R&M Biometrics, Nashville, Tn.) image analysis system. Using this system, microscope images of algal cells were displayed on a computer monitor via a TV camera. An ancillory digitizing tablet (Hipad) was then used to measure cell axes. Cell biovolumes were then calculated using formulae for the nearest geometric solid. Total biovolume of each taxon within each sample was determined by multiplying the mean cell volume by the cell number.

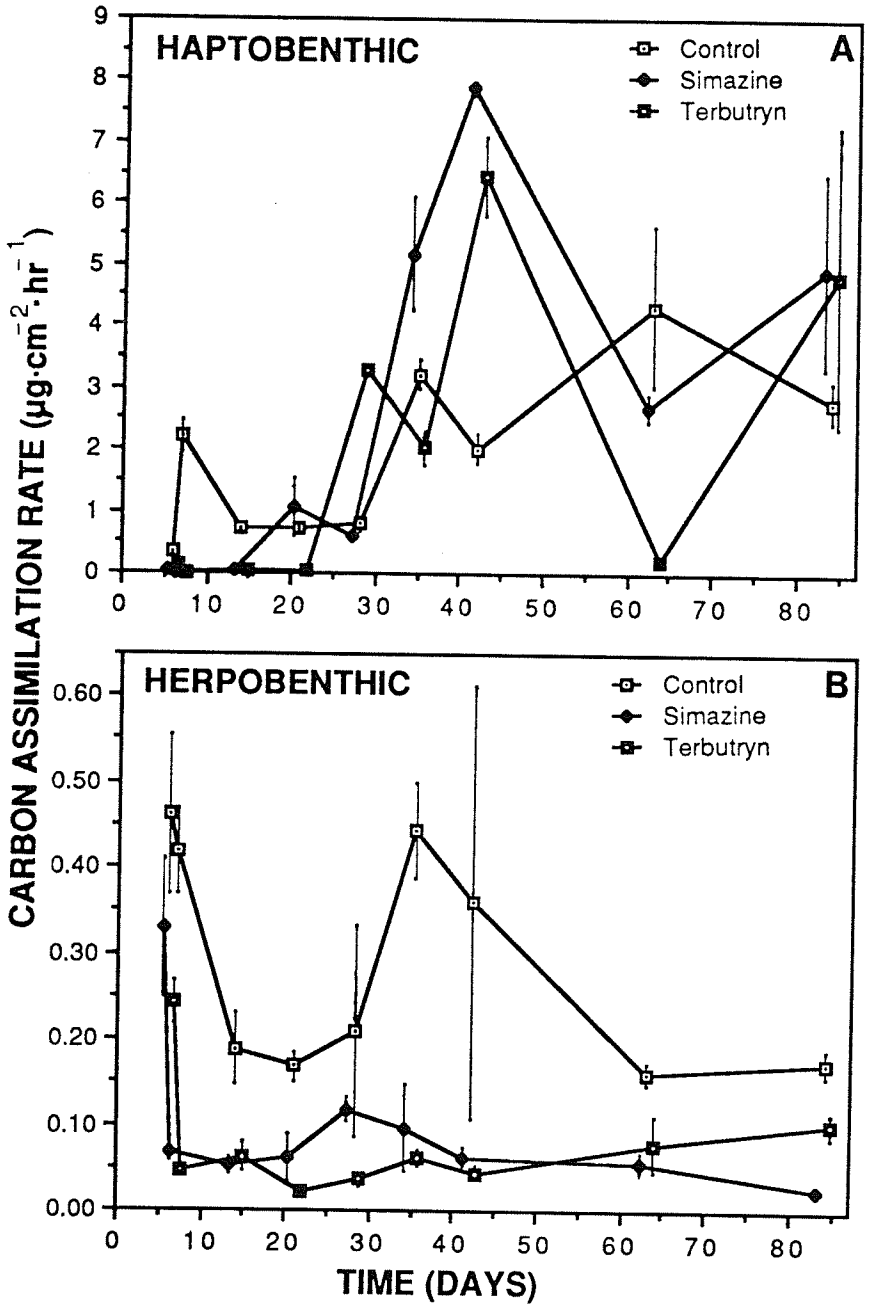
Nonmetric multidimensional scaling using Euclidean distance was used to graphically describe dissimilarity of the haptobenthic communities between each of three treatment over the six sampling periods. Data matrices were created using species composition with both cell numbers and total

biovolumes. A two dimensional graphic representation presents the dissimilarity of each community to all other communities on each sample date in each treatment.

2.4 RESULTS

The influence of simazine and terbutryn on carbon assimilation rates were found to differ between the haptobenthic algae (Fig. 2A), colonizing the acrylic substrata and the herpobenthic algae (Fig. 2B) living in the marsh sediment. After herbicide addition on day six, photosynthetic rates in the treated haptobenthic communities were depressed for approximently two weeks. Between days 30 and 53, productivity rates of the two herbicide treated haptobenthic communities greatly exceeded that measured in the control enclosure. This was followed by a sharp declines in productivity until day 63. On the final productivity sampling date (day 84), carbon fixation rates were once again higher than that observed in the control. Productivity rates of the control haptobenthic community did not demonstrate the same fluctuating patterns observed in the treated enclosures. Productivity rates in the treated herpobenthic enclosures however, were never found to exceed those observed in the controls (Fig. 2B). The productivity peak identified on day 37 in the control enclosure did not occur in either herbicide treated enclosure. The 2.0 $mg \cdot L^{-1}$ simazine and the 0.01 $mg \cdot L^{-1}$ terbutryn treatments did not

Figure 2: Carbon assimilation rates of haptobenthic (A) and herpobenthic (B) periphyton in the the control enclosures, and in enclosures treated with 2.0 $mg \cdot L^{-1}$ simazine and 0.01 $mg \cdot L^{-1}$ terbutryn over an 84 day experimental period. Herbicides were added on day 6. Error bars are the SE of replicates (n=3).


HAPTOBENTHIC
Control
Simazine
Terbutryn
A
HERPOBENTHIC
Control
Simazine
Terbutryn
B
CARBON ASSIMILATION RATE (µg·cm⁻²·hr⁻¹)
TIME (DAYS)
0
1
2
3
4
5
6
7
8
9
0.00
0.10
0.20
0.30
0.40
0.50
0.60
10
20
30
40
50
60
70
80

appear to be substantially different from one another in their effect on photosynthesis of both communities, even though the application concentration of simazine was 200X that of terbutryn.

The trends of haptobenthos chlorophyll a content (Fig. 3) were found to closely follow trends observed in carbon assimilation rates (Fig. 2A). After day 25, chlorophyll a content of the herbicide treated samples begin to surpassed that of the control, however the sharp decline observed in the productivity rate in the simazine enclosure was accompanied by a more moderate decline in pigment content. During this time, chlorophyll a content in the terbutryn treated community was substantially reduced, following trends observed in carbon assimilation rates.

Dissolved oxygen concentrations in all four treated enclosures followed a similar pattern (Fig. 4B, C). After herbicide addition on day 6, dissolved oxygen levels dropped dramatically. After day 25, levels were found to exceed those measured for the controls (Fig 4A), however after day 35 the oxygen levels in the treated enclosures generally remained lower than that of the controls.

Dissolved ammonia concentrations in all four herbicide treated enclosures (Fig 5B, C) were substantially greater than control values (Fig. 5A). Three days following herbicide addition (day 9), ammonia levels in all four

Figure 3: Chlorophyll *a* levels of haptobenthic periphyton in the control enclosure and in enclosures treated with 2.0 $mg \cdot L^{-1}$ simazine and 0.01 $mg \cdot L^{-1}$ terbutryn over an 84 day experimental period. Herbicides were added on day 6. Error bars are SE of replicates (n=3).

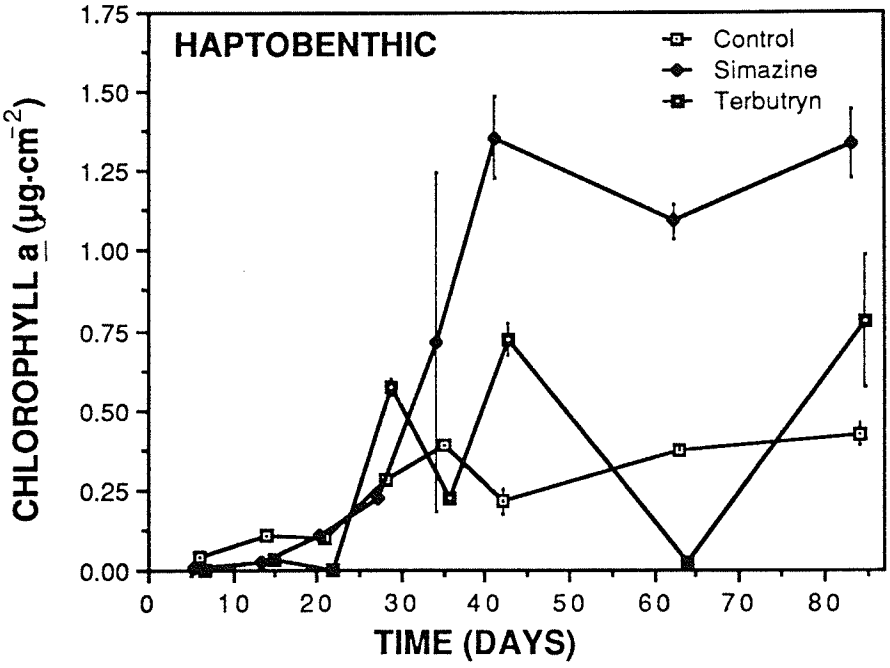

HAPTOBENTHIC
Control
Simazine
Terbutryn
CHLOROPHYLL a (µg·cm⁻²)
TIME (DAYS)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
50
60
70
80

Figure 4: Dissolved oxygen in the control enclosures (A) and in enclosures treated with 2.0 $mg \cdot L^{-1}$ simazine (B) and 0.01 $mg \cdot L^{-1}$ terbutryn (C), over an 86 day experimental period. Herbicides were added on day 6.

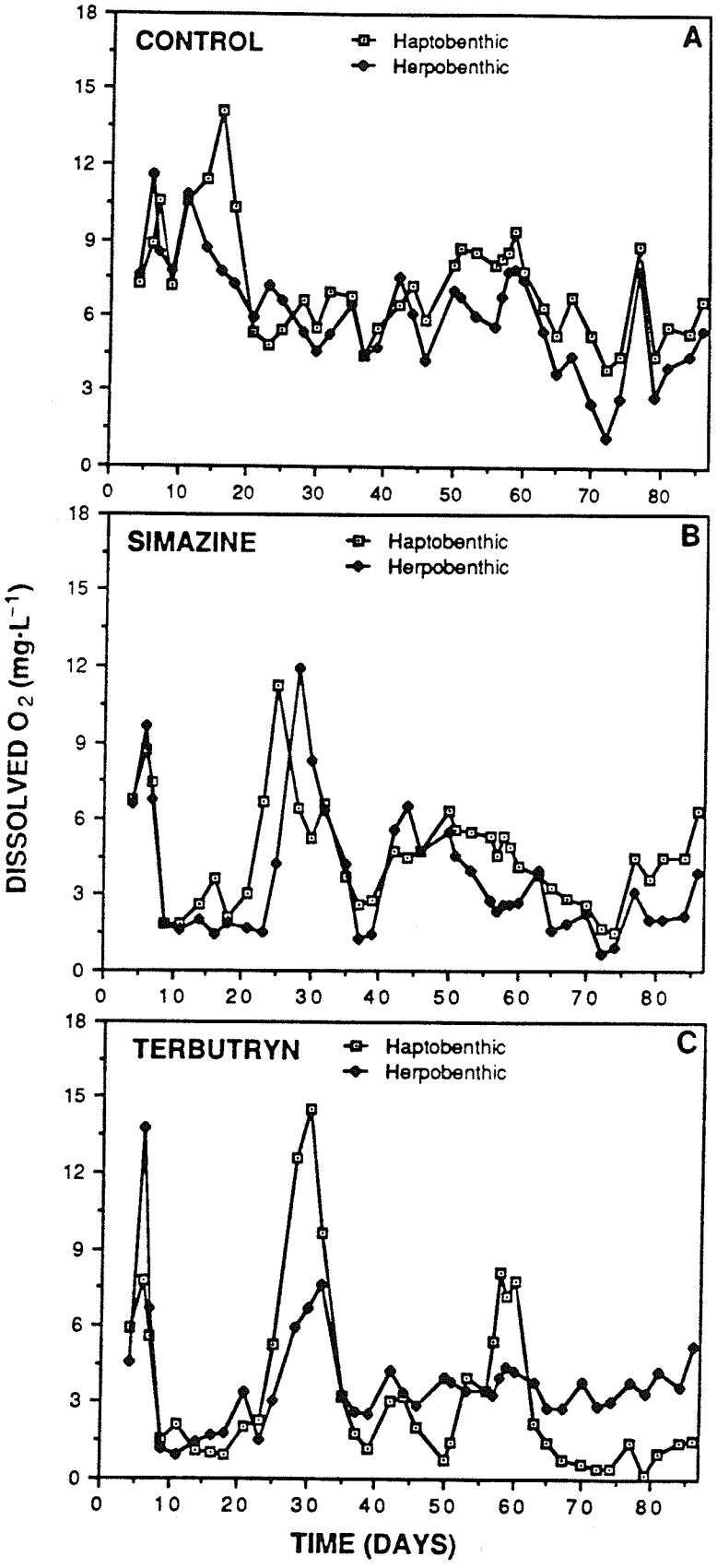
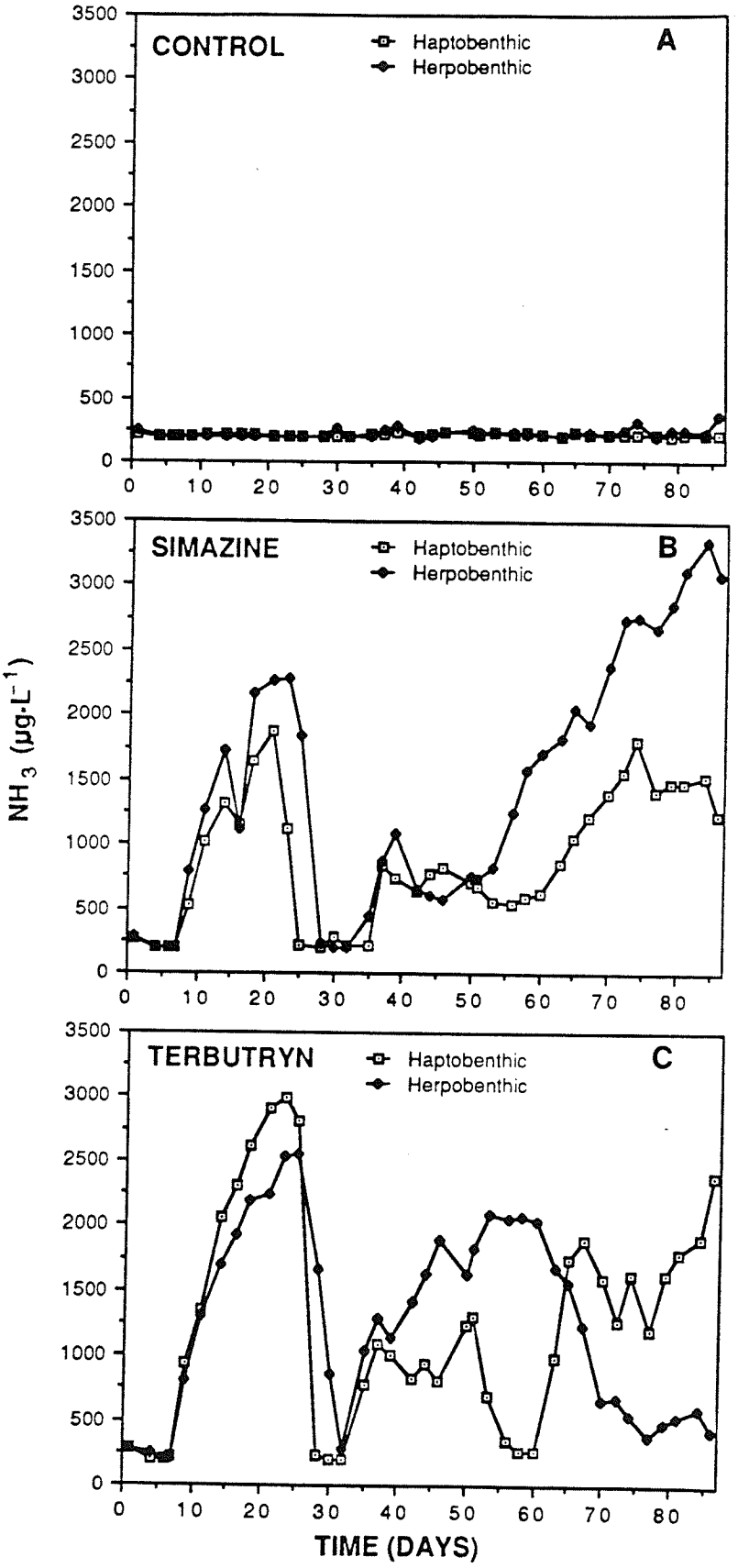

CONTROL
Haptobenthic
Herpobenthic
A
SIMAZINE
Haptobenthic
Herpobenthic
B
TERBUTRYN
Haptobenthic
Herpobenthic
C
DISSOLVED O2 (mg·L-1)
TIME (DAYS)
0
3
6
9
12
15
18
0
10
20
30
40
50
60
70
80

Figure 5: Ammonia concentrations in the control enclosures (A) and in enclosures treated with 2.0 $mg \cdot L^{-1}$ simazine (B) and 0.01 $mg \cdot L^{-1}$ terbutryn (C) over an 86 day experimental period. Herbicides were added on day 6.

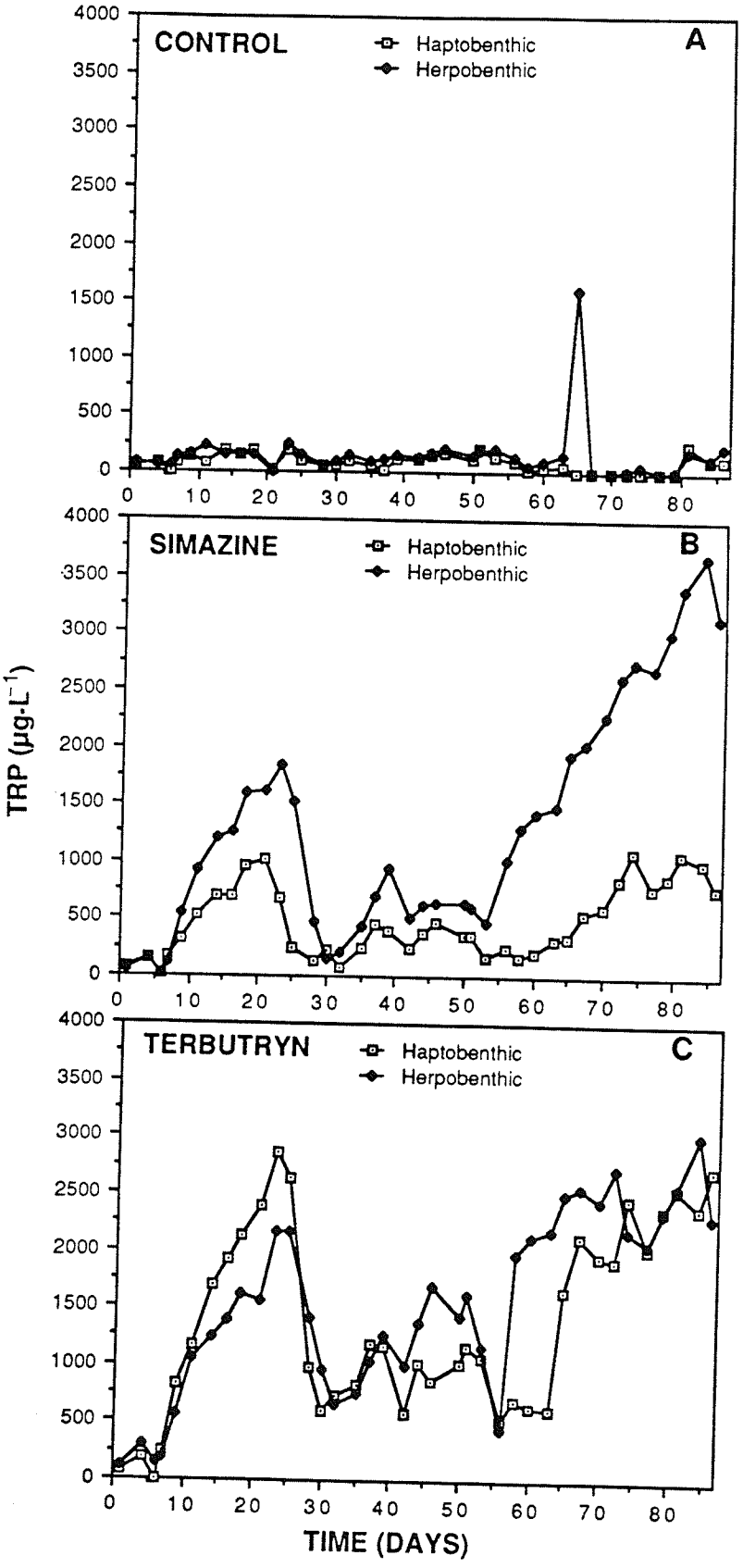

CONTROL
Haptobenthic
Herpobenthic
A
SIMAZINE
Haptobenthic
Herpobenthic
B
TERBUTRYN
Haptobenthic
Herpobenthic
C
NH_3 (µg·L^{-1})
TIME (DAYS)
0
500
1000
1500
2000
2500
3000
3500
0
10
20
30
40
50
60
70
80

treated enclosures increased rapidly. By day 25, concentrations decreased to levels comparable to that recorded for the controls. Within days, ammonia levels in the simazine treated water were again much higher than in the controls, and remained high for the duration of the experiment. Ammonia in the terbutryn enclosures also returned to levels in excess of controls, but fluctuated more than in the simazine enclosures.

Total reactive phosphorus concentrations (TRP) followed trends very similar to those previously described for ammonia (Fig. 5). Treated enclosures (Fig. 6B, C) were shown to be consistently higher in TRP than in control enclosures (Fig. 6A), however the haptobenthic simazine treated enclosure was found to have a consistently lower TPR than that found in the herpobenthic simazine treated enclosure.

The relationship between nutrient concentrations and carbon assimilation rates in haptobenthic herbicide treated enclosures were evaluated. Principal components analysis was applied to TRP and ammonia concentrations in both treated enclosures. The eigenvalues for these analyses in the simazine and terbutryn enclosures were 98% and 95% respectively. Component scores from these analyses were plotted against carbon assimilation rates, incorporating a three week assimilation lag period. The coefficient of

Figure 6: Total reactive phosphorus concentrations (TRP) in the control enclosures (A) and in enclosures treated with 2.0 $mg \cdot L^{-1}$ simazine (B) and 0.01 $mg \cdot L^{-1}$ terbutryn (C) over an 86 day experimental period. Herbicides were added on day 6.

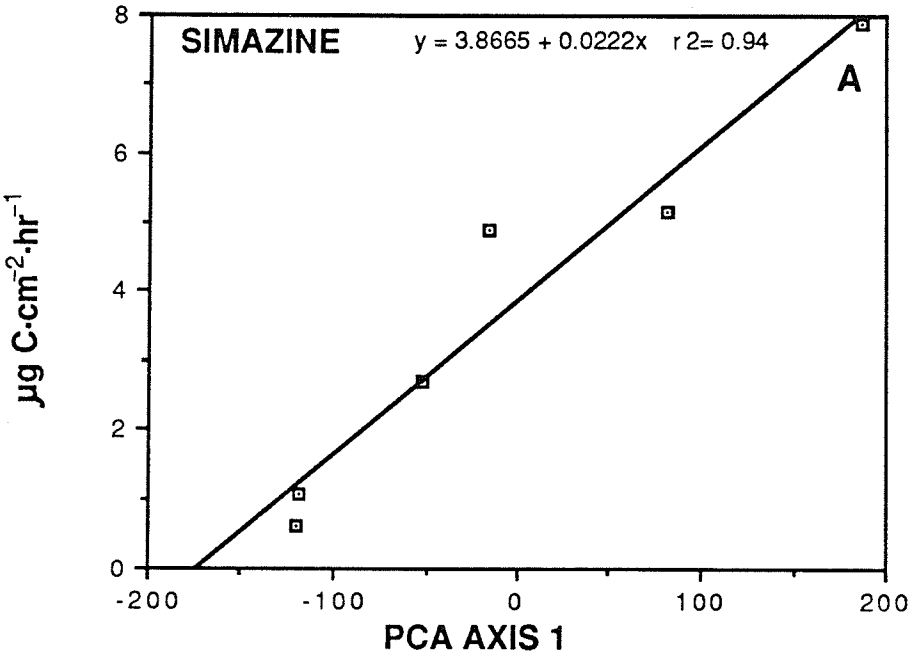

CONTROL
Haptobenthic
Herpobenthic
A
SIMAZINE
Haptobenthic
Herpobenthic
B
TERBUTRYN
Haptobenthic
Herpobenthic
C
TRP (µg·L⁻¹)
TIME (DAYS)
0
500
1000
1500
2000
2500
3000
3500
4000
10
20
30
40
50
60
70
80

determination for these parameters in the simazine enclosure (Fig. 7A) was significant (r^2=.94, $p<.01$), however this relationship in the terbutryn treatment (Fig. 7B) was poorer (r^2=.52, $p>.10$).

Silicon concentrations in the herbicide treated enclosures (Fig. 8B, C) did not appear to differ greatly from the control enclosure (Fig. 8A) during the first 50 days of the experiment. After this time, silicon levels fluctuate, although concentrations in the treated enclosures were not consistently higher or lower than that of the control.

Simazine concentrations within the enclosed water declined rapidly during the first week following application (Fig. 9). A more gradual reduction in simazine concentration was observed from day 15 to day 87. Final concentrations were 0.155 and 0.225 $mg \cdot L^{-1}$ in the haptobenthic and herbobenthic enclosures respectively, 7.75 % and 11.3 % of the original concentration. On day 91 terbutryn concentrations in the haptobenthic and herpobenthic enclosures were found to be 0.00500 and 0.00315 $mg \cdot L^{-1}$ respectiviely, or 50 % and 32 % of the original quantity added. These data suggest that terbutryn may be a more persistent herbicide within the water column.

Figure 7: The relationship between carbon fixation rate and component scores from a PCA conducted on ammonia and TRP concentration data, incorporating a three week delay. Samples were collected from haptobenthic enclosures treated with 2.0 $mg \cdot L^{-1}$ simazine (A) and 0.01 $mg \cdot L^{-1}$ terbutryn (B).


SIMAZINE
y = 3.8665 + 0.0222x r 2= 0.94
A
µg C·cm⁻²·hr⁻¹
PCA AXIS 1
8
6
4
2
0
-200
-100
0
100
200

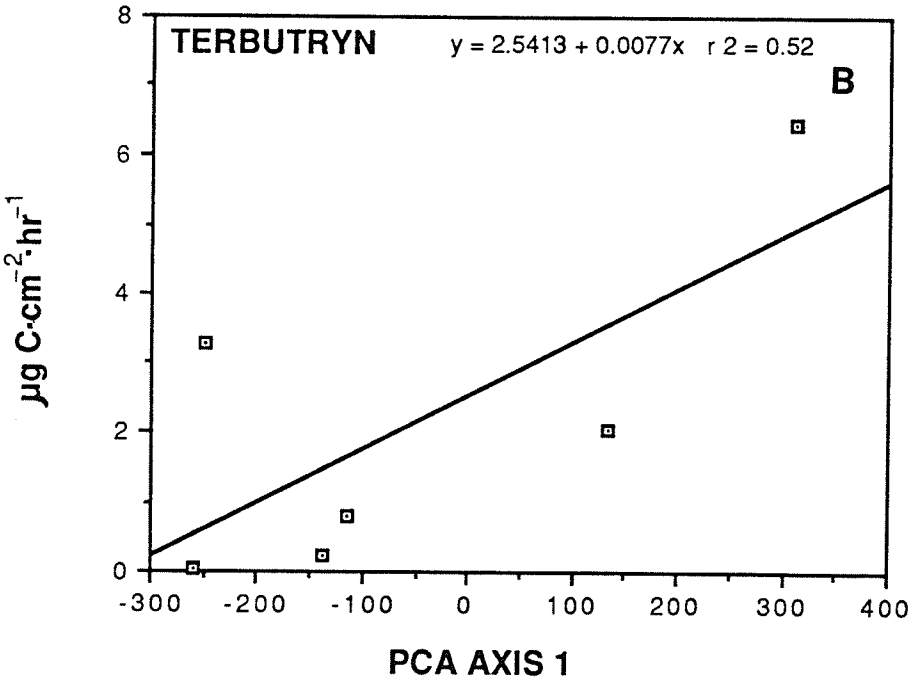

TERBUTRYN
y = 2.5413 + 0.0077x r 2 = 0.52
B
µg C·cm⁻²·hr⁻¹
PCA AXIS 1
8
6
4
2
0
-300
-200
-100
0
100
200
300
400

Figure 8: Silicon concentrations in the control enclosures (A) and in enclosures treated with 2.0 $mg \cdot L^{-1}$ simazine (B) and 0.01 $mg \cdot L^{-1}$ terbutryn (C) over an 86 day experimental period. Herbicides were added on day 6.

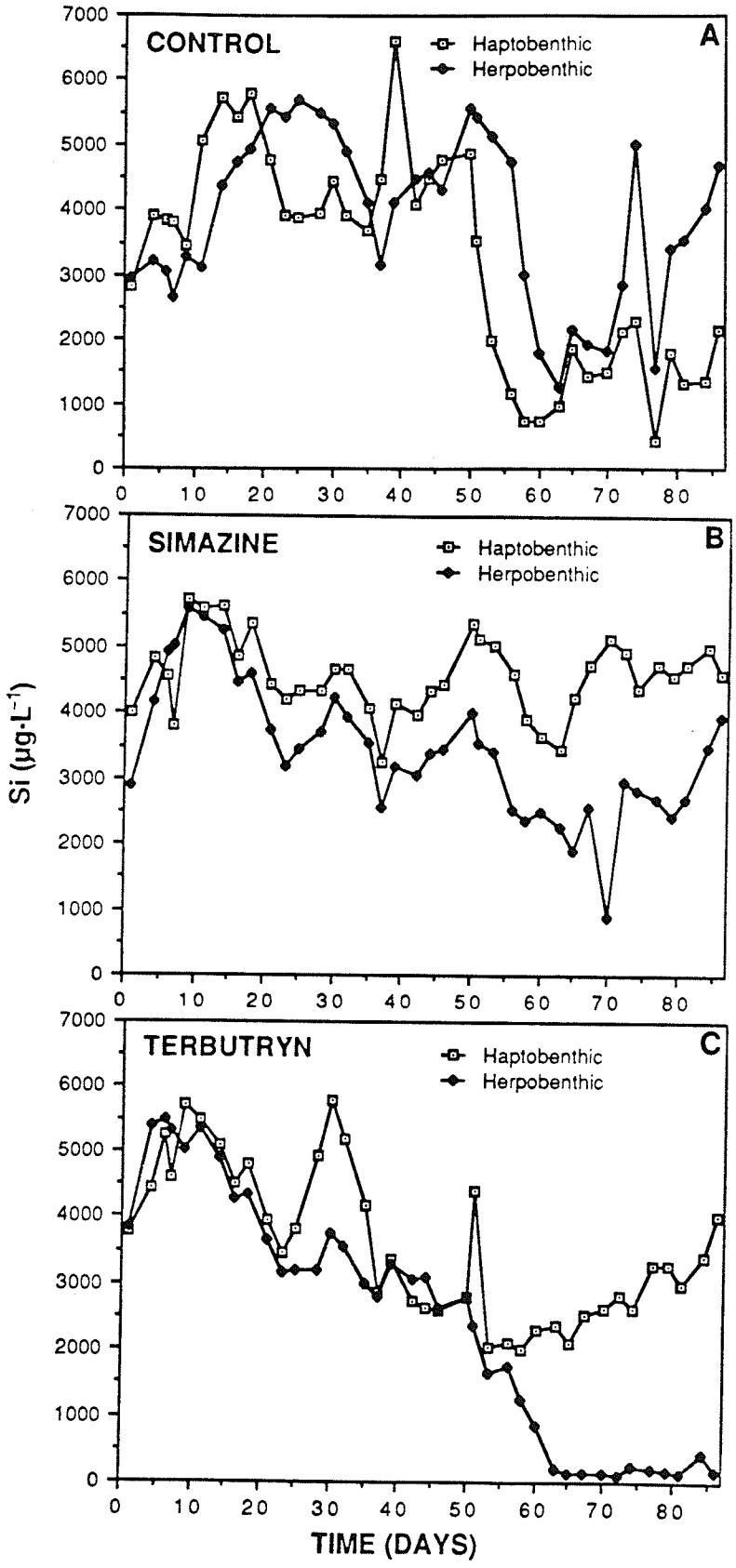
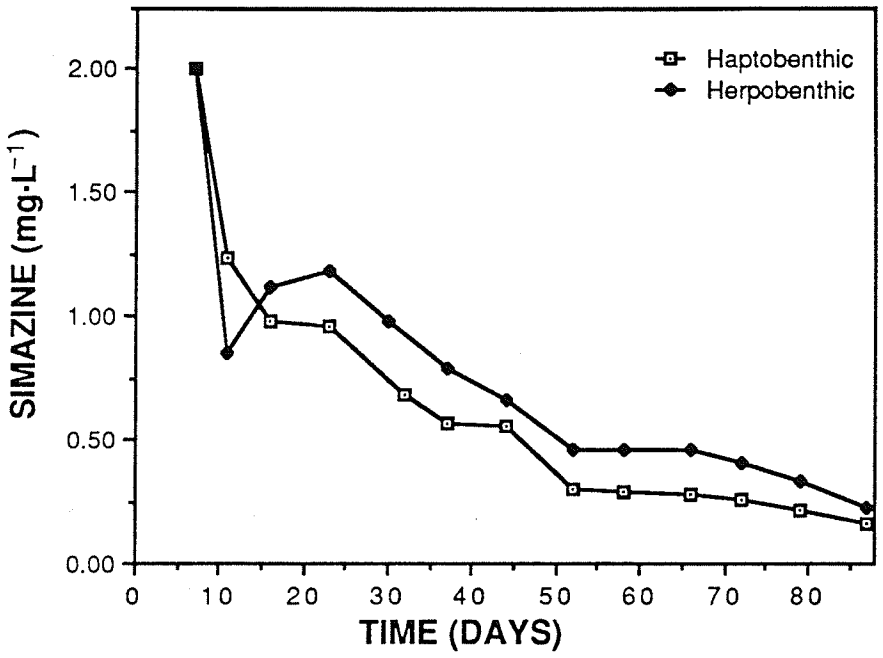

CONTROL
Haptobenthic
Herpobenthic
A
SIMAZINE
Haptobenthic
Herpobenthic
B
TERBUTRYN
Haptobenthic
Herpobenthic
C
Si (µg·L-1)
TIME (DAYS)
0
1000
2000
3000
4000
5000
6000
7000
0
10
20
30
40
50
60
70
80

Figure 9: Simazine concentrations over an 87 day experimental period.


Haptobenthic
Herpobenthic
SIMAZINE (mg·L^{-1})
2.00
1.50
1.00
0.50
0.00
0
10
20
30
40
50
60
70
80
TIME (DAYS)

Although herbicide exposure to haptobenthic algae did not result in consistent decreases in total cell densities through the experiment (Fig. 10), dramatically less biovolume was produced in both herbicide treatments, when compared with the control (Fig. 11). Unlike the productivity recovery observed after day 25, biovolume in the treated enclosures remained depressed through the entire experiment. At no time after herbicide addition, did biovolumes of thesimazine or terbutryn treated haptobenthic community exceed 35 % of the biovolume recorded for the control. The differences in the observed biovolumes appear to be related to the differences in the species compositon. After day 42, the control (Fig. 12A) was dominated by relatively large Chlorophyta taxa whereas simazine (Fig. 12B) and terbutryn (Fig. 12C) treated samples were dominated by smaller Bacillariophyta taxa. Mean biovolumes/cell for all species for each sampling period in the control, simazine and terbutryn treatments were calculated as 3050, 1464 and 216 μm^3 respectively.

Nonmetric multidimensional scaling, which circumvents the linearity assumption of metric ordination methods (Kenkel and Orloci, 1986), was used to illustrate dissimilarity of haptobenthic community structure between treatments based on abundance and biovolumes of taxa. Using total cell numbers (Fig. 13A) and total biovolume (Fig. 13B)

Figure 10: Haptobenthic algal density over an 84 day experimental period in the control enclosure and in enclosures treated with 2.0 $mg \cdot L^{-1}$ simazine and 0.01 $mg \cdot L^{-1}$ terbutryn. Herbicides were added on day 6.

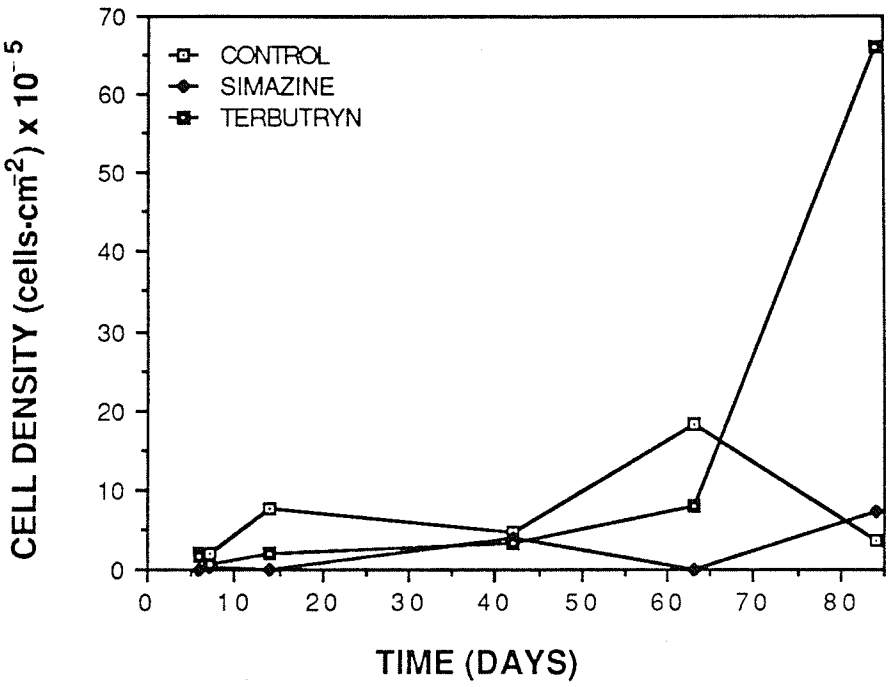

CONTROL
SIMAZINE
TERBUTRYN
CELL DENSITY (cells-cm^{-2}) x 10^{-5}
TIME (DAYS)
0
10
20
30
40
50
60
70
80

Figure 11. Haptobenthic algal biovolume over an 84 day experimental period in the control enclosure and in enclosures treated with 2.0 $mg \cdot L^{-1}$ simazine and 0.01 $mg \cdot L^{-1}$ terbutryn. Herbicides were added on day 6.

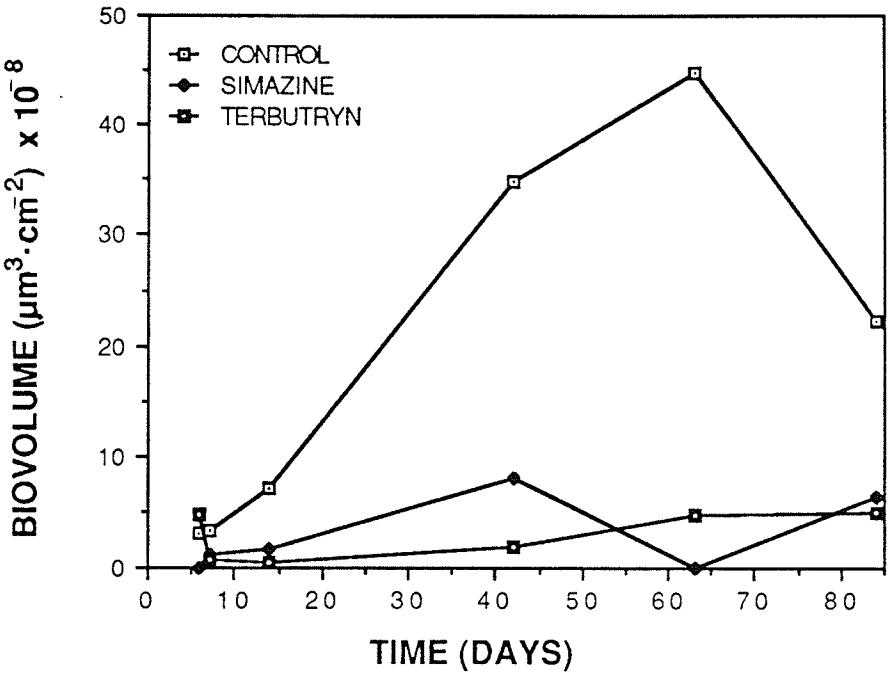

CONTROL
SIMAZINE
TERBUTRYN
BIOVOLUME (µm³·cm⁻²) x 10⁻⁸
TIME (DAYS)
0
10
20
30
40
50
60
70
80

Figure 12: Changes in the composition of the haptobenthic algal community over an 84 day experimental period in the control enclosure and in enclosures treated with 2.0 $mg \cdot L^{-1}$ simazine and 0.01 $mg \cdot L^{-1}$ terbutryn. Herbicides were added on on day 6.

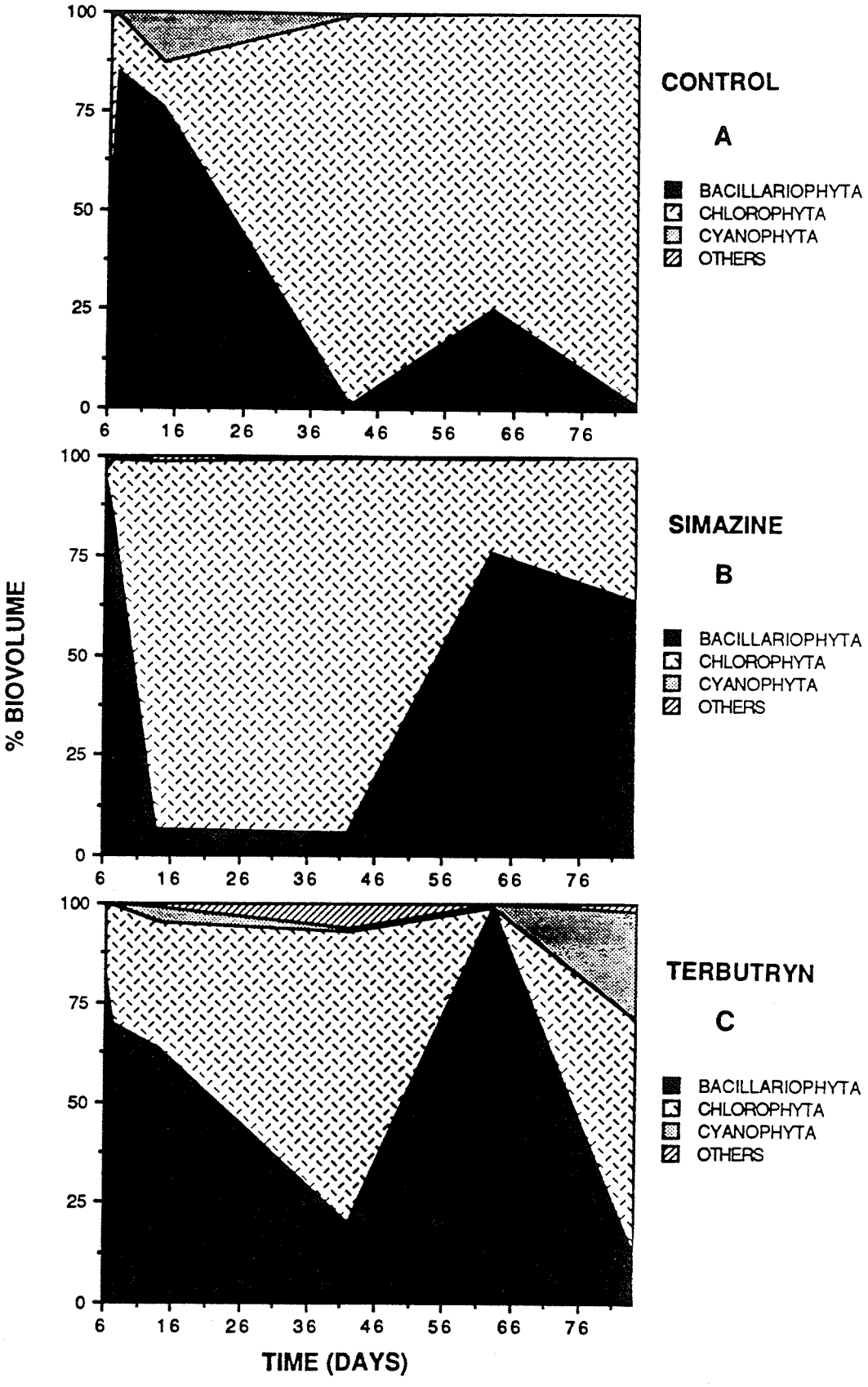
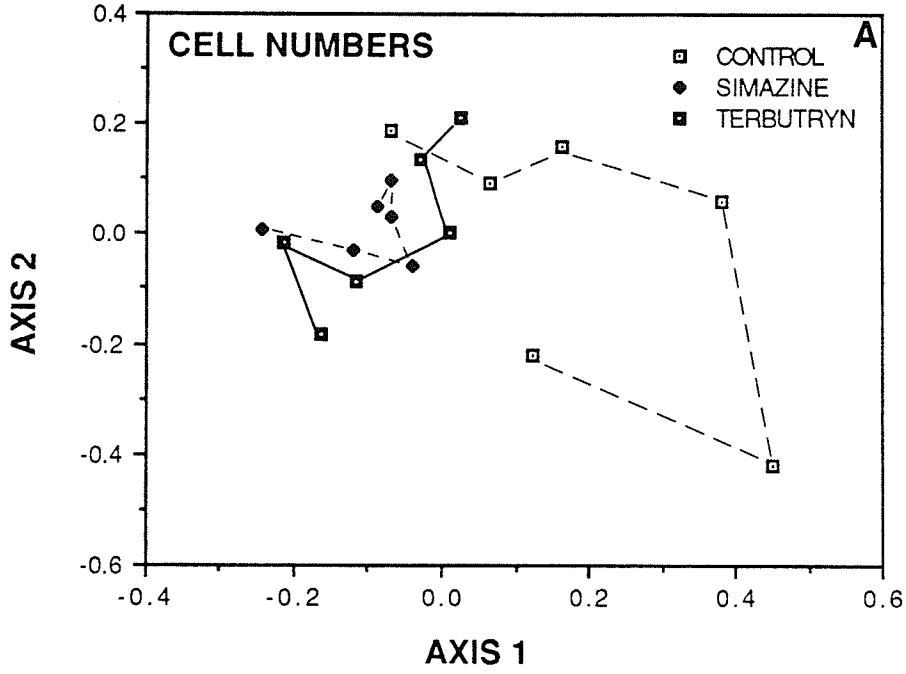
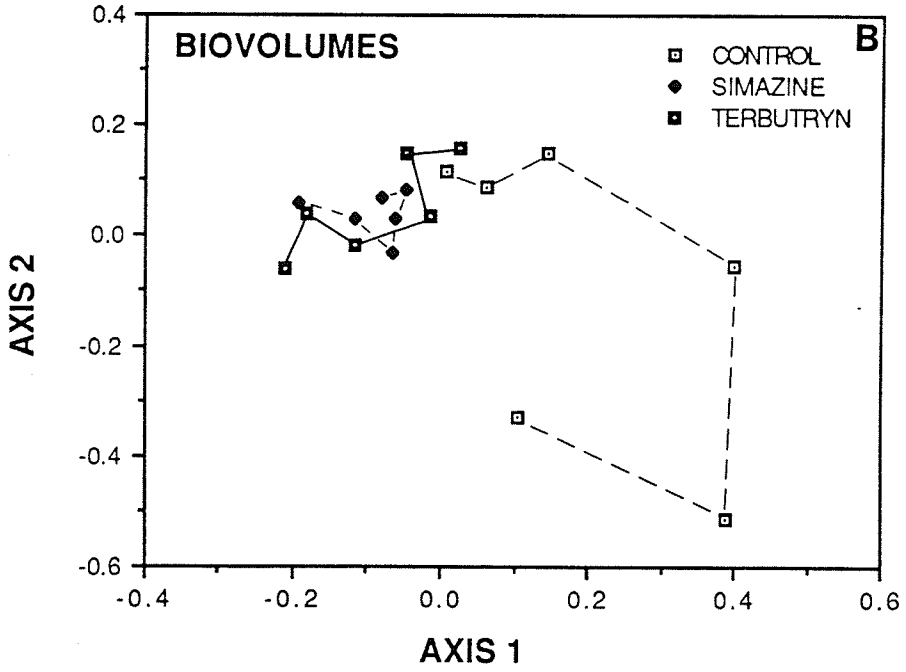

CONTROL
A
SIMAZINE
B
TERBUTRYN
C
BACILLARIOPHYTA
CHLOROPHYTA
CYANOPHYTA
OTHERS
% BIOVOLUME
TIME (DAYS)
0
25
50
75
100
6
16
26
36
46
56
66
76

Figure 13. Nonmetric multidimensional scaling of Euclidean distance between cell numbers (A) and biovolumes (B) of haptobenthic algal communities in the control enclosure and in enclosures treated with 2.0 $mg \cdot L^{-1}$ simazine and 0.01 $mg \cdot L^{-1}$ terbutryn over an 84 day experimental period (6 sampling intervals).

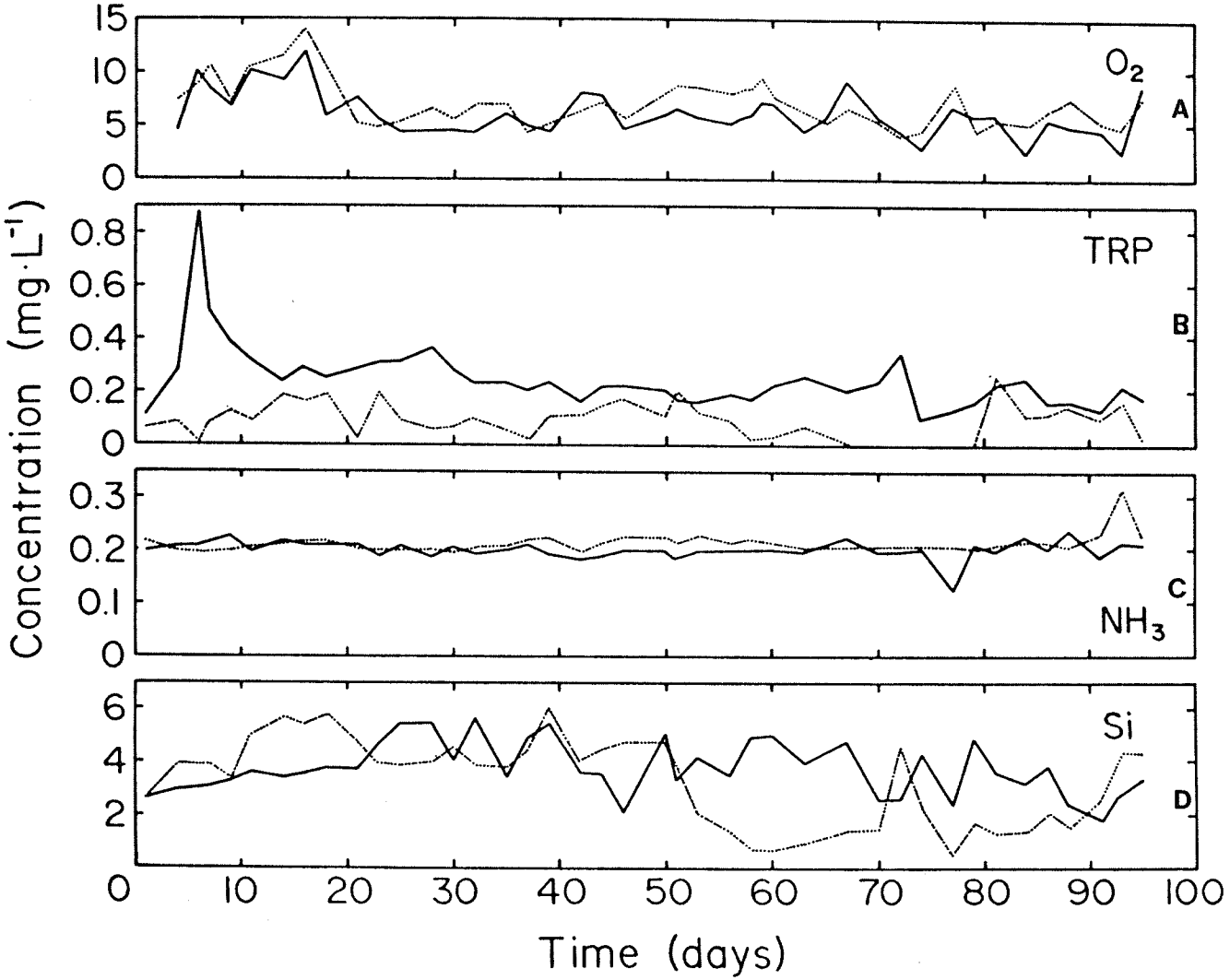

CELL NUMBERS
A
CONTROL
SIMAZINE
TERBUTRYN
AXIS 2
AXIS 1
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6


BIOVOLUMES
B
CONTROL
SIMAZINE
TERBUTRYN
AXIS 2
AXIS 1
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6

differences between the treatments are presented temporally in a two dimensional ordination scale. Data from the beginning of the experiment are represented by the points near the top of the plot. In both figures, the herbicide treated communities follow a pattern similar to one another yet quite different from that presented by the control. The biovolume plot (Fig. 13B) which better describes algal biomass accentuates the trend observed in the cell number plot (Fig. 13A).

In order to identify the appearance of any enclosure effects during this study, concentrations of dissolved oxygen, total reactive phosphorus (TRP), ammonia and silicon were measured throughout the experiment, both within the enclosed water and in the adjacent marsh (Goldsborough et al., 1986). Although considerable variation was evident (Fig. 14A), the overall mean enclosure oxygen level was statistically higher than that of the surrounding water (7.06 and 5.85 $mg \cdot L^{-1}$ respectively, $p=0.0001$). Ammonia levels were slightly lower in the marsh than in the enclosed water ($p=0.02$), although the mean differed by only 10 $\mu g \cdot L^{-1}$ (Fig. 14C). Silicon concentrations demonstrated widespead temporal variability (Fig. 14D) but were not significantly different ($p=0.1146$). Measurements of total reactive phosphorus (TRP) indicate an enclosure trend, with levels in the surrounding marsh ca. four times that within the

Figure 14: Concentrations of dissolved oxygen (A), total reactive phosphorus (B), ammonia (C) and silicon (D) in the control enclosures (·····) in comparison to levels in the surrounding water (———) over a 97 day experimental period.


15
10
5
0
O_2
A
0.8
0.6
0.4
0.2
0
TRP
B
0.3
0.2
0.1
0
C
NH_3
6
4
2
0
Si
D
Concentration (mg·L⁻¹)
0
10
20
30
40
50
60
70
80
90
100
Time (days)

enclosed water (Fig. 14B), although conclusions based on the significance of the test ($p=0.0550$) cannot be made.

2.5 DISCUSSION

In most experimental sites, large scale toxicological experiments are not feasible, thereby creating the need for a replicable enclosure system to allow for intensive investigations into treatment effects. In areas, such as the Delta Marsh, *in-situ* enclosures must be adapted to tolerate fluctuating water levels which occur due to set-ups in adjacent Lake Manitoba, however enclosures used for periphyton research must also be designed to prevent wall shading. The telescoping enclosure described in this study proved to meet the requirements needed for an effective periphyton toxicological study, and would appear to be suitable for studies lasting a minimum of 85 days. The small size of these enclosures allows them to be easily transported, placed in position, and removed for further use. For effective use of such enclosures, it is recommended that unenclosed water and control enclosed water be monitored to identify the appearance of enclosure effects which could, if unidentified, interfere with the interpretation of treatment results. Consequently, for this experiment, concentrations of oxygen, TRP, ammonia and silicon were monitored both inside and outside of enclosures. The higher mean oxygen level recorded in the

enclosed water (Fig. 14A) may have been a result of lower turbidity, lower BOD or higher oxygen production. The mean ammonia levels (Fig. 14C) were found to differ by only 10 $\mu g \cdot L^{-1}$ and it seems unlikely that this difference is biologically significant. Although silicon concentrations (Fig. 14D) were not significantly different, TRP concentrations (Fig. 14B) were considerably higher outside the enclosures. These higher concentrations of TRP may partially be explained by the analytical technique which did not differentiate particulate from dissolved phosphorous. Sediments within the enclosures were much less vulnerable to disturbance by wave action, whereas unenclosed water may have contained a higher percentage of particulates due to sediment distrurbance by wind or fish activity (eg. carp). At another experimental site within the Delta Marsh, photosynthetic rates and algal biomass (chlorophyll *a*) of haptobenthic periphyton have been measured within similar enclosures and in the surrounding water (Goldsborough *et al.*, 1986). In these experiments, mean photosynthetic rates were significantly higher within enclosures (2.7649 and 2.2829 $\mu g.cm^{-2} \cdot hr^{-1}$ respectively, $p=0.0001$), however this difference only became apparent after 44 days of periphyton development. It is suspected that with increased architectural complexity of the periphytic community over time, the loosely attached filamentous green algae and globose masses of blue-green algae would become vulnerable

to detachment through physical and biological disturbances, which were more likely to occur in the unenclosed site.

The artificial substrata used in this experiment proved to have number of advantages over more commonly used glass slides (Weitzel et al, 1979; Hoagland, 1983; Herman et al., 1986; Hamilton et al., 1987), coverslips (Meier et al., 1983), acetate strips (Hooper-Reid and Robinson, 1978; Shamess et al., 1985) or natural substrata. Extruded acrylic rods are relatively inexpensive (ca. $0.40 Can./meter) and can be positioned vertically into the sediment negating the need for additional support. By prescoring the rods, specific areas along the entire length of the rod can be sampled with minimal disturbance of the algae. The diameter of the rods makes them convenient for incubation in productivity experiments without the need for scraping the substratum and risking cell rupture and disruption of the structural integrity of the community. This is essential since the complex architecture of the periphytic community would seem likely to differentially influence carbon assimilation rates depending upon the amount of shading and the amount of water movement occurring within the community. Acrylic rods easily dissolve in xylene based scintillation fluors, again eliminating the need for scraping periphyton from the substrata. The acrylic rods do not, of course, exactly simulate natural substrata and some differences in

community composition may be expected (eg. Shamess et al, 1985).

In this study the degree of triazine toxicity appears to be at least partially dependent on the spatial location of the algal community. Productivity of the herpobenthic community living within the sediment (Fig. 2b) was inhibited to a much greater extent than the haptobenthic community sampled from the upper portion of the artifical substrata positioned within the water column (Fig. 2a). The mean assimilation rates of the haptobenthic communities in the simazine and terbutryn treatments were 130% and 99% (respectively) of that measured for the control. In contrast, the mean assimilation rates of the herpobenthic communities in the simazine and terbutryn treatments were only 34% and 27% (respectively) of that recorded for the control. It appears that the treatment response and recovery rates are related to the physical characteristics of the triazine herbicides. Triazine herbicides are known to readily absorb to organic matter (Nicholls et al., 1984), therefore it was not unexpected to see a very fast initial disappearance of simazine within the water column, during the first week (Fig. 9). The rates of simazine disappearance from the water column became much slower after day 11. This pattern of herbicide loss was similar to rates of terbutryn loss from farm ponds (Muir et al., 1981). In their study,

both terbutryn and its degradation products were detectable in the sediments 61 weeks after treatment. At an application rate of 100 $\mu g \cdot L^{-1}$, residue concentrations reached a maximum of 1.4 $\mu g \cdot g^{-1}$ 84 days after treatment. Another study monitored the persistence of simazine applied to irrigation ditches. Three years following initial application, aproximently 50 % of the simazine could still be accounted for in the soil of the ditch sides and bottoms (Smith et al., 1975). Glotfelty et al. (1984) found adsorption constants for simazine on high organic bottom sediments to be linear over a 0.01 - 1.0 $mg \cdot L^{-1}$ concentration range, with these values being generally higher than values normally reported for soil. The concentration of simazine applied to ponds water in Missouri was found to be directly related to the concentration ending up in the sediments (Mauck et al., 1976). Although these concentrations declined shortly after application, residues of 0.16 $\mu g \cdot g^{-1}$ were still present in the sediments 456 days after a 3.0 $\mu g \cdot mL^{-1}$ treatment. Higher concentrations of simazine were found in the water column after a strong wind, suggesting that the herbicide can quite easily disassociate with the sediment with any physical disturbance. Based on the results of these studies it seems probable that adsorption of the herbicide to the rich organic sediments of the Delta Marsh may have resulted in a much greater inhibition to the photosynthetic herpobenthic community living within it. It is recognized that walls of

the PVC enclosure may have adsorbed some of the herbicide, and this emphasizes the importance of determining actual versus predicted herbicide concentrations. It is hypothesized that the levels of herbicide in the sediment remained high enough to inhibit primary productivity within this community, throughout the experimental period. In addition, loss of dissolved herbicide to the sediment may have permitted the re-establishment of the haptobenthic community colonizing the acrylic substrata.

The accelerated recovery of productivity rates (Fig. 2a) and elevated pigment levels (Fig. 3) of the herbicide treated haptobenthic communities after day 30 beyond control values appeared to be related to the concentration of dissolved nutrients. Within days of herbicide treatment, very high concentrations of dissolved ammonia and total reactive phosphorous (TRP) were recorded (Fig. 5, 6). Previous studies have correlated herbicide treatment with a number of secondary effects. Documented increases in ammonia (Walker, 1964), phosphate and nitrate (Anderson, 1981; Murphy et al., 1981;) have been attributed solely to the decomposition of vegetation. Since no macrophytes were present in our enclosures, the nutrient pulse cannot be attributed to macrophyte death and decomposition. Dissolved oxygen levels (Fig. 4) measured within the water column were low during periods of high ammonia and phosphorous

concentration (Fig. 5, 6). These high ammonia levels were likely contributing to the low oxygen conditions through ammonia oxidation. Other researchers have also reported low oxygen conditions after herbicide application (eg. Murphy et al., 1981; Wingfield and Johnson, 1981), and it seems probable that these low oxygen conditions may have been stimulating nutrient release from the sediments (Mortimer 1941, 1942). Goldsborough & Robinson (1983) proposed that increased nutrient levels after triazine treatment may be associated with the inhibition of the herpobenthic epipelic algae which normally intercept nutrients as they flux from the sediments. Our study supports this hypothesis, as herpobenthic algae remained inhibited throughout the experimental period. In a shallow clearwater lake, it was demonstrated that a benthic algal community was capable of preventing the flux of NH4-N from the sediment to the overlying lake water (Jansson, 1980). The results of our study suggest that the increased nutrient levels recorded after herbicide treatment were in response to both low oxygen conditions and decelerated use of nutrients by the photosynthetically inhibited herpobenthic community.

After day 30, the treated haptobenthic algae appeared to be no longer inhibited (Fig 2a), and consequently were likely able to take advantage of the increased supply of available nutrients in the water column. The dramatic

decrease in ammonia and TRP levels in the treated enclosures after day 25 (Fig. 5, 6), seems to be explained by the nutrient demand of the algae in these enclosures. The apparent utilization of these additional nutrients by the algae suggests that algae in the Delta Marsh are, to some degree, nutrient limited. Once these nutrient levels returned to the levels recorded for the control, carbon assimilation rates (Fig. 2A) and chlorophyll a content (Fig. 3) also declined rapidly. Increased nutrient levels after day 35. once again was followed by an increased rate of production within this community. Peak TRP and ammonia concentrations and peak carbon assimilation rates were separated by a three week lag period. Component scores from a TRP/NH_3 principal components analysis regressed against carbon assimilation rates, incorporating the three week delay. seemed to verify this well for simazine treatment (Fig. 7A) but less well for terbutryn treatment (Fig. 7B). The poorer relationship found for terbutryn treatment was related to the high carbon assimilation value on day 28. It is possible that the community at that time was responding to the increased nutrient supply which occurred after day 7, with a shorter lag phase.

Although very similar increased nutrient levels were recorded in the herpobenthic treated enclosures, no obvious accelerated carbon assimilation rates were measured within

this community. Nutrient limitation within the marsh sediment may not have been present or more likely, herbicide levels may still have been inhibiting the herpobenthos. The similar fluctuating trend of TRP and ammonia is likely attributable to the utilization of these nutrients by phytoplankton, and periphyton growing attached to the enclosure walls and the sediment sampling tubes.

Within the haptobenthic enclosures, terbutryn treatment resulted in higher mean levels of ammonia and TRP than did the simazine treatment, however productivity rate recovery was higher in the simazine treatments. It is possible that the inhibitory level of the herbicides was higher within the terbutryn enclosure and the algal community was therefore unable to take full advantage of the increased supply of nutrients.

Goldsborough and Robinson (1983) report that after one week of exposure, a 1.0 $mg \cdot L^{-1}$ simazine treatment to haptobenthic periphyton resulted in a reduction in chlorophyll *a* to 10% of that in the control, and carbon assimilation rates were reduced to less than 5% of the control. In our experiment, after two weeks of exposure, a level at approximently 1.0 $mg \cdot L^{-1}$ simazine in the haptobenthic enclosure did not appear to inhibit carbon assimilation or chlorophyll *a* content when compared to the control. The difference in these results may be related to

the variation in exposure time between the two experiments. The extra week of exposure time in this study may have given the community (primarily unicellular Chlorophytes) time to adapt to the presence of the toxicant. Goldsborough and Robinson (1987) have found that periphyton can develop resistance towards simazine within seven days at exposure levels over 0.8 $mg \cdot L^{-1}$. Successional changes towards more resistant species has also been documented in lake phytoplankton, exposed to atrazine treatment (deNoyelles et al., 1982). In addition, the higher nutrient levels in the our study, may have contributed to this faster recovery.

Silicon concentrations in the enclosure water did not follow trends observed for ammonia and TRP. Both the untreated (Fig. 8A) and treated enclosures (Fig. 8B, C) demonstrated dramatic fluctuations over the length of the experiment. Fluctuations in these levels are likely related to not only the density of haptobenthic and herpobenthic diatoms (Fig. 12) but also to the density of diatoms in the phytoplankton community which was not monitored in this study..

Although herbicide exposure had only a short term inhibitory influence on haptobenthic carbon asssimilation rates and chlorophyll a accumulation, its influence on algal biovolume and species compositon was longer lasting. Total cell numbers over the duration of the experiment (Fig. 10)

indicate that herbicide exposure resulted in only slightly lower numbers up until day 63. On the last sample date (day 84) haptobenthic cell numbers in the simazine treated enclosure were slightly higher than the control, while the terbutryn enclosure had substantially higher numbers. Due to the size variation which exists amongst algal species, a more meaningful indication of herbicide influence on community biomass can be seen by looking at biovolumes (Fig. 11). Herbicide exposure was found to greatly reduce the total biovolume throughout the experiment in both treatments. The variation between the cell number and biovolume trends, in the later part of the experiment, is explained by the differences in the species compositon between treated and untreated haptobenthic samples. The smaller taxa in the treated enclosures had much higher carbon assimilation rates (Fig. 2A) following high nutrient exposure, but much lower biovolumes (Fig. 11). Community doubling times were calculated on day 42 in each treatment. At this time, the biovolume in the control was dominated by large filamentous Chlorophytes (98.7%), in particular Oedogoniales which made up 48% of the community biovolume. Doubling time for this group was 477 hours. On this same sampling day, the simazine treatment was dominated by smaller Chlorophytes (93.9%), primarily unicellular coccoid greens which made up 87% of the total biovolume. Community doubling time was 23.2 hours. The terbutryn treatment had a

similar community structure with 73% of the biovolume represented by Chlorophytes, 72% of which consisted of unicellular coccoid greens. An extremely fast doubling time of 6.91 hrs was calculated for these organisms. After day 42, the biovolume of the control enclosure was found to be dominated by relatively large Chlorophytes (eg. Chaetophora sp., Oedogonium sp.), whereas the simazine and terbutryn enclosures were dominated by small diatom species (eg. Cocconeis placentula Ehr., Epithemia adnata (Kutz.)Breb., Nitzschia palea (Kutz.)W.Sm., Navicula twymaniana Arch.).

Figure 12 presents the relative percentage of three algal divisions over the sampling period in the control and treated enclosures. Cyanophytes were found to be present in the control during the first half of the experiment, but did not reach significant biovolumes in the terbutryn enclosure until the end of the experiment. These biovolume data indicate that the simazine treatment presented conditions more favorable to diatom species than to chlorophytes, and the terbutryn treatment emphasized this trend to an even greater extent. The results of nonmetric multidimensional scaling also suggests the the composition and abundance of both herbicide treated haptobenthic communities changed dramatically after treatment and remained distinctly different throughout an 84 day period (Fig. 13). At another study site within the Delta Marsh, a short term herbicide

exposure was shown to have a long lasting influence on species composition (Goldsborough and Robinson, 1986). In that study, filamentous green algae were also found to be inhibited to a greater extent than other taxa, whereas diatoms, particularly C. placentula, dominated the simazine and terbutryn treated enclosures. They found a blue green alga of the order Chamaesiphonales also made up a large proportion of the total biovolume in the highest terbutryn treatments. A study investigating the impact of atrazine on lake periphyton also found a shift from a chlorophyte to a diatom dominated community (Hamilton et al., 1987). Herman et al. (1986) found that the major impact of atrazine treatment on lake periphyton was to reduce the biomass of the Chlorophyta, however Scenedesmus acutiformis was affected the least of all species examined. The results of our study support the general trend of chlorophytes being the most susceptible group to triazine exposure, however it does appear that individual species within this group may be tolerant even at high herbicide concentrations.

The short term inhibition of productivity, the changes in the chemical composition of the water, and the long term species compositional changes may all contribute to a significant impact on other biological components of the marsh ecosystem. Shifts in insect species composition and emergence times were found to occur in ponds treated with

2.0 $mg \cdot L^{-1}$ atrazine (Dewey, 1986). Atrazine treatment affected the insect community indirectly by reduction of the food source (periphyton, macrophytes) and to some extent their habitat (macrophytes).

2.6 CONCLUSIONS

The results of this study suggest that the biological impact of triazine treatment is complex, and it is not possible to evaluate toxicological effects by measuring physiological or community structural responses alone. The results also demonstrate that algal communities may respond differently, depending on their spatial location within the treatment area. Although the inhibited functional responses of the haptobenthic algae recovered shortly after treatment, this recovery proved to be short lived as the community appeared to be responding to enhanced nutrient conditions resulting from secondary effects of the treatment. The primary and secondary effects resulting from herbicide exposure were very similar for the methylthio-triazine terbutryn treatment and the chloro-triazine simazine treatment, despite the large differences in the application concentrations. An extended monitoring period is needed to evaluate if the communities are eventually able to functionally and structurally recover to what is observed under untreated conditions. It is also essential to assess

what impact these changes have on other biological components of the ecosystem.

CHAPTER 3

THE APPLICATION OF AN AUTOMATED GRAIN DENSITY MICROAUTORADIOGRAPHY PROCEDURE TO THE STUDY OF ALGAL PRODUCTIVITY

3.1 ABSTRACT

This investigation describes an automated quantitative grain density microautoradiogrphy procedure. The effects of Lugol's iodine preservative on isotope leakage from algal cells was examined. The quantity of label lost was found to range between 20.5% in a large diatom species to 56.4% in the a small diatom species. The applicability of using this technique to evaluate intra and interspecific variation in algal response to triazine herbicide exposure *in-situ* is discussed.

3.2 INTRODUCTION

In the investigation of factors influencing algal productivity, most research has reported whole community or whole population responses, giving us little information on the differential responses of individual species. Unlike conventional scintillation counting methods for the assay of radioactive isotopes, which can only measure incorporation into whole populations and communities, microautoradiography allows the study of the incorporation of some radioisotopes into individual cells. Both autoradiography and pulse

counting techniques are based on the release of ionizing radiation from the isotope, however the detection is quite different. Scintillation counting involves the absorption of radiation by a scintillator, with the amount of fluorescence by the scintillator relating directly to the amount of radioactivity in the sample. In contrast, autoradiographic techniques detect radiation through the use of photographic emulsions. The radioactive emissions recorded by the creation of latent images amoungst the silver grains of photographic emulsion, can be used to quantify radioisotope incorporation. As a result, autoradiography can be used to evaluate the relative contribution of each individual to community assimilation.

Both nuclear track autoradiography (NTA) and grain density autoradiography (GDA) have been used in limnological studies. These techniques involve the use of photographic emulsions, although in the case of NTA a thicker and usually more sensitive emulsion is applied. Consequently, in NTA, emissions from low energy beta particles produce a track of silver grains originating from the labelled cell. Since these emissions may produce tracks in any plane, tracks are located by focussing through the entire thickness of the emulsion. The number of tracks counted can then be used to determine the amount of label assimilated (Knoechel and Kalff, 1976a):

$$DPM(C) = \frac{T \times 2.0}{M \times 0.86 \times 0.98 \times 0.01y} \quad (1)$$

where: DPM(C) is the dpm·cell^{-1}; T is the number of tracks recorded per cell, with each track consisting of at least 4 grains arising from within 5 µm of the cell (Knoechel and Kalff, 1976a); 2.0 corrects for beta emissions which are directed downward into the glass slide (which supports the labelled cells) and therefore cannot create a track; M is the emulsion exposure time (minutes); 0.86 is the correction factor applied when using C^{14} to account for beta particles with insufficient energy to create a track (Levi and Rogers, 1963); 0.98 corrects for latent image erasure occurring during exposure (Knoechel and Kalff, 1976a); and 0.01y corrects for the internal absorption of beta particles by individual cells, where y=100.0-0.98x, and x is the mean cell dimension in µm (Knoechel and Kalff, 1976b). The derived dpm·cell^{-1} data can then be used to calculate actual assimilation rates as per scintillation counting. Grain density autoradiography follows a similar principal, however when using this technique, a very thin layer of nuclear emulsion is applied to the slide. As a result, beta emmissions arising from the labelled cells are recorded as individual grains as opposed to tracks. The thinner the emulsion, the closer is the correspondence between the number of grains and the number of beta emissions. Using this technique dpm·cell^{-1} are derived as in equation 1

excluding the 2.0 and 0.86 correction factors which are not required in this calculation.

Although GDA has been utilized quite extensively, concern has arisen over its use as a quantifiable method of estimating isotope assimilation. Knoechel and Kalff (1976b) argue that some GDA users have not accounted for self-absorption nor latent image erasure. They also argue that error arises from not including grain counts from a large enough radius around the cell, and from not correcting for background grain production resulting from heat, pressure, chemography or from dark assimilation. In defence of the use of quantitative GDA, Paerl and Stull (1979) report that a high correlation between GDA and NTA can be achieved. Davenport and Maquire (1984) also report that GDA can be quantifiable if self-absorption, latent image erasure and cell geometry are taken into consideration. They suggest that GDA may be a more favorable technique since it can provide a better measure of intraspecific distribution of productivity rates because of its ability to show a larger response gradient. It would appear that both methods can be used successfully to measure isotope incorporation provided the appropriate correction factors are applied.

Some of the advantages and disadvantages associated with both techniques are as follows. Considering material costs and time investment, GDA is favored as a smaller

quantity of emulsion is required, and emulsion drying times and developing times are considerably shorter. The thinner emulsion used in GDA also provides better resolution of labelled cells for taxonomic identification. Since only single grain production as opposed to track production is required, a wider range of low energy isotopes can be used in GDA (eg. H_3). As discussed by Davenport and Maguire (1984), GDA is particularly advantageous when investigating intraspecific variation of primary production, since a wider optimum labelling range can be used. With NTA a larger range of exposure times or incubation periods must be used to evaluate cells which have wide ranges of metabolic rates, since the optimum number of tracks per cell is under three (Knoechel and Kalff, 1979). The time consuming process of recording tracks or grains, and the uncertain accuracy involved in manual counting is a serious disadvantage associated with both techniques. However, because GDA results in grain production in a single focal plane, automated counting devices can be employed to reduce the time required to count grains and it may quite likely reduce counting error. One of the major advantages of using NTA, is that this technique does not require a correction for background grain production or chemographic grain production since it is only necessary to count the number of tracks and not the total number of grains. When using GDA, control

unlabelled cells or dark incubated cells should be used to correct for background grain production.

Both of these techniques have been used in aquatic biology primarily in the study of phytoplankton and bacteria, however it would appear that the technique has more widespead applicability. To date, researchers have used autoradiography to evaluate inorganic carbon assimilation rates of phytoplankton (Maquire and Neill, 1971; Watt, 1971; Gutel'makher, 1973; Stull et al., 1973; Paerl, 1977, 1978a; Descolas-Gros, 1980; Ogawa and Ichimura, 1981), and their heterotrophic potential (Saunders, 1972). Knoechel and deNoyelles (1980) have used NTA to determine the influence of light and phosphorous enrichment on the carbon fixation rates of hypolimnetic phytoplankton. GDA has been used by Friebele et al. (1978) to investigate the relationship between phytoplankton size and the rate of phosphate uptake. GDA has also been utilized quite extensively to examine bacterial heterotrophic activity (Paerl, 1974; Hoppe, 1976; Paerl, 1978b; Chocair and Albright, 1980; Novitsky, 1983; Marcussen et al., 1984), and Paerl (1983) has used GDA to determine the partitioning of CO_2 in a colonial cyanobacterium as it relates to the promotion of surface scums. Autoradiography has only recently been utilized for periphyton research. Pip and Robinson (1982a, 1982b) used NTA to analyse the dynamics of individual epiphytic

periphyton productivity by quantifying both inorganic and organic carbon assimilation rates. Carbon assimilation rates of epiphytic algae, as they relate to cell surface area, has been investigated using this same technique (Robinson and Pip, 1983). The species specific productivity rates of epilithic periphyton has also been examined using NTA (Duthie and Hamilton, 1983). To date, only two investigations have used autoradiography in toxicological research. Bruno et al. (1982) used GDA to monitor the uptake and biotransformation of benzo(a)pyrene in periphytic algal communities, and Hamilton et al. (1987) have used NTA to assess the impact of atrazine on the productivity rates of lake periphyton.

One of the problems associated with the use of autoradiography has been the differential influence of algal preservatives on both the leakage of isotope from labelled cells, and their promotion of chemography. Silver and Davoll (1978) were first to report substantial losses in the activity of cells treated with some commonly used preservatives. Subsequent to that research being published, conflicting evidence on the influence of these preservatives had been presented (Rogers, 1979; Holtby and Knoechel, 1981; Smith and Kalff, 1983; Davenport and Maguire, 1984; Paerl, 1984). Consequently, the development of non-preservation techniques (eg. Paerl, 1984) will be advantageous to the

futher use of microautoradiography in limnological investigations.

The intents of this study are to firstly demonstrate the use of an automated quantitative grain density microautoradiography procedure, and secondly, to evaluate the effects of Lugol's iodine preservative on cell leakage in such a procedure. This paper also discusses the applicability of the technique in in-situ algal toxicity studies.

3.3 METHODS

In the first portion of this study, an experiment was conducted to evaluate the relationship between disintegration rates calculated from scintillation counting, and grain production recorded from grain density microautoradiography. Unialgal cultures of Nitzschia sp., Synedra sp., Chorella sp. and Chlamydomonas sp. were grown in 500 mL of modified WC medium (Guillard and Lorenzen, 1972) with a sterile aeration system. Light intensity and culture temperatures were maintained at 92 $\mu E \cdot m^{-2} \cdot s^{-1}$ and 16°C. Cultures in exponential growth phase were thoroughly mixed and 12-25 mL samples were dispensed into culture tubes and incubated with 1.0 mL of $NaH^{14}CO_3$ corresponding to an activity of 1,744,328 dpm. For each species, incubation periods of 30, 60 and 120 minutes were used under the above

growth conditions. At the end of each incubation period, one 25 mL sample was preserved with Lugol's iodine and three samples were used as replicates for scintillation counting. Labelled cells for scintillation counting were filtered onto 0.45 µm cellulose nitrate membrane filters, rinsed with deionized water, fumed for one minute over concentrated HCl, then placed into 10.0 mL of Scinti verse (Fisher Scientific) scintillation fluor. After 24 hours, sample activity was determined using a Picker Liquimat 220 counter. Colour quenching was corrected by using the channels ratio method (Wang and Willis, 1965). Counting efficiency was 74-90%. Cell densities of each culture were determined using a Coulter Counter (Model B) and data were expressed as $dpm \cdot cell^{-1}$

After a brief fixation period, labelled cells preserved for autoradiography were gently washed six times with 25 mL of deionized water using a syringe as a settling chamber. The washing, conducted over a three day period was intended to remove the unincorporated isotope and prevent chemographic grain production from the acidic Lugol's preservative. Following the sixth rinse, the cells were washed with a 100 $mg \cdot L^{-1}$ gelatin (Knoechel and Kalff, 1976a) solution to facilitate the adhesion of cells onto glass slides. Labelled cells were sedimented onto one half of a number of slides and control cells (preserved unlabelled

cells) onto the other to permit background correction to be made for each slide. Each slide was inscribed using a lead pencil so that they could be identified easily after the developing procedures were complete. Slides with frosted ends were used so that the upper surface of the slide could be distinguished from the bottom surface when working in the darkroom.

Sedimented slides were coated in 38°C NTB-2 (Kodak) nuclear emulsion diluted 1:1 with filtered deionized distilled water (Paerl, 1978a, 1984). Dipped slides were quickly placed on ice cooled enamel trays for one hour to facilitate the gelling and even distribution of the emulsion on the slide (Knoechel and Kalff, 1976a). Gelled slides were removed from the ice trays and were left to dry for an additional hour before being placed in lightproof boxes containing silica gel desiccant. A Wratten #2 safelight was used during emulsion mixing, with all other steps being conducted in complete darkness. All emulsion coated slides were stored at 5°C to minimize fading of the latent image and to reduce bacterial growth. The exposure periods for the four species ranged from 46 to 69 hours.

Exposed autoradiographs were placed in acrylic holders and developed in full strength Kodak D-19 developer for three minutes, followed by two minutes in a 1% acetic acid stop bath and a five minute fixation in both 30% and 10%

sodium thiosulphate. Slides were twice rinsed in deionized water for a total of 15 minutes. All solutions were prefiltered and brought to room temperature before use.

Developed slides were air dried and viewed at 400X magnification using a Leitz Diavert phase contrast microscope. Grain production was scored within an approximate 10 μm radius of the cell boundaries using a Bioquant II (R&M Biometrics, Nashville, Tn.) microdensitometry image analysis system. Using this system, cell images with surrounding grains were displayed on a computer monitor via a TV monitor. An ancillory digitizing tablet (Hipad) was used to delineate the area to be counted. For each slide viewed, 50 labelled cells and 50 control cells were counted, and dpm·cell^{-1} was derived as follows:

$$DPM(C) = \frac{N - C}{T \times 0.98 \times 0.01y} \qquad (2)$$

where: DPM(C) is the corrected dpm·cell^{-1}, N is the grains count for the labelled cells, C is the mean number of grains for the control cells, T is the time of emulsion exposure (minutes), 0.98 corrects for latent image erasure occurring during exposure (Knoechel and Kalff, 1976a), and y corrects for internal absorption of the grains within the cell. For the calculation of y, we used the relationship derived by Knoechel and Kalff (1976b) from Hendler's (1959) data:

$$y = 100 - 0.98x \quad (3)$$

where: y is the percent efficiency of track counts and x is the mean cell dimension (μm). Two comparisons of dpm·cell^{-1} determined by GDA (using the Bioquant II) and dpm·cell^{-1} determined by scintillation counting were conducted for each species.

In-situ simazine and terbutryn treated haptobenthic algae were used to evaluate the applicability of this grain density technique in the elucidation of differential toxicological responses. The study site was located in the Blind Channel of the Delta Marsh on the southern end of Lake Manitoba, Canada (99°W 19′W, 50° 7′N). Enclosures containing colonized acrylic substrata, were treated with triazine herbicides. One enclosure was used as a control receiving no herbicide treatment. Of the remaining two enclosures herbicide enclosures, one received 2.0 mg·L^{-1} simazine [2-chloro-4,6-bis(ethylamino)-s-triazine] and the other was treated with 0.01 mg·L^{-1} terbutryn [2-(tert -butylamino)-4-(ethyla mino)-6-(me thylthio)-s -triazine] . Herbicide dissolution occurred after six days of periphytic community development on artifical substrata.

Algal cells used for GDA determinations were collected from segments of acrylic substrata located at 10-20 cm water depth within each of the three treatment enclosures (Chapter 2). From each treatment on each of three sample dates

(04/06/84, 11/06/84 and 09/07/84), three rod segments were randomly sampled, of which two were used for C^{14} light assimilation measurements and one used to correct for dark assimilation. Each algal sample was placed in a culture tube containing 25 mL of enclosure water twice prefiltered (Whatman GF/C and Sartorius 0.45 µm cellulose nitrate) and transported back to the laboratory. Samples were inoculated with 1.0 mL of standardized $NAH^{14}CO_3$ and incubated in a growth chamber for two or four hours at a light intensity of 168 $\mu E \cdot m^{-2} \cdot s^{-1}$ and a temperature of 22°C. Following incubation, cells were detached from the acrylic substratum using a fine paint brush and a rubber policeman. Cells were then vacuum filtered through 0.45 µm cellulose nitrate filters, washed with deionized water, then resuspended into 20 mL of deionized using a fine paint brush. The resuspended cells were again filtered through a new 0.45 µm filter, rinsed, then fumed for one minute over concentrated HCL to remove residual inorganic C^{14}. Each of the filters supporting the unpreserved labelled cells was then inverted onto one end of a clean microscope slide. Using a fine paint brush, cells were transferred from the filter paper to the slide. The corresponding dark incubated samples were also prepared as above and transferred to the other end of the same slide. The inclusion of dark and light incubated cells on a single slide assured that both were subjected to identical treatment and permitted grain counts to be

corrected for dark assimilation and background grain density within a single treatment. A number of slides from each treatment, for both incubation times were prepared so that autoradiograph exposure times, and subsequent grain density could be varied. Slides containing the dried labelled algal cells were placed in dust-proof boxes until the nuclear emulsion was applied. The emulsion coating and developing conditions were as described above. All slides prepared from a single sampling period were emulsion coated on the same day to maintain consistency in emulsion thickness. The Bioquant II system was again used to count grain production and it was also employed to measure cell dimensions for the determination of cell volumes. Algal biovolumes were determined from the measurement of several cells , with calculations based on the closest geometric solid. Inorganic carbon assimilation rates per cell ($\mu g\ C \cdot cell^{-1} \cdot hr^{-1}$) and per um^3 ($\mu g\ C \cdot um^{-3} \cdot hr^{-1}$) were calculated for each species as follows:

$$\mu g\ C\ fixed \cdot cell^{-1} \cdot hr^{-1} = \frac{DPM(C) \times C \times 1.05}{DPM(T) \times T} \qquad (4)$$

$$\mu g\ C\ fixed \cdot um^{-3} \cdot hr^{-1} = \frac{DPM(C) \times C \times 1.05}{DPM(T) \times V \times T} \qquad (5)$$

where: DPM(C) is the specific activity of the algal cell determined from equation 2, C is the dissolved inorganic carbon present in the incubation water determined from

alkalinity (APHA, 1980), 1.05 is the C^{14} uptake discrimination factor, DPM(T) is the total specific activity of the isotope added, V is the biovolume (μm3) of the cell, and T is the incubation time (hr). Assimilation rates of species in herbicide treated and non-treated enclosures were compared.

During a later sampling period (20/08/84), samples of both light and dark incubated cells were preserved in a (2%) Lugol's iodine solution following a four hour C^{14} incubation period to evaluate the effects of preservation on isotope leakage from labelled cells, by comparison with cells prepared using the non-preserved technique developed during this study. Samples were preserved for 15 days then gently washed six times with 25 mL of deionized water using a syringe as a settling chamber. The washing was conducted over a three day period and was intended to reduce chemographic grain production from the acidic Lugol's preservative. Drops of cell suspensions from light and dark incubations were allowed to dry at opposite ends of clean microscope slides, then stored in dust proof boxes until the nuclear emulsion was applied. The procedure used to apply the emulsion and the developing conditions for both the preserved and non-preserved samples, were as described above. Developing times between 333 and 487.3 hrs were found suitable for grain counting. Three diatom species (Navicula

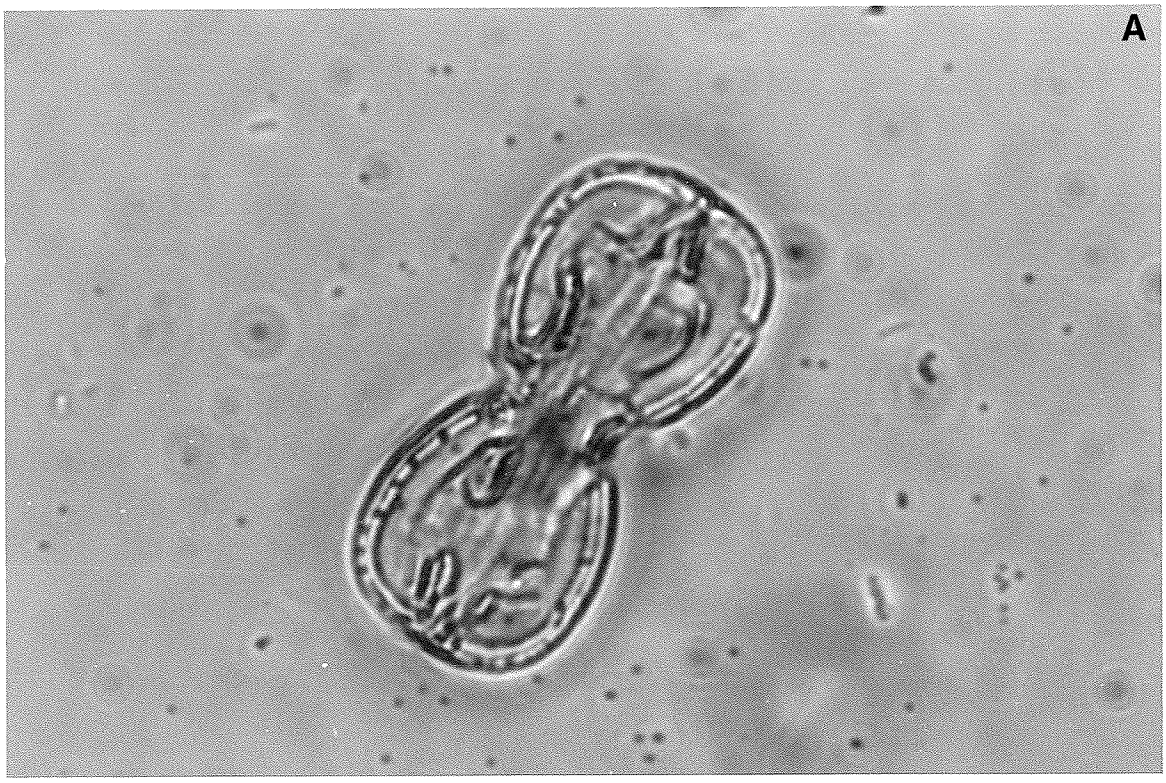
twymaniana, Nitzschia palea and Cocconeis placentula) representing a range of surface area to volume ratios were used to evaluate the the effects of Lugol's iodine preservative on cell leakage and subsquequent loss of the labelled carbon. Between 38 and 66 cells of each species were used to calculate the mean dpm·cell^{-1} as described above (Equation 2). The percent of label lost due to preservation was determined by assuming the non-preserved cells had 100% retention of the label. The relationship between cell volume and loss of label was also examined.

3.3 RESULTS and DISCUSSION

Although autoradiography is a valuable tool in understanding the kinetics of assimilation processes by individual micro-organisms, the amount of time required to complete analysis may partially explain its limited use in limnology. Manual counting of grains is not only extremely time consuming, but it also limits the total number of grains associated with a single cell that can be accurately enumerated. The computerized image analysis system used here, was found to be an efficient and accurate alternative to manual counting. The associated digitizing tablet (Hipad) allows delineation of a cell as well as a 10 µm boundary around the cell. Cell images viewed through a microscope, are transferred via a TV camera to a microcomputer (Fig.

15). Once the area to be counted has been outlined, the image is scanned and the grain count is displayed (Fig. 16). Since the system must be calibrated to particles within a narrow range of densities it cannot distinguish between silver grains and similarly or more dense artifacts. It is therefore necessary to visibly observe the scans to ensure that the correct number of grains have been counted. Where artifacts are present, the hipad can be used to exclude this area from the scan, or alternatively, the counts attributable to this artifact can be subtracted from the total count. For this reason it is important that the autoradiography slides are relatively free of non-cellular artifacts such as sediment or dust. We have found that the double membrane-filtration technique described earlier, effectively eliminates the majority of small artifacts which would normally be found on slides when the more commonly used cell settling technique is used. We have also found that even with phase contrast microscopy, some algal cells are too dense to allow the use of the automated system. For such cells manual counts of the grains overlying the cell must then be added to the grains that were counted in the 10 um radius using the Bioquant II. Despite these limitations, we have found the automated counting system to be an attractive tool for use in autoradiographic investigations.

Figure 15: Grain density autoradiograph of a dark incubated (A) and light incubated (B) Amphiprora cell.


A

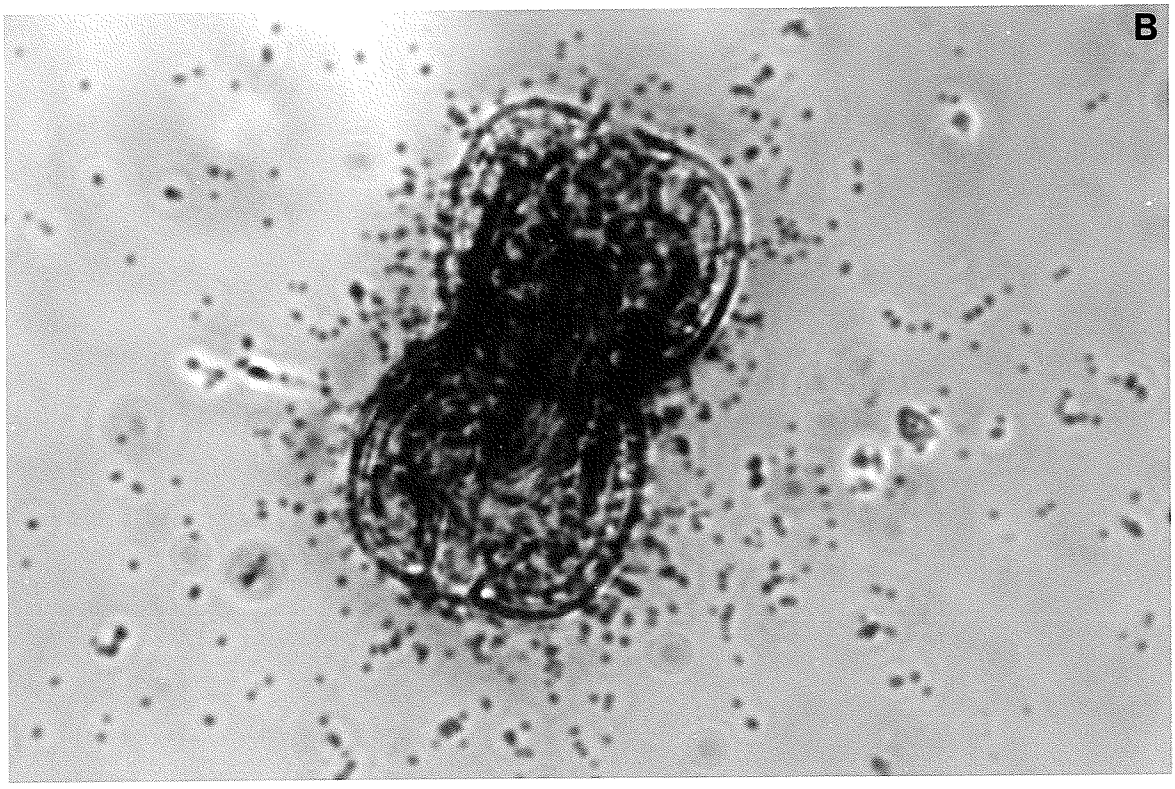
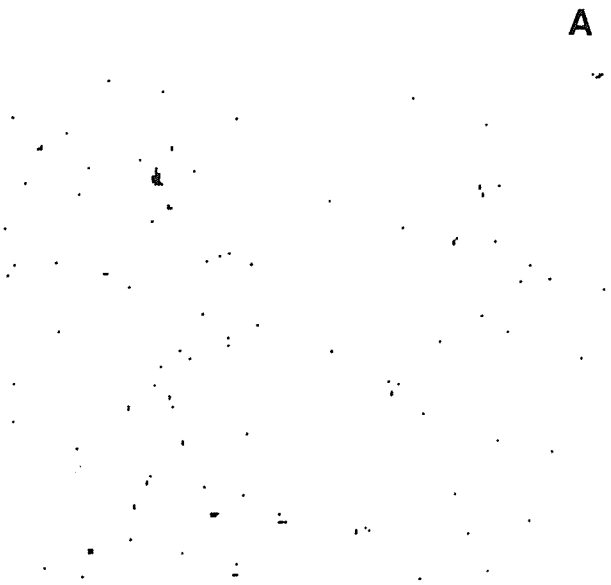

B

Figure 16: Bioquant II grain density image of the dark incubated (A) and light incubated (B) Amphiprora cell shown in Figure 15.


A


B

Table 1 presents dpm·cell^{-1} determined from scintillation counting and dpm·cell^{-1} calculated from GDA. Although the standard deviations of the grain density counts were often high, compared to those recorded from scintillation counting, there were no significant differences between the means of any of the four species. The large standard deviation would appear to be related to differences in intraspecific metabolic rates, which cannot be detected in the liquid scintillation samples. In the batch cultures used in this experiment, cells in lag, stationary or death phase would not be actively incorporating labelled carbon, whereas cells in exponential growth phase would show a greater assimilation rate. For example, intraspecific variability was particularly evident on the Chorella sp. culture, as grain production was found to range between 0 and 300 counts per cell in a 69 hr. exposure period. The effect of the brief exposure to Lugol's preservative on isotope leakage cannot be assessed.

The correlation between dpm·cell^{-1} calculated from scintillation counting and from GDA was found to be high (r^2=0.988). From the regression line (y=1.275x-0.007136) the relationship indicates that one distintegration corresponds to approximently 1.27 grains, indicating that tracks or partial tracks may have formed. Although the results of this experiment suggest that the relationship between

TABLE 1

A comparison of dpm·cell^{-1} calculated from scintillation counting with dpm·cell^{-1} calculated from grain density autoradiography.

Species	Scintillation counting dpm		Grain density counting		
	$\bar{x}$	s	$\bar{x}$	s	p>* (n)
Chlamydomonas sp.	0.389	0.0373	0.455	0.343	.35 (53)
	0.454	0.1004	0.600	0.342	.20 (53)
Chlorella sp.	0.0160	0.0002	0.0239	0.0220	.25 (53)
	0.0160	0.0002	0.0158	0.0226	.45 (52)
Synedra sp.	0.101	0.0166	0.0943	0.0551	.40 (52)
	0.101	0.0166	0.159	0.0861	.10 (53)
Nitzschia sp.	0.0454	0.0027	0.0409	0.0382	.40 (53)
	0.0454	0.0027	0.0447	0.0760	.45 (54)

* t test significance level.

distintegration and grain production is linear, it is advisable to test this relationship for each new experiment, as variation in emulsion thickness would be expected to influence grain production. The use of stripping film as an alternative to liquid emulsion, may eliminate fluctuations in the emulsion thickness. The type and quantity of preservative used may also influence this relationship. The relationship between disintegrations and grain production can be determined as outlined in this study, or by including cells of known specific activity to individual autoradiographs. The average grain production of these cells can then be used to establish the relationship between specific activity and grain production for all cells within the sample.

Although there are conflicting reports of the effects of preservatives on isotope leakage from labelled cells, there appears to be enough information to suggest that non-preservation techniques are advantageous. Substantial loss of filter-retainable radioactive label is reported when cells were preserved in gluteraldehyde, formaldehyde and non-acidified Lugol's iodine, however the magnitude of this loss varied considerably (Silver and Davoll, 1978). Holtby and Knoechel (1981) have reported a 40% loss of C^{14} from phytoplankton three hours after treatment with Lugol's iodine, although Davenport and Maguire (1984) report less

than a 5% loss of the label. A combination of a very brief fixation by Lugol's iodine with collection of cells on polycarbonate filters was found to minimize the amount of tracer leaked (Smith and Kalff, 1983). Other researchers (Descolas-Gros, 1980; Bruno et al., 1982; Pip and Robinson, 1982a, 1982b; Duthie and Hamilton, 1983; Robinson and Pip, 1983; Hamilton et al., 1987) have also used various concentrations of Lugol's iodine as a preservative in autoradiographic studies, making it difficult to compare assimilation rates due to the uncertainty in the quantity of isotope leakage. The results of our study suggest that non-preserved samples can have considerably higher activity than those preserved with Lugol's iodine (Table 2). Of the three haptobenthic diatoms used, the smallest species, Navicula twymaniana had the greatest leakage of isotope (56.4%), whereas the larger diatom, Cocconeis placentula demonstrated the least (20.5%). The relationship between cell volume and amount of leakage was very good (r^2=.999), as was the relationship correlation between % leakage and dpm·μm^3 (r^2=.986), although only three species were used in these correlations. The conflicting results presented in the literature suggest that the amount of leakage resulting from preservation is not a constant and may in fact be influenced by a number of factors. The choice of preservative used, as well as its concentration appear to influence the amount of leakage observed. Filtration or centrifugation may also

TABLE 2

Influence of Lugol's preservative on isotope leakage from three haptobenthic diatoms.

Species	dpm·cell^{-1} Preserved cells $\bar{x}$	s	Non-preserved cells $\bar{x}$	s	p* (n)	volume (μm^3)	% loss
Navicula twymaniana Arch.	0.389	0.393	0.893	0.569	p<.005 (100)	55.9	56.4%
Nitzschia palea (Keutz.)W.Sm.	1.823	1.180	2.755	1.390	p<.005 (86)	301.	33.8%
Cocconeis placentula Ehr.	2.387	1.605	3.008	1.469	p<.025 (131)	445.	20.5%

* t test significance level.

differentially contribute to label loss. The morphological and physiological nature of the algal cells would seem also to be an important consideration. Small delicate cells in poor physical condition would be expected to be most susceptible to leakage, although even the relatively large diatom Cocconeis placentula which has a rigid silica-impregnated cell wall, demonstrated 20% leakage in this study. Due to the inability to predict a consistent correction factor, non-preservation techniques appear to be most acceptable. Early non-preservation techniques involved filtering labelled algal cells onto membrane filters then clearing these filters with exposure to acetone fumes (Watt, 1971; Paerl, 1974; Paerl, 1977; Paerl and Stull, 1979; Ogawa and Ichimura, 1981), or cedarwood oil (Saunders, 1972; Stull et al., 1973). However, as Knoechel and Kalff (1976b) point out, acetone is commonly used to extract algal pigments and therefore one might expect either leakage and or translocation of the label when cells are exposed to this solvent. More recently, Paerl (1984) suggested using a freeze fixation technique that involves immersing the filters containing the labelled cells in liquid nitrogen. C^{14} retention in these samples was higher than samples preserved with 1% glutaraldehyde, 1% ethyl alcohol, 1.5% formaldehyde or with 1% Lugol's iodine.

Our study presents a modified non-preservation procedure which eliminates the acetone fuming step and does not require the use of liquid nitrogen. We have also found that a number of slides supporting both labelled and dark incubated cells can be prepared from a single membrane filtration.

Using this non-preservation technique, autoradiographs of haptobenthic algae sampled from control, simazine and terbutryn treated enclosures were made 1, 8 and 36 days following herbicide application (Table 3, 4). Although identical emulsion coating procedures were followed for slides from all three sample dates, it is recognized that slight differences in emulsion thickness may have occurred. These differences, would however, only influence the absolute carbon assimilation values, and not comparisons made on any given sample day. Due to the large size variation which exists between the species examined, carbon assimilation rates were expressed on a per unit volume basis (ng $C \cdot \mu m^{-3} \cdot hr^{-1}$), as well as on a per cell basis (pg $C \cdot cell^{-1} \cdot hr^{-1}$). These data suggest that both intra- and inter-specific variability can be quantified. Assimilation rates expressed on a biovolume basis, of all treated species examined, demonstrated lower assimilation rates than those in the control one day following herbicide treatment (Table 3). For example, Synedra acus in the simazine and terbutryn

TABLE 3

Species specific carbon assimilation rates* per μm^3 of haptobenthic algae in the control encolsure and in enclosures treated with 2.0 $mg \cdot L^{-1}$ simazine and 0.01 $mg \cdot L^{-1}$ terbutryn.

Species	Days following treatment					
	1		8		36	
	$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE
Synedra acus Keutz.						
control	8.62	1.63	2.42	0.978	-	
simazine	0.0446	0.00714	0.906	0.237	-	
terbutryn	0.0112	0.00261	0.366	0.0676	-	
Synedra ulna (Nitz.)Ehr.						
control	-		0.735	0.144	-	
simazine	0.00899	0.00270	0.5515	0.130	-	
terbutryn	-		0.2869	0.0936	-	
Synedra delicatissima W.Sm.						
control	10.1	1.76	-		-	
simazine	0.112	0.0125	-		-	
terbutryn	0.0286	0.00858	-		-	
Nitzschia palea (Keutz.)W.Sm.						
control	-		5.55	1.45	1.58	0.367
simazine	-		5.59	1.75	5.67	0.519
terbutryn	-		2.06	0.779	3.60	0.3801
Nitzschia acicularis W.Sm.						
control	-		2.79	0.533	-	
simazine	0.150	0.0160	5.28	1.29	-	
terbutryn	-		3.34	0.694	-	
Cyclotella meneghenlana Keutz.						
control	0.463	0.115	0.311	0.0492	-	
simazine	0.0329	0.00680	0.151	0.0548	-	
terbutryn	0.00347	0.000758	0.271	0.0502	-	
Coccoid Chlorophytes						
control	1.17	0.130	1.62	0.691	-	
simazine	0.120	0.0367	0.0760	0.0112	0.497	0.128
terbutryn	0.258	0.122	0.3185	0.0651	0.641	0.110
Anacystis sp.						
control	2.066	0.780	0.619	0.207	-	
simazine	0.0825	0.0204	0.454	0.0983	-	
terbutryn	0.0154	0.00416	2.94	0.506	0.459	0.118

* Carbon assimilation rates and standard errors in nanograms of carbon per μm^3 per hour.

TABLE 4

Species specific carbon assimilation rates* per cell for haptobenthic algae in the control enclosure and in enclosures treated with 2.0 $mg \cdot L^{-1}$ simazine and 0.01 $mg \cdot L^{-1}$ terbutryn treatments

Species	Days following treatment					
	1		8		36	
	$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE
Synedra acus Keutz.						
control	11.72	2.417	4.545	1.838	-	
simazine	0.0844	0.0135	1.158	0.3030	-	
terbutryn	0.0161	0.00376	1.085	0.2006	-	
Synedra ulna (Nitz.)Ehr.						
control	-		9.752	1.906	-	
simazine	0.1539	0.0462	7.599	1.791	-	
terbutryn	-		8.921	2.910	-	
Synedra delicatissima W.Sm.						
control	14.479	2.531	-		-	
simazine	0.1242	0.01384	-		-	
terbutryn	0.0280	0.00839	-		-	
Nitzschia palea (Keutz.)W.Sm.						
control	-		0.7757	0.2030	0.2235	0.0517
simazine	-		1.587	0.4974	1.226	0.1121
terbutryn	-		0.7106	0.2693	1.143	0.1207
Nitzschia acicularis W.Sm.						
control	-		0.6551	0.1250	-	
simazine	0.02508	0.00268	0.9952	0.2424	-	
terbutryn	-		3.960	0.8225	-	
Cyclotella meneghenlana Keutz.						
control	0.9818	0.2428	0.2560	0.04046	-	
simazine	0.01873	0.003875	0.1294	0.04682	-	
terbutryn	0.00311	0.000681	0.3967	0.07353	-	
Coccoid Chlorophytes						
control	2.368	0.2636	2.170	0.9927	-	
simazine	0.08876	0.00272	0.1351	0.01997	0.6857	0.1759
terbutryn	0.3047	0.1439	0.6440	0.1316	1.724	0.296
Anacystis sp.						
control	0.4974	0.1739	0.03791	0.01269	-	
simazine	0.00398	0.000986	0.03081	0.006681	-	
terbutryn	0.000902	0.000243	0.1549	0.02670	0.07712	0.01983

* Carbon assimilation rates and standard errors in picograms of carbon per cell per hour.

enclosures had assimilation rates which were 0.5% and 0.1% of that measured in the control. Using this technique it is possible to follow the recovery of individual species over time. In this experiment by day 8, assimilation rates measured for this same species had increased to 37% of the control in the simazine treatment and to 15% in the terbutryn. Synedа ulna also demonstrated lower rates in the treated enclosures 8 days after treatment, however the magnitude of this inhibition was less than for S. acus. Nitzschia palea which was not present in sufficient numbers to score on day 1, was inhibited only by the terbutryn treatment 8 days after treatment. This species had however, much higher assimilation rates in both treated enclosures 36 days after application. This result may be related to the higher nutrient concentrations in the herbicide enclosures, which appeared to be stimulating community productivity. (Chapter 2). Nitzschia acicularis did not appear to be inhibited by either herbicide 8 days after treatment, however 28 days later, this species was not found in either treatment, and cell densities in the control enclosure were very low. The centric diatom Cyclotella meneghenіana also appeared to be recovering 8 days following treatment, it too, however, was absent from herbicide treated samples collected 28 days later. Coccoid Chlorophytes showed similar inhibition both 1 and 8 days following treatment. Although

insufficient numbers of control cells could be found to estimate assimilation rates, the herbicide treated species were shown to have much higher assimilation rates on day 36 than measured 28 days earlier. A small blue green alga, Anacystis sp. also demonstrated inhibition directly after herbicide treatment, although by the day 8, the assimilation rate of this species in the simazine enclosure was 73% of that measured in the control, and terbutryn exposed cells had assimilation rates 4.75 x higher. The species specific carbon assimilation rates expressed on a per cell basis (Table 4) are perhaps not as informative as those based on biovolume (Table 3), although they do contain considerably less error due to the difficulty involved in accurately calculating cell volumes. The largest species demonstrated the highest assimilation rates. As a result, Anacystis sp. appears to have a low assimilation rate, whereas on a per um^3 basis this species was often more productive than others.

Hamilton et al (1987) have used nuclear track autoradiography (NTA) to evaluate the effects of atrazine on lake periphyton. They found that NTA could also be used to evaluate responses of periphyton exposed to a photosynthetic inhibitor. In their study the productivity of the larger filamentous algae, specifically Stigeoclonium sp., Spirogyra sp., Mougeotia sp. and Oedogonium sp. were most affected. In

general, triazine herbicides have been shown to be most phytotoxic to green algae (Hawxby et al., 1977; Goldsborough and Robinson, 1986; Herman et al., 1986; Hamilton et al., 1987, however a number of studies have identified species within this group which appear to be tolerant or possibly resistant (Vance and Smith, 1969; deNoyelles et al., 1982; Galloway and Mets, 1984; Hamilton et al, 1987). Triazine resistance in species such as corn, sorghum and sugarcane is attributable to rapid metabolism of the herbicide. Resistance in some other species is believed to involve a modification in a protein at the herbicide binding site at photosystem II which reduces triazine binding affinity (Arntzen et al., 1982). Our results suggest that those species which were actively incorporating inorganic carbon on day 8 (eg. Nitzschia palea, Nitzschia acicularis, Anacystis sp.), even while exposed to relatively high concentrations of herbicide, may have been using one of these forms of triazine resistance or tolerance during that one week of exposure. By intensively following a single population after herbicide exposure, it may be possible to determine whether the entire population is inherently resistant or whether resistance is induced in selected individuals within the exposed population. The pattern of intraspecific variability may provide some insight into the inherent frequency or the induction of herbicide resistance.

Although GDA analysis can provide information on species-specific responses when evaluating in-situ toxicological effects, the use of GDA also has practical limitations. High intra-specific variability, which may occur in the presence of a toxicant, requires that a large number of cells be counted to detect statistical differences. The quantity of intact dead cells in a sample will influence assimilation estimates. This problem may become especially evident with haptobenthic diatoms since a large proportion of dead cells may remain attached to the substratum. In field experiments with high species diversity, it may be necessary to limit the number of species studied. In our study, where over 74 species were present through the experimental period, it was necessary to select only a few representative species to examine the feasibility of using GDA for in-situ toxicity studies on haptobenthic periphyton. Community composition has been demonstrated to shift in the presence of triazine herbicides (Chapter 2; Goldsborough and Robinson, 1986) thereby making it difficult to make comparisons between treated and untreated individuals of the same species over time. Secondary effects of herbicide treatment (Chapter 2) may also make it more difficult to assess the direct impact of the herbicide on the algal population. In spite of these considerations, quantitative GDA can be used to provide the

information required to assess the effects of toxicants on individual algal species.

3.4 CONCLUSIONS

This study has provided additional support for the use of grain density microautoradiography in aquatic research. Inter and intra-specific variation in isotope assimilation rates can be quantified provided that the appropriate correction factors are applied. Non-preservation autoradioagraphy techniques, such as the one described in this study, should be utilized since the quantity of label lost due to leakage is not yet predictable. Computerized image analysis with automated grain counting was found to be time efficient, however enumerating grain production from very dense cells required some manual counting. GDA can be used successfully to detect species specific influences of toxicants within mixed algal communities.

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