MICROCOSM ASSESSMENT OF THE EFFECTS OF MONENSIN, 10:2 SATURATED FLUOROTELOMER CARBOXYLIC ACID, AND ATRAZINE ON AQUATIC MACROPHYTES AND RESPONSES OF INDIVIDUALS VERSUS ASSEMBLAGES

by

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ABSTRACT

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The phytotoxicities of monensin, the 10:2 saturated fluorotelomer carboxylic acid (10:2 FTCA), and atrazine to freshwater macrophytes were investigated in three microcosm-based assessments. Both responses of plants grown as individuals in "cone-tainers" and those grown in mixed and monocultures were examined to permit comparison of the toxicological sensitivities of macrophytes under each planting design, and investigation of whether interactions between neighbours may modify plant response to an environmental contaminant. Exposures of monensin and the 10:2 FTCA at environmentally relevant concentrations were found to produce few significant effects in the higher aquatic plants across all growth conditions, thus direct comparisons of effective concentrations were not conducted. Significant differences between relative growth rates (RGR) of plant grown in assemblages versus individually indirectly indicate that over longer exposure durations toxicity may be underestimated using the individual "cone-tainer" method. RGRs and sensitivities of plants to atrazine were found to be in the same range across planting methods, demonstrating that responses of aquatic plants in the individual-test system reflected those observed in model populations and two-species communities. A lack of observed relations between plants in the mixed and monoculture tests, however, meant that the potential for modification of toxicity through plant interactions was not investigated.

PREFACE

Chapters 2, 3 and 4 of this thesis are organized as manuscripts to be submitted for publication in scientific journals. For this reason, there is some repetition of introductory and methodological material. All chapters and the associated manuscripts were written by Erin McGregor, as indicated by primary authorship listed below.

Chapter 2:

McGregor, E.B., Solomon, K.R., Hanson, M.L. 2007. Monensin is not toxic to aquatic macrophytes at environmentally relevant concentrations. *Arch Environ Contam Toxicol* 43:541-551.

Chapter 3:

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Chapter 4:

McGregor, E.B., Solomon, K.R., Hanson, M.L. 2008. Evaluating the effects of planting system design on the toxicological sensitivity of *Myriophyllum spicatum and Elodea canadensis* to a chemical stressor : a case study using atrazine. *Aquatic Tox.* Submitted January 2008.

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Acronym	Definition
EC_x	effective concentration causing an impact of x % from control on an organism with exposure to a toxicant
NOEC	no observed effect concentration
Monensin	monensin sodium salt, $C_{36}H_{61}NaO_{11}$
FTOH	fluorotelomer alcohol, $CF_3(CF_2)_nCH_2\ CH_2OH$ n = 3, 5, 7, …
FTCA	saturated fluorotelomer carboxylic acid, $CF_3(CF_2)_nCH_2CO_2H$ n = 3, 5, 7,…
FTUCA	unsaturated fluorotelomer carboxylic acid, $CF_3(CF_2)_{n-1}CF{=}CHCO_2H$ n = 3, 5, 7,…
PFCA	perfluorinated carboxylic acid, $CF_3(CF_2)_nCO_2H$ n = 1, 2, 3, …
PFDA	perfluorodecanoic acid, $CF_3(CF_2)_8CO_2H$

1 INTRODUCTION

1.1 AQUATIC PLANTS IN ECOTOXICOLGICAL TESTING

The evaluation of phytotoxicity is an important consideration in the assessment of the risk an environmental contaminant poses to ecological communities (Davy et al., 2001). Although aquatic macrophytes were once considered less sensitive to toxicants than animal species, including invertebrates and fish (Kenaga and Moolenaar, 1979; Lytle and Lytle, 2001), review of the literature has identified numerous chemical stressors that prove more toxic to freshwater plants than fauna (Lewis 1995; Roshon et al., 1999). As macrophytes play a significant role within the freshwater ecosystem by contributing to primary production, the generation of oxygen, and the cycling of nutrients (Wiegleb, 1988), protection of aquatic plant communities may prove essential to maintaining the structure and function of an ecological system. The interactions of aquatic macrophytes with other organisms are also important, providing food and habitat for epiphytic bacteria, periphyton, invertebrates, fish, and waterfowl (Carpenter and Lodge, 1986). Many aspects of the physical environment are influenced by the presence of aquatic plants such as light penetration, temperature patterns, and water flow (Newman, 2004). They also help to stabilize sediments thus reducing turbidity, erosion, and improving overall water quality (Madsen et al., 2001). The introduction of chemical compounds to the aquatic environment may result in adverse impacts on non-target vascular plants through both direct and indirect toxicity. In turn, impacts on the plant community may produce severe alteration of an ecosystem though indirect contaminant effects to higher trophic levels (Fleeger et al., 2003; Relyea and Hoverman, 2006).

For these reasons, primary producers are required as part of pre-registration testing of pesticides in North America (Davy et al., 2001). Pesticides represent a significant group of environmental contaminants that may enter aquatic ecosystems by intentional release or through more common unintentional routes such as spray drift and surface run-off (Leonard, 1988). The current pesticide registration process is administered through The United States Environmental Protection Agency (EPA) and the Canadian Pest Management Regulatory Agency (PMRA) and relies on a three-tier evaluation system. Laboratory based toxicity testing must be conducted on one floating aquatic macrophyte, *Lemna* sp., as well as four algae species including *Pseudokirchneria subcapitata*, *Anabaena flosaquae*, *Navicula pelliculosa* and *Skeletonema costatum*. Progression between the first two tiers of the evaluation system occurs when a 50% or greater inhibitory effect on growth is observed in test species at the maximum label dosage, while the third tier of testing, involving the use of field-level assessments, is only required on a case-by-case basis (Davy et al., 2001).

This registration protocol has received criticism surrounding the under-representation of higher aquatic plants. Testing requirements are focused on a single floating species while both emergent and submersed species are entirely excluded. There is also concern that *Lemna* sp. and algal varieties may not reasonably serve as surrogates for all other higher aquatic plants (Peterson et al., 1994; Lewis, 1995; Vervliet-Scheebaum et al., 2006). Consequently, recommendations have been made for implementation of a set of refined guidelines for non-target plant toxicity testing (Boutin et al., 1995; Davy et al., 2001; Lytle and Lytle, 2001). The new system is based on a four-tier testing structure that utilizes additional test species and test endpoints. While

the first level is based on a deterministic approach, a probabilistic risk assessment process is utilized in the higher tiers with the suggestion that microcosm-based multiple species tests are used when deemed necessary.

With this proposal, there has been a move in the literature towards the development and validation of various methods to assess the response of aquatic macrophytes to chemical pollutants. In addition to the standard methods available for conducting laboratory toxicity tests using *Lemna gibba* (ASTM, 1999a) and *Myriophyllum sibiricum* (ASTM, 1999b), studies have utilized other species such as *Elodea canadensis*, *Myriophyllum heterophyllum, Myriophyllum spicatum*, *Ceratophyllum dermersum*, *Egeria densa, Najas* sp., and *Potamogeton* sp., for both laboratory assays and microcosm based assessments (Jones and Estes, 1984; Detenbeck et al., 1996; Roshon and Stephenson, 1997; Fairchild et al., 1998; Hanson et al., 2001; Davies et al., 2003; Hanson et al., 2006).

1.1.1 Microcosm studies

The utility of microcosm experiments has been demonstrated with their successful application in the investigation of both the effects of contaminants on biological communities (Giddings et al., 2002; Van den Brink et al., 2005), and the environmental fate of various pollutants (Shaw and Kennedy, 1996; Graham et al., 1999). While microcosm systems are designed to mimic natural environments they are not a perfect representation and may lack components such as a shallow littoral zone and fish or macrophyte communities (Williams et al., 2002). However, they are considered a useful intermediate between laboratory-based testing and full-scale field assessments, with numerous advantages (Caquet et al., 1996). The simplicity of single-species laboratory-

based toxicity testing does not allow for detection of indirect effects due to the interactions that occur within complex natural environments (Van den Brink et al., 2005). However, model lentic ecosystems, such as those used in our studies (Figure 1.1), allow for testing of multiple species from several trophic levels and thereby allow for the measurement of indirect effects that may occur due to changes in food supply, water quality, and habitat, among other things (Solomon, 1996). These simulated field studies may also provide more realistic exposure scenarios as compared to laboratory testing, under which chemical compounds have the potential to partition, degrade and dissipate as they would in the environment (Liber et al., 1993). Microcosm testing allows for replication between test units and permits researchers to capture the responses of organisms to a range of stressor concentrations, which may not be achieved in large-scale field investigations (Graney et al., 1995). The drawbacks to microcosm-based assessments have also been reviewed (Shaw and Kennedy, 1996), and include cost considerations, limitations in the number of replicates, and the variability of results associated with time and test locations.

Several benefits of microcosm studies are specific to the investigation of effects of a contaminant on aquatic plants. Macrophytes grown in the laboratory are exposed to artificial light conditions and the addition of nutrients and sugar to growth containers that may confound results, while under semi-field conditions plants experience natural light and resource acquisition. Another advantage is that the response of macrophytes may be observed over the course of a complete growth cycle, and the recovery of plant populations and communities may be monitored over time. When aquatic plants are grown in model ecosystems they are also exposed to natural stressors such as grazing,

competition for resources, and abiotic factors that may modify their response to chemical toxicants through indirect effects (Shipley and Keddy, 1988; Fleeger et al., 2003; Hanson et al., 2006).

In the past, microcosm based assessments rarely focused on the impacts of environmental contaminants on the vegetation component of the aquatic ecosystem, but rather macrophytes were assessed as a secondary objective of the research (Huggins et al., 1993). According to these methods, plants are grown directly from pond or stream bottom-sediments and macrophyte growth and species composition are largely uncontrolled. Response of the plant community to an environmental contaminant may be estimated by measurement of the percent reduction in plant cover or final stand biomass (Van den Brink et al., 1997; Van Geest, 1999). This type of evaluation allows researchers to make general observations about the toxicity of a compound to the aquatic vegetation however, more specific endpoints are often not examined and effective concentrations are seldom calculated. Due to high variability between test systems, both within and between treatments, this method does not lend well to reproduction of results (Coors et al., 2006).

More controlled methods have been developed in order to evaluate the response of aquatic macrophtyes to a chemical stressor whereby plants are grown in sediment-filled containers and placed into a larger model ecosystem (Hanson et al., 2001; Davies et al., 2003). This approach allows for regulation of the number of plants added to each microcosm, the species types, the planting configuration, and attains lower variability among systems (Coors, 2006). A study conducted by Hanson et al. (2003) compared the variation of microcosm toxicity data for *Myriophyllum* spp. grown in individual test

containers within experimental ponds, to the variation of toxicity data from standard laboratory assays and found that variation was not significantly different. They concluded that under these semi-field test conditions most of the observed variation was inherent in the plants, indicating that this method may fittingly reduce variation caused by test design, resulting in a statistically sensitive assay.

1.1.2 Individual level toxicity testing

The term ecotoxicology was first formally defined by Truhaut (1977) to be the branch of science focused on investigating the toxic effects of environmental contaminants to ecosystem constituents, conducted in an integrative context. The general objective of ecotoxicological research is to understand and predict effects of pollutants on populations and communities under realistic exposure scenarios (Chapman, 2002). In practice however, the emphasis of research is on understanding the effects of contaminants at lower levels of biological organization, monitoring biochemical and organism level responses (Clements and Kiffney, 1994), and most traditionally using single-species toxicity bioassays (Preston, 2002). The underlying assumption is that organism-level toxicity tests can be reliably used to predict effects at higher levels of biological organization including populations, multi-species, communities, and ecosystem responses (Cairns, 1983). The application of ecotoxicological data in the ecological risk assessment process commonly relies on this tenet despite concern over the minimal amount of research available demonstrating a causal relationship between responses at different levels of organization (Clements and Kiffney, 1994; Preston, 2002), and the fact that research methods have progressed substantially beyond simple single-species laboratory testing (Calow and Forbes, 2003). Proponents of a more ecological

viewpoint reason that the interactions between abiotic and biotic elements of a system are more complex than can be predicted from the examination of the individual ecosystem components (Cairns, 1983). Thus, the ecotoxicological practice of monitoring individual life-history traits, such as survival, growth, and reproduction of organisms is deemed an oversimplification, discounting the dynamic nature of communities and potentially overlooking complex ecosystem interactions (Calow and Forbes, 2003).

There is evidence that the response of organisms to an environmental contaminant may be influenced by the associations occurring within an ecosystem, including both intra-species interactions such as population density effects (Simkiss et al. 1993, Sibly et al. 2000), and inter-species interactions, such as competition and mutualism (Preston 2002). For example, a study conducted investigating the effect of the interaction between zinc toxicity and population density dependence on the population growth rate of the potworm *Enchytreaus doerjesi*, noted synergistic effects between the variables at the two lowest population densities, while at higher initial densities both stressors acted independently (Kramarz et al., 2005). The researchers emphasize that bioassays conducted utilizing different intial densities may yield very different estimates of effective concentrations, illustrating that intra-species interactions may play a role in modifying the response to a toxicant. In another study, laboratory testing conducted to determine the short-term toxicity of cadmium, mercury and pentachlorophenol to freshwater oligochaetes, *Limnodrilus hoffneisteri* and *Tubifex tubifex*, found that responses of mixed species differed significantly from individually tested species (Chapman et al., 1982). Comparison of the 96-hour lethal concentrations indicated that the species were less tolerant of contaminants when tested in pure culture rather than in

mixed, demonstrating reduced toxicity to a chemical stressor associated with inter-species interactions. These results are consistent with ecological findings from an examination of seagrass communities that indicates, generally, increasing species richness is linked to an increase in community function, and a greater capacity to retain function under disturbance (Duarte, 2000).

Concern around monitoring endpoints at an individual-level is associated with the potential for these tests to provide an over- or under-estimation of effective concentrations to higher levels of organization (Forbes and Calow, 1999). While it has been suggested that seemingly insignificant effects on individual life-history traits may be magnified at the population level to produce large imacts (Halbach et al., 1983), others suggest that effects gradually accumulate up the levels of biological organization according to the duration and magnitude of the effects (Lin et al., 2005). Review of the literature, examining 41 studies in which both population growth rate (*r*) and individual-level responses were measured revealed that *r* was commonly equally or less sensitive to toxicants as one or more of the individual-level responses, implying that magnification of effects at the population level did not occur. Additionally, the most sensitive individual-level variable proved to be inconsistent (Forbes and Calow, 1999). The literature reviewed however, consists of data almost exclusively from populations that were not density-limited (Forbes and Calow, 2001). This makes it difficult to generalize whether over- or under-estimation of toxicant effects to populations are likely from individual-level observations, as most nature populations do experience density dependence over the long-term (Forbes and Calow, 2001).

While there is concern over the emphasis of individual-level testing within the field of ecotoxicology, these types of tests have proven to be of incredible utility (Chapman, 2002; Calow and Forbes, 2003), especially as an initial toxicity screen for the wide-range of new chemicals entering the environment. Supporters of the single-species approach argue that this type data has been used in ecological risk assessment for years and has protected ecosystems from harm with great success (Cairns, 1984), however, others suggest that this is largely due to the use of vastly over-protective application factors that account for uncertainties in the analyses and are not based on scientifically sound theory (Cairns, 1984; Calow and Forbes, 2003). In order to evaluate whether the individual-level approach provides information that is useful for understanding effects on ecosystem structures and functions and data that is appropriate for use in the ecological risk assessment process, a data-based comparison of the sensitivity of chronic single-species tests and experimental ecosystem investigations was conducted (Versteeg et al., 1999). Toxicity data for a suite of environmental contaminants (n=11), including pesticides, metals, surfactants, organics and inorganic compounds, was evaluated to determine whether a substantial overlap in the distribution of the single-species and model ecosystem data exists, thus indicating whether extrapolating from the single-species to the ecosystem level is appropriate. Based on this analysis researchers found that a sufficiently large dataset (> 5 species) of individual-based responses could be used to generate concentrations protective of the experimental ecosystem. With these results the authors also acknowledge that there are assumptions built into the analysis such as the use of model ecosystem data as a surrogate for natural systems, and that it is worthwhile

to continue work on the development and refinement of approaches other than single-species method.

The phytotoxicity of environmental contaminants is commonly evaluated using the reductionist approach, measuring organism-level responses such as growth rate and pigment concentrations of individuals in order to predict the response of the larger plant community. Standard laboratory-based assays for assessing the response of *Myriophyllum* spp. to potential chemical stressors rely on these methods (ASTM 1999b), as do other non-standardized laboratory tests (Turgut and Fomin, 2001; Knauer et al., 2006). Aquatic macrophytes are grown in isolated test units and do not interact among or between species. The results of these individual-based tests are used to inform the lower tiers of ecological risk assessment. Simulated field level assessments used in the higher tiers of risk assessment, such as outdoor microcosm evaluations, strive to provide more environmental realism while they too may follow the same pattern. Macrophytes may be grown within a larger model ecosystem, but are still planted in pots as individuals thereby limiting potential interactions between organisms and possibly growth rate (Detenbeck et al., 1996; Hanson et al., 2001; Davies et al., 2003; Brain et al., 2005a; Brain et al., 2005b; Hanson et al., 2006). While these individual planting methods are used in attempts to reduce variability within the test systems, there is no confirmation in the literature that the responses of individually grown plants characterize those of more realistically grown plant populations and communities.

1.1.3 Test species and selected endpoints

Vascular aquatic plants may be categorized from an ecotoxicological perspective both taxonomically and based on morphological features. Belonging to the Division of

Angiosperms, aquatic macrophytes fall into three Classes including the dicotyledons, the magnoliids, and most frequently, the monocotyledons (Cronk and Fennessy, 2001). The plants may belong to a variety of Subclasses, Orders, and about 20 diverse Families (Wetzel, 2001). Aquatic plants are also commonly classified according to their growth form with simple categories assigned by Sculthorpe (1967) to include emergent, submerged, floating, and floating-leaved plants. It has been demonstrated that both taxonomic and growth form differences between macrophyte species may result in differential sensitivities to environmental contaminants based on physiology (mode of actions) or route of exposure.

A review of the literature by Fletcher et al. (1990) established that notable differences in sensitivity to a toxicant may exist between various taxonomic groups of terrestrial plants. The analysis found that responses of species within the same genera are generally more similar than responses of plants from differing genera. They also concluded that the sensitivity of a plant to an environmental contaminant is generally more influenced by taxonomic differences than by the conditions under which testing was conducted, such as greenhouse versus field testing. The minimal range of differences between laboratory and field responses, due largely to the stationary nature of plants, implies that the extrapolation between laboratory data and the field is a reasonable practice with consideration given for potential variability due to environmental differences.

A laboratory based investigation was conducted by Fairchild et al. (1998) to evaluate the sensitivity of five species of aquatic macrophytes and six species of algae to four common herbicides, including two from each of the triazine and acetanilide

compound groups. They determined that sensitivities to the herbicides were significantly different between macrophyte species, spanning a range of more than two orders of magnitude. Generally, *Ceratophyllum demersum* were found to be most sensitive, *Lemna minor* and *Myriophyllum heterophyllum* were found to be least sensitive, and *Elodea canadensis* and *Najas* sp. were in the middle range. However, this ranking was not consistent across all herbicides tested. The wide variability in responses of non-target aquatic plants to herbicide exposure indicates that the use of a single macrophyte species to characterize the range of potential effects is largely inappropriate. Davies et al. (1999) found similar results in a mesocosm based investigation that evaluated the relative sensitivities of four macrophyte species to six herbicides. The investigators selected macrophyte species from a variety of plant types including a submerged monocotyledon, an emergent monocotyledon, a floating monocotyledon, and a submerged dicotyledon. As well, herbicides were chosen to represent a range of chemical structures and modes of action, none of which were common to those investigated by Fairchild et al. (1998). The study found large differences in sensitivities of the test species and noted that *Lemna* sp. was not the most sensitive species overall. Cedergreen et al. (2004) also tested the sensitivity of twelve aquatic plants to a single herbicide, metsulfuron-methyl, monitoring relative growth rate and specific leaf area over 14 days of exposure. The calculated IC_{50}s varied 56-fold between the evaluated species. They also noted that the faster growing plant species with a small exposed leaf area were more sensitive to the herbicide. Another study investigated whether the morphology of five aquatic test species influenced their sensitivity to three commercial herbicides (Veit and Moser, 2004). The researchers observed that taxonomically related species may react differently to a

chemical stressor. Yet, overall, the growth form of the macrophytes appeared to be more important in determining plant sensitivity. Due to the variability and unpredictability of aquatic macrophyte responses, researchers have emphasized the need to evaluate effects of environmental contaminants on a suite of non-target aquatic plants, including plants from a variety of taxonomic and morphological classes in order to ensure a comprehensive evaluation of potential risk (Lewis, 1995; Lytle and Lytle, 2001; Hanson and Arts, 2007).

Our studies utilized *Myriophyllum spicatum* L., *Elodea canadensis* Michx., *Egeria densa* Planch., and *Lemna gibba*, in a microcosm-based multiple-species testing scheme in order to evaluate the phytotoxicity of several environmental contaminants. The four species are described below and were selected based on their availability, ease of maintaining and planting, validated use within the field of ecotoxicology, ecological relevance in the region of south-eastern Ontario, Canada, where the outdoor microcosm testing was conducted, and taxonomic (monocotyledons and dicotyledons) and morphological (floating and rooted submersed) forms. *E. canadensis* and *M. spicatum* were used in our model population and community tests due to their natural coexistence in aquatic plant communities (Nichols and Shaw, 1986) and their tendency to grown under similar physio-chemical conditions (Abernethy et al., 1996).

1.1.3.1 Floating species: Lemna gibba

Occupying the interface between air and water, the floating aquatic macrophytes are an important test group in the assessment of effects of an environmental contaminant. These non-rooted plants may be exposed to contaminants through both aerial and aquatic routes thus making them appropriate for testing of compounds that create surface films or

enter the environment through spray drift (Taraldsen and Norberg-King 1990). *Lemna* sp. or duckweed, a genus of free-floating macrophytes, are the mostly widely utilized aquatic plants in toxicity testing, partly because they are required under pre-registration testing standards of pesticides in North America (Davy et al., 2001). Several standard procedures are available that outline laboratory methods for conducting static toxicity tests with duckweed, including methods from the USEPA (1996), the American Public Health Association (APHA, 1998), Environmental Canada (1999), and the American Society for Testing and Materials (ASTM, 1999a).

Lemna gibba L. (G-3), Gibbous duckweed, is a globally distributed C-3 monocotyledon of the Lemnaceae family (Hillman, 1961). *L. gibba* are a fast-growing species of small vascular macrophytes that propagate largely through rapid vegetative growth and to a lesser extent through sexual reproduction (Cronk and Fennessy, 2001). Through these means the plants form dense mats on the water's surface, providing habitat for invertebrates and food for waterfowl (Newmaster et al., 1997). Gibbous duckweed consist of two parts, frond and root, while lacking true leaves and stems (Hillman, 1961). These long-day plants have a reduced root system that is thought to serve as anchors to keep frond upright (Davy et al., 2001). Within a laboratory setting, the small size of the plants and their fast-growing nature make them simple and inexpensive to culture while large enough that researchers may conduct nondestructive visual evaluations of their response to a contaminant (Wang, 1990). *L. gibba* have a high capacity for assimilation of various chemical compounds, with rapid uptake from the test medium and demonstrated bioconcentration (Greenberg et al., 1992). They are especially susceptible to surface-active agents and hydrophobic compounds located at the water surface (Wang,

1990). Additionally, the vegetative propagation of the plants also helps to ensure their genetic similarity within laboratory cultures (Hillman, 1961) thus reducing the potential variability of plant responses to test compounds. Due to their widespread occurrence, ecological importance, and simiplicity of laboratory toxicity methods for these plants, *L. gibba* are considered a favourable test species. Their use in the assessment of phytotoxicity has been extensively reviewed in the literature (Greenberg et al., 1992), and standardized methods for laboratory based testing are currently available (ASTM, 1999a). While the sensitivity of *Lemna* sp. to a chemical stressor often fall within the same range as other aquatic plants (Fairchild et al., 1998), there is regulatory concern that the observed response may not always be representative of effects to other aquatic plants, such as submerged, emergent or dicotyledonous macrophytes (Davies et al., 2003).

1.1.3.2 Vascular submerged species: *Myriophyllum spicatum, Elodea canadensis,* and *Egeria densa*

Rooted submerged plants are adapted to thrive in the aquatic environment under oxygen, carbon dioxide and light limited conditions (Cronk and Fennessy, 2001). Most above sediment components, including photosynthetic tissues, are completely submersed beneath the surface of the water and below ground elements are rooted in the sediments. In order to deal with environmental limitations submerged plants characteristically have thin and highly divided leaves, a reduced or absent cuticle, minimal woody tissue, the presence of aerenchyma tissue for improved buoyancy and gas exchange, and chloroplasts that are located in the outer epidermal cells (Cronk and Fennessy, 2001; Wetzel, 2001). These adaptations may lead to differential routes of uptake and sensitivity of the plants to aquatic pollutants compared with terrestrial and floating species (Davy et

al., 2001). Submersed macrophytes may come into contact with chemical contaminants through two routes, including exposure of the entire above ground surface of the plants through the water column, and exposure of root systems to contaminants that have partitioned out of the water column and into the bottom sediments (Lewis, 1995).

Due to the distinct routes of potential contaminant exposure and their ecological importance, the inclusion of a rooted submergent, *Myriophyllum sibiricum* Komarov, has been recommended as part of the updated methods for evaluating the response of non-target aquatic plants to pesticides (Davy et al., 2001). A standardized 14-day static axenic test method is available for *M. sibiricum*, through the American Society for Testing and Materials (ASTM, 1999b). It is not widely used, with only three published reports following this method (Roshon et al., 1996; Roshon et al., 1999; Hanson and Solomon, 2004), though it may also be applied to *M. spicatum* (Hanson and Solomon, 2004). Several varieties of submerged macrophytes have also been used successfully in the assessment of phytotoxicity, including *Myriophyllum spicatum* L., *Elodea canadensis* Michx., and *Egeria densa* Planch., although species specific standardized methods have yet to be developed.

Myriophyllum spicatum L., Eurasian watermilfoil, is a rooted dicotyledon and a member of the Haloragaceae Family. Largely considered a weed species, the plant originated in Europe, Asia, and North Africa, but is now widely present across North America and found in Ontario, Quebec and British Columbia (Aiken et al., 1979; Creed, 1998). As a disturbance tolerant species (Abernethy et al., 1996), Eurasian watermilfoil is found in a wide variety of abiotic conditions ranging from oligotrophic to eutrophic waters, depths of 0.5 to 10 m, and pH conditions of 5.4 to 11 (Aiken et al., 1979).

However, nutrient rich waterways and depths of 1 to 4 m are optimal for growth (Nichols and Shaw, 1986; Cronk and Fennessy, 2001). *M. spicatum* plant shoots are extensively branched, range from 0.5 – 7 m in length, and have leaves that are usually arranged in whorls of 4 with 10 to 26 pairs of leaf divisions. Plants grow tall to compensate for light attenuation (Cronk and Fennessy, 2001), with the majority of Eurasian watermilfoil biomass found at the surface of the water (Titus and Adams, 1979) forming dense canopies that are capable of shading out native plant species (Dale and Gillespie, 1977). *M. spicatum* is a perennial plant that overwinters as an evergreen, or by dying back in the fall to a root crown with unexpanded shoots, and forming new axillary buds in the early spring (Aiken et al., 1979). Propagation occurs largely through vegetative regeneration, using shoot fragments as a rapid and effective means of dispersal (Valley and Newman, 1998). To a lesser extent, the plants also spread through sexual monoecious reproduction (Valley and Newman, 1998; Cronk and Fennessy, 2001). Like other rooted vascular species, *M. spicatum* contributes to the freshwater ecosystem by influencing the abundance and composition of fish and invertebrate species (Newman, 2004), and by releasing allelopathic polyphenols that may inhibit the growth of other aquatic organisms (Nakai, 2000). *M. spicatum* has been used fairly commonly in the assessment of phytotoxicity, with tests conducted both in the laboratory (Christopher and Bird, 1992; McCann, et al. 2000; Hanson and Solomon, 2004) and under semi-field conditions (Hanson et al., 2001; Davies et al., 2003; Marwood et al., 2003; Brain et al., 2005a; Coors et al., 2006).

Elodea canadensis Michx., Canadian waterweed, is a rooted submergent with cosmopolitan distribution. It is a perennial monocotyledon of the Hydrocharitaceae

Family, originating in North America (Nichols and Shaw 1986; Spicer and Catling 1988). Like Eurasian watermilfoil, Canadian waterweed is considered an invasive weedy species, causing aquatic weed problems in regions where it has invaded (Spicer and Catling 1988). It was introduced to Europe in the 19th century and has spread to regions of Asia, Africa and Australia (Bowmer et al. 1995). In Canada, *E. canadensis* is the most widespread and common native species, found in temperate regions including the Great Lakes area and the St. Lawrence Valley (Cook and Urmi-Konig, 1985; Spicer and Catling 1988). The plants spread primarily through vegetative reproduction (Nichols and Shaw 1986), with the rapid dispersal of stem fragments occurring through wave action, water currents, waterfowl transport, and human activities (Cook, 1985). Canadian waterweed consists of herbaceous stems with dichotomously branching elongate leafy shoots. Leaves occur in whorls of three on the middle and upper stem branches (Spicer and Catling 1988; Bowmer et al. 1995). Plants overwinter by producing dormant shoot apices in the fall that re-establishing as leafy stems in the spring (Bowmer et al. 1995). Common growth conditions are similar to those required by *M. spicatum*, including water depths between 4 and 8 m, a pH range of 6.5 – 10, and fine bottom sediments (Spicer and Catling 1988). While both species are most commonly found in hard, nutrient rich, and alkaline waters, experiments have suggested that *E. canadensis* is generally more competitive that *M. spicatum*. Eurasian watermilfoil was found less tolerant of shade-stress, cutting-disturbance and deep water conditions than Canadian waterweed (Sheldon and Boylen 1977; Abernethy et al. 1996). As mentioned, in terms of sensitivity to various herbicides *E. canadensis* was found in the middle range compared with a suite of other macrophyte species (Fairchild et al. 1998). While not used as extensively as

Myriophyllum spp. in the assessment of phytotoxicity, the response of *E. canadensis* to environmental contaminants has been evaluated both in the laboratory (Brown and Rattigan 1979; Mal et al. 2002; Cedergreen et al. 2004; Knauer et al. 2006) and under semi-field conditions (Netherland et al. 1997).

An ecological study was conducted by Abernethy et al. (1996) in which *E. canadensis* and *M. spicatum* were grown in mono- and mixed cultures to investigate the response of the plants to artificially-imposed stress, disturbance, and interspecific competition. The study found that when grown in mixed culture *M. spicatum* proved to be the less competitive species, displaying a significant loss in biomass. Under these test conditions a significant interspecific interaction was observed between species, implying that there is potential for modification of the response of these plants to an environmental pollutant when grown in the same plant community. Conversely, indirect effects of toxicity of a contaminant on species interactions may also be observed (Preston, 2002).

Egeria densa Planch., Brazilian waterweed, is a perennial monocotyledon also belonging to the Hydrocharitaceae Family. The species is native to South America (Roberts et al. 1999), with a growth form much similar to that of *E. canadensis* but distinguished by larger leaves in whorls of four or five (Bowmer et al. 1995). *E. densa* is an invasive submergent, introduced to North American through the aquarium trade (Washington State Department of Ecology, 2007) and is considered a prominent weed in some regions of the United States (Getsinger and Dillon, 1984). Like *E. canadensis*, Brazilian waterweed reproduces through both flowering and vegetative stem fragmentation, is found to overwinter as root crowns (Getsinger and Dillon 1984; Haramoto and Ikusima 1988), and like *M. spicatum* tends to form a dense canopy at the

water surface (Getsinger and Dillon 1984). Although not used extensively in the field of ecotoxicology, several recent studies have successfully utilized Brazilian waterweed in semi-field based testing (Hanson et al. 2006; Skogerboe 2006). While the plants are not currently found in the environment of southern Ontario, we are interesting in observing how well the species grows under these test conditions. Additionally, *E. densa* was used in our field studies due to the ease with which it is established and maintained, and because it is readily available.

1.1.3.3 Endpoint selection

A number of effect measures may be monitored in the evaluation of toxicity of an environmental contaminant to aquatic macrophytes. These include various anatomical or morphological endpoints such as length and biomass of roots, shoots, stems, leaves, number of nodes and fronds, growth rates, numbers of individuals, chlorosis and necrosism, seed germination, as well as biochemical and physiological effect parameters including changes in pigment concentrations, production of stress proteins, enzyme activity, levels of oxygen production and carbon fixation, among other endpoints (Jana and Choudhuri, 1982; Lewis et al., 1995; Roshon et al., 1999; Hanson et al., 2001; Lytle and Lytle, 2001). Firstly, for a measurement endpoint to be of use it must be toxicologically sensitive to the contaminant, susceptible to chemical exposure and demonstrating a measureable response, and thus allowing for calculation of effective concentrations (EC_xs). Additionally, endpoints of interest should demonstrate low variability in order for statistical discenment between treatment-related changes in the system and natural variation. The greater the variability associated with an endpoint, the lower the statistical power of the analysis, and the greater the likelihood of committing a

Type II error (Hanson et al., 2003). Preferably, endpoints selected for assessment should demonstrate both toxicological and statistical sensitivity.

Currently, the somatic endpoints are more commonly monitored (Davy et al., 2001) while not always the most statistically sensitive, as observed in a microcosm-based investigation assessing the effects of tylosin on *L. gibba* and *M. spicatum* (Brain et al., 2005a). The researchers found that statistical power was generally lower for somatic endpoints than pigment measures assessed in the two species of macrophytes. Also, in terms of statistical sensitivity *M. spicatum* was found to be less variable than *L. gibba*. In a larger investigation assessing the endpoint variation associated with *Myriophyllum* spp. microcosm toxicity data conducted by Hanson et al. (2003), researchers found differing results to those presented by Brain et al. (2005a). Node number and plant length were consistently the most statistically powerful of the tested endpoints for both *M. spicatum* and *M. sibiricum*, while pigment concentrations, biomass measures and root lengths were less statistically sensitive endpoints. These results correspond with another microcosm based toxicity test in which pigment endpoints were found to be less statistically sensitive endpoints in *M. sibiricum,* when compared to the somatic endpoints (Brain et al., 2005b). Under laboratory conditions Knauer et al. (2006) also evaluated the sensitivity of various effect measures for detecting differences from the control. Generally, the most sensitive endpoints of those evaluated in *E. canadensis* and *M. spicatum* were total plant length, shoot length, and wet biomass, while side shoot length and total root lengths were less sensitive. In a semi-field assessment of the toxicity of oxytetracycline to *E. densa* and *Ceratophyllum demersum* L., it was found that the strongest responses in *E. densa* were associated with plant length and root development endpoints (Hanson et al., 2006).

Making generalizations regarding which endpoints are, as a rule, the most statistically sensitive and thus allow for greater detectability of differences from controls, is not entirely appropriate as endpoint sensitivities have been found to vary with test conditions, toxicants and between species (Versteeg et al., 1999). While plant length is commonly determined to be a sensitive effect measure for various test compounds and species, it is not recommended that length is universally used as a endpoint across all chemical toxicants and exposure scenarios. Instead, it has been suggested that a suite of endpoints are utilized in investigation of phytoxicity (ASTM 1999a; ASTM 1999b; Davy et al., 2001), as was done in our toxicological tests conducted using *L. gibba* and individually grown submersed plants.

Due to constraints on time and manageability of sampling associated with monitoring a multitude of test endpoints on plants grown in populations and communities, we found it necessary to monitor fewer endpoints under these conditions. As measurements of the biomass of plants are commonly used as a reflection of macrophyte productivity (Wetzel, 2001), root and shoot biomass were included as endpoints in our pure and mixed culture plant tests. Relative growth rates (RGRs) may be easily calculated from the biomass data and used as an additional effect measure. In general, the greater the RGR and the longer the test duration, the lower the concentration of toxicant need to impact a specific growth rate and the more sensitive a toxicity test conducted on aquatic macrophytes may be to a chemical stressor (Huebert and Shay, 1993). For this reason, the RGR of plants may be used to facilitate an indirect comparison of the potential toxicological sensitivity of plants grown under various toxicity test conditions.

1.2 COMPOUNDS OF INTEREST

In recent years, several classes of chemicals have emerged as important aquatic contaminants; these include veterinary and human pharmaceuticals and fluorinated organic compounds (Halling-Sorensen et al., 1998; Martin et al., 2004a). While the pesticide registration process attempts to identify and reduce potential ecological impacts of compounds that are purposely applied purposely in the environment, many other types of contaminants are unknowingly released into aquatic ecosystems and *a priori* toxicity testing is often not conducted. The impact of these compounds on non-target aquatic organisms may be largely unknown. This is the case for monensin, an ionophore antibiotic, as well as the 10:2 saturated fluorotelomer carboxylic acid (10:2 FTCA), a stable intermediary breakdown product in the degradation of fluorotelomer alcohols (FTOH) to perfluorinated carboxylic acids (PFCA).

In a recent report prioritizing veterinary pharmaceuticals based on their usage, toxicity profile, and potential to reach the environment in the United Kingdom, researchers classified monensin as a high priority for detailed risk assessment due to high usage, a high toxicity profile classification, and an unknown potential to reach the environment (Capleton et al., 2006). This demonstrates the existing concern in the scientific community over the potential non-target effects of the compound. Widely used older pesticides may also come under increased scrutiny as new information regarding their ecotoxicity emerges. Atrazine, a herbicidal compound used in North American agricultural systems for almost fifty years (Solomon et al., 1996), has come under renewed interest with the publication of research suggesting unanticipated toxicity of the

compound may exist when it is a component of pesticide mixtures in the environment (Hayes et al., 2006).

1.2.1 Monensin

1.2.1.1 Use Pattern

Monensin, first described by Haney and Hoehn in 1967, is a naturally occurring antibiotic that is derived through the fermentation of a strain of the microorganism *Streptomyces cinnamonensis* (Agtarap et al., 1967; Elanco, 1989), and demonstrates microbiological action against many gram-positive bacteria, fungi, and protozoa (Cha et al., 2005). Monensin sodium salt (Table 1.2) is the commercial form of the compound, produced during the manufacturing process by exposing monensin to sodium ions during a pH adjustment phase of production (Elanco, 1989). The compound is exclusively used in veterinary applications, commonly administered as a food additive for growth promotion and as a coccidiostat in the poultry and cattle industries (Matabudul et al., 2001). While specific statistics on usage of agricultural pharmaceuticals in North America are not available, it was estimated that in 1998 approximately 600 000 kg and 900 000 kg of monensin were used in the United States poultry beef and poultry industries, respectively (UOCS, 2001). The usage rates in Canada are likely to be similar on a proportional basis, as the level of monensin use per animal is comparable between Canada and the United States. Canada's cattle numbers over the last 3 years are between 15-16.5% the size of the United States (NASS, 2005a) and the poultry industry (broiler chicken and turkey production) was found to be about 20% the size of the United States

in 2004 (NASS, 2005b; Statistics Canada, 2005). Based on these numbers, a rough estimate of usage in Canada would be between 270 000 and 280 000 kg.

1.2.1.2 Physical and Chemical Properties

Monensin belongs to the group of organic chemicals known as the ionophores (Pressman, 1985), defined by their ability to selectively induce ion passage across biological membranes through the formation of dynamically reversible cation complexes (Pressman, 1976; Pressman and Fahim, 1982). These lipid-soluble compounds consist of two subclasses including the neutral and carboxylic ionophores, the latter group transferring cations as electrically neutral complexes (Pressman, 1985). Monensin is a monocarboxylic polyether, and consists of four compounds shown in Table 1.3: factor A, B, C, and D (Elanco, 1989). Monensin factor A accounts for more than 90% of the total microbiologically active material (Carlson and Mabury, 2006). The molecule consists of a quasilinear array of heterocyclic rings when in the uncomplexed, protonated state (Pressman, 1969). At the appropriately high pH, hydrogen bonding occurs between the carboxyl group at the head of the molecule and one or two hydroxyl groups at the tail, folding the molecule into a 24 atom ring (Pressman and Fahim, 1982). This crystal structure wraps around a cation forming an antiporter complex (Agtarap et al., 1967), with hydrogen bonding occurring between the ether oxygens, the hydroxyl oxygens, and the carboxylic acid oxygen of the polyether, and the monovalent cation (McGuffey et al., 2001). These reversible bonds are only produced when monensin in its ionic form, as the charged carboxylate drives the formation of a complex in order to achieve electrostatic neutralization of the cation. The chelation cavity formed by this structure is relatively small, partly explaining why monensin has a preference for monovalent cations over

divalent, and a ten times stronger affinity for Na+ over K+ (Pressman and Fahim, 1982). The general selectivity of monensin acid is Na>K>Rb>Li>Cs (Pressman, 1969), most readily forming complexes with sodium ions.

Transfer of ions occurs when the mobile complex attaches to a grampositive microorganism, becomes solubilized in the bilipid cell membrane, and exchanges the monovalent cation for a proton (Pressman, 1976; Russell and Strobel, 1989). Along with the cellular influx of Na+ and H+, rapid movement of K+ out of the cell occurs due to concentration gradients. The disturbance of ionic equilibrium and the reduction of pH levels within the cell, cause the organism to utilize energy in attempt to restore cellular balance. However, the cell quickly exhausts ATP and is unable to grow and divide. The severity of the antibiotic effects depends on the sensitivity of the microorganisms to monensin (McGuffey et al., 2001)

The mostly hydrocarbon-like shell of the molecule demonstrates in part why monensin is only moderately water soluble, but soluble in most organic solvents (Elanco, 1989). The solubility of monensin in water is determined to range from 4.8 to 8.9 mg/L, with lower solubility in low pH environments (Lissemore et al., 2006). At pH values ranging between 7 and 9, as found in the freshwater microcosms, over 99% of monensin is expected to be ionized (Sassman and Lee, 2007). Other known physiochemical properties are presented in Table 1.4.

1.2.1.3 Sources and Fate

Monensin may enter the environment at several possible locations including the release of compound at the manufacturing site, at the feed mixing location, and at the use site. The amount of compound released at the manufacturing and mixing locations is

likely insignificant due to regulatory controls (Elanco, 1989) however, monitoring data collected to confirm this are unavailable. At the use site, monensin is administered to poultry and cattle as a feed additive. Studies have indicated that orally administered monensin is partly absorbed by the target species, metabolized to a varying extent via O-demethylation, hydroxylation and/or decarboxylation (Donoho et al., 1978), excreted in the bile, and almost exclusively eliminated in the feces (Herber and Van Duyn, 1969; Herberg et al., 1978; Donoho, 1984). A proportion of the administered dose is excreted in the feces as relatively inactive metabolites, and up to 50% of the oral dose is excreted as parent molecule (Donoho et al., 1978; Donoho, 1984). The land application of manure containing the unchanged compound serves as the most likely source of monensin to the environment (Halling-Sorenson et al., 1998).

Once in agricultural soil the degradation of monensin is fairly rapid, with a dissipation half-life estimated to range between 1.2 and 3.8 days depending on soil properties (Carlson and Mabury, 2006; Sassman and Lee, 2007). The compound also shows a propensity for sorption to soils and sediments (Carlson and Mabury, 2006; Kim and Carlson, 2006), with a log K_{OW} of 2.8 to 4.2 (Elanco, 1989), and a reported sorption coefficient (K_d) between 1.09 and 78.6 L/kg, as determined through investigation using various agricultural soils (Sassman and Lee, 2007). These properties indicate that monensin has relatively low potential for mobility and is thereby unlikely to leach through soils and into groundwater (Carlson and Mabury 2006). However, despite the potential for breakdown and sorption of monensin in soils, the compound is detected in surface waters and sediments around areas of intense agricultural activity (Hao et al., 2006, Kim and Carlson, 2006, Lissemore et al., 2006). Particulate-bound monensin is

most likely moving into the aquatic environment following rainfall or irrigation events associated with surface run-off waters (Lissemore et al. 2006). Additionally, although spreading of manure on agricultural soils occurs only periodically over the course of the year, monensin demonstrates the ability to move readily in and out of solution depending on the existing conditions and may desorb from sediments (Lissemore 2005) thus potentially facilitating a semi-continuous release of compound in surface waters.

To a much lesser extent monensin may reach the aquatic environment through several other routes. The direct input of animal waste to surface waters (Lissemore et al. 2006), and the leakage or overflow of animal waste storage structures (Kolpin et al. 2002) or inappropriate disposal of used containers and unused feed additive, leading to over-land flow of compound to the aquatic environment may occur (Boxall et al., 2003). Due to the high molecular weight and melting point of monensin, presented in Table 1.4, atmospheric transport of the compound is unlikely (Elanco, 1989).

Lissemore (2005) investigated the potential fate of monensin in aquatic and soil systems using several methods, including laboratory-based research examining abiotic and biotic degradation of the compound, computer-based degradation modeling, and an outdoor microcosm-based fate study. Experiments investigating abiotic degradation, conducted over 219 days and according to United States Environmental Protection Agency (US EPA) protocols, demonstrated very slow hydrolysis rates for the compound under ambient conditions. The shortest hydrolytic half-life of the compound was found to be about 34 weeks at a pH of 4, while at a pH as high as 9 no measurable degradation occurred. Hydrolysis rates were found to increase in the presence of $CuSO_4$, an environmentally relevant nucleophile, indicating the conservative nature of the laboratory

based test. A study conducted over 30 days found similar results, with no hydrolysis of the compound observed at a range of pH values (Elanco, 1989). The biotic degradation of monensin was also investigated, conducted over a 130-day study duration using fresh manure from an organic beef operation as inoculum. Measurable degradation of monensin was not observed over the course of the study. While these results are seemingly conflicting with those of Carlson and Mabury (2006) and Sassman and Lee (2007), the author notes that there is potential that microorganisms may not have adapted under the test conditions to synthesize enough degradative enzymes needed to catalyze degradation of the compound (Lissemore, 2005). Computer model results also predicted very slow biotic degradation of monensin. In general, the researchers demonstrated that monensin is quite resistant to both abiotic and biotic degradation under laboratory conditions (Lissemore, 2005). However, the author notes that phytolysis may contribute significantly to the degradation of the compound in surface waters, as supported by results from the Atmospheric Oxidation Program for Microsoft Windows (AOPWIN), although not experimentally investigated.

In the microcosm-based investigation of the environmental fate of monensin, concentrations of compound in the water-column and sediments were monitored over 35 days, and were found to be fairly stable under semi-field conditions (Lissemore, 2005; Hillis et al., 2007). An average of about 70% reduction in surface water concentration of monensin was observed over the study duration, with similar rates of decline across all treatment levels. Results from the model ecosystem test were in general agreement with laboratory findings, while discrepancies were deemed likely a reflection of degradation of compound via photolysis occurring in the field (Lissemore, 2005). Using their

experimental data, the half-life of monensin in the water column is calculated to be about 20 ± 5 days. Measurements of the compound in the sediment conducted on days 8 and 21, found that concentrations were an order of magnitude higher than surface water concentrations, indicating that sorption did occur within the microcosms. Researchers also found that an increase in water column concentrations observed at Day 22 were likely associated with desorption of monensin from sediments due to increased water temperatures, thus indicating that sediment bound compound should not be considered entirely unavailable to aquatic organisms.

1.2.1.4 Environmental Concentrations

Concentrations of monensin have been measured in the aquatic environment in several recent reports, detected both in sediments and surface waters. A study conducted in Southern Ontario, Canada, by Lissemore et al. (2006) surveyed the occurrence of 28 pharmaceuticals in the surface waters at seven agricultural sites within the Grand River watershed. Monensin was determined to be one of the most frequently detected analytes under observation, found present in about 75% of the water samples at concentrations ranging from 6.2 to 1172 ng/L. The detection of monensin was found to vary temporally according to manure and biosolid application times (Hao et al., 2006; Lissemore et al., 2006). Monensin was also detected in a study conducted by Kim and Carlson (2006) investigating the occurrence of ionophores along the Cache la Poudre River, Northern Colorado. They sampled for pharmaceuticals at several sites along the river associated with differing land uses, detecting monensin only at those sites that were considered agriculturally-influenced. The highest concentration of monensin detected in the surface water was 0.036 μg/L, and in the sediment at approximately 32 μg/kg. The tendency for

monensin to sorb to organic carbon and other components of the solid phase was demonstrated by the significantly higher concentrations of compound present in the sediments compared to the overlying water, found at a concentration of almost three orders of magnitude greater than in the river at particular sample sites.

1.2.1.5 Toxicity

Monensin toxicity is highly species dependent with a narrow range of doses proving safe and effective for growth promotion and prevention of coccidiosis in target species, while causing toxic responses in horses and turkeys at similar dose ranges (Matsuoka 1976; Langston et al., 1985). The interspecies variation in monensin toxicity is thought to be related to differences in distribution and clearance of the compound and differences in plasma binding proteins (Donoho, 1984). Additionally, the catalytic efficiency of monensin breakdown appears to be inversely proportionally to toxicity of the compound in chicks, cattle, pigs, rats and horses, suggesting that species-specific metabolism of the compound influences toxicity (Nebbia et al., 2001).

Only two published studies are available investigating the effects of monensin on non-target aquatic organisms, both conducted on the lower levels of the aquatic community. Brain et al. (2004) conducted a seven-day static-renewal laboratory test assessing the toxicity of monensin towards *Lemna gibba*, reporting an EC_{50} of 998 μg/L for growth. They also noted visible injury, including beige/white banding, frond separation, and misshapen and underdeveloped fronds in the 1000 μg/L treatment and to a lesser extent in the 300 μg/L treatment. The other investigation was conducted in outdoor aquatic microcosms, assessing the changes in zooplankton assemblages when exposed to the compound over 50 days at concentrations ranging from 0.5 to 500 μg/L

(Hillis et al., 2007). They found that monensin did not affect community structure, although changes within specific Orders were observed. Significant decreases in the species richness of Rotifera and Cladocera occurred at the highest treatment (500 µg/L), beginning at days 8 and 28, respectively, and lasting until the end of the study. A decrease in the abundance of Rotifera populations was observed for the 50 µg/L treatments on only day 8, while in the highest treatment a significant decrease in Rotifera occurred from day 3 until the end of the study. In addition, a large decrease in larval Copepoda nauplii abundance was detected by day 14 in the 500 µg/L treatment, and population recovery did not occur by day 50. A concentration-dependent increase in Ostracoda abundance was observed on days 28 and 50, and days 14 to 50 for the 50 and 500 µg/L treatments, respectively. The significant changes observed in zooplankton populations were deemed likely due to indirect effects of monensin on the algal community, as indicated by changes in the chlorophyll-*a* concentrations within the microcosms over the course of the study. In the same study researchers noted visual observations of impact to plant material, including algae and *M. spicatum*, when exposed to monensin concentrations of 50 and 500 µg/L (Lissemore, 2005). The additional assessment of phytotoxicity of monensin to submersed rooted plants is warranted, as these plants may be exposed to the compound via water and sediment concentrations. As field-level observations indicate that aquatic macrophytes may be more sensitive to the antibiotic than initial laboratory based investigation suggested, further study investigating phytotoxicity of this compound is warranted.

1.2.2 10:2 saturated fluorotelomer carboxylic acid

Long-chain perfluorinated carboxylic acids (PFCAs), belonging to the class of fluorinated organic compounds, have been recognized as persistent and bioaccumulative environmental contaminants (Martin et al., 2003). They are globally distributed compounds, detected in biological systems including surface waters (Hansen et al., 2002; Boulanger, 2004) and biota (De Silva and Mabury, 2004b; Martin et al., 2004). Although the origin of the acids in the environment are not well defined, it is suggested that the degradation of fluorotelomer alcohols (FTOHs) via biodegradation (Dinglasan et al., 2004; Wang et al., 2005a) and atmospheric oxidation (Ellis et al., 2004) may serve as a relevant source of PFCAs (Lange, 2002; Dinglasan et al., 2004; Ellis et al., 2004; Wang et al., 2005a; Wang et al., 2006b). In addition, direct release of PFCAs salts used during perfluorinated polymer production may also serve as a potential source (Prevedouros et al., 2006). FTOHs are named according to the telomorization process by which they are produced, manufactured for use in the synthesis of other fluorinated organics and in order to be in incorporated into a variety of other commercial materials (Ellis and Mabury, 2003).

1.2.2.1 Sources

Several studies have demonstrated that the degradation of FTOHs via abiotic and biotic pathways, ultimately producing PFCAs, also generate corresponding-length fluorotelomer carboxylic acids as stable intermediary products. Both the saturated form of fluorotelomer carboxylic acids (FTCAs: $CF_3(CF_2)_nCH_2CO_2H$ n = 3, 5, 7, ...) and unsaturated fluorotelomer carboxylic acids (FTUCAs: $CF_3(CF_2)_{n-1}CF{=}CHCO_2H$ n = 3, 5, 7,

…), have been identified as breakdown products, with production of the unsaturated molecule associated with the elimination of the HF group from the α-carbon, perhaps via abiotic hydrolysis or biotically through enzyme oxidation (Dinglasan et al., 2004), instead forming a double-bond with the γ carbon (Scott et al., 2006). A study investigating the microbial degradation of 8:2 FTOH, found that fluorotelomer carboxylic acids account for up to about 50% of the mass balance of the original FTOH, representing a significant portion of degradation product (Dinglasan et al., 2004). Similarly, production of the saturated acid and unsaturated acids have been found to account for up to 27% and 6%, respectively, of the original FTOH mass after 28 days in a sewage sludge degradation study (Wang et al., 2005b). Atmospheric oxidation of FTOHs may also produce significant amounts of fluorotelomer carboxylic acids demonstrated in a smog chamber reaction study between FTOH and chlorine radicals, in which up to 26% of the starting material was transformed to the telomer acids (Ellis et al., 2003). Additionally, the metabolic biotransformation of FTOHs in biota may yield these acids. Hagen et al. (1981) detected the production of 8:2 FTCA when a single oral dose of 8:2 FTOH was administered to adult male rats. Saturated and unsaturated fluorotelomer carboxylic acids were also formed as metabolic products by isolated rat hepatocytes (Martin et al., 2005) and detected in plasma samples in additional rodent FTOH-exposure studies (Kudo et al., 2005; Fasano et al., 2006). Research has mainly focused on investigating the transformation pathways of 8:2 FTOH, although it is expected that the 10:2 FTOH may degrade similarly and produce corresponding chain length metabolites including the 10:2 FTCA, 10:2 FTUCA and the perfluorodecanoic acid (Dinglasan et al., 2004), though this has yet to be confirmed.

1.2.2.2 Physical and Chemical Properties

Fluorotelomer carboxylate molecules consist of a fluorinated alkyl chain connected to an ethanoic (-$CH_2C[O]OH$) functional group, and are named according to the molecular ratio of fluorinated carbons within the chain to hydrogenated carbon atoms. Several general properties of the fluorinated surfactant class of chemicals are applicable to the FTCAs and FTUCAs, imparted by the fluorinated tail chain. Surfactants are defined as surface-active molecules that are capable of efficiently lowering surface tension by selective adsorption at an interface (Kissa, 2001). Function of these chemicals is largely due to their amphiphilic nature, composed of a water soluble hydrophilic component and a water-insoluble hydrophobic component. This characteristic structure is evident in the fluorinated class of surfactants, with molecules made up of a hydrophillic head group located in position 1 of a fluorinated alkyl tail chain. The chain may be partially fluorinated, as is the case in FTOHs and fluorinated carboxylic acids, or perfluorinated in which the alkyl chain group is saturated with fluorine atoms. The substitution of hydrogen for fluorine atoms on the alkyl tail produces several unique properties. Unlike conventional oleophillic surfactants, fluorinated surfactants exhibit added oil repellency of the already hydrophobic tail chain (Patrick, 1971) and demonstrate stability under extreme environments, due in part to the high energy of the carbon-fluorine (C-F) bond (Fielding, 1979). The C-F single bond strength is reported to be ~484kJ/mol, the strongest observed in organic chemistry, lending resistance to breakdown in thermal, acidic, and basic environments, as well as degradation due to reducing and oxidizing agents (Kissa, 2001; Stock et al., 2004a;). Also, a strong polarity is observed between the C-F bonds due to the high electronegativity of fluorine (Key,

1997). Although fluorine is relatively small (1.47 Å), it is much larger then hydrogen (1.20 Å), resulting in fluorinated tail chains that are much bulkier and less flexible than equivalent hydro-carbon chains (Eaton and Smart, 1990). Additionally, a repellent sheath around the tail chain is formed by the tightly packed electron cloud around the carbon backbone increasing rigidity and molecular stability (Stock et al., 2004a).

The extent of fluorination of the alkyl chain influences the characteristics of the molecule, with increasing fluorination resulting in increased chemical stability (Stock et al., 2004; Kissa, 2001). Changes in the geometry and properties of the molecule are also observed as fluorinated surfactant carbon chain length is altered in order to release internal strain (Ellis and Mabury, 2003). A zigzag conformation is assumed when the tail chain is made up 8 or less carbons, zigzag and helical when the chain is 10 carbons long, and completely helical when the length increases to 12 + carbons (Wang et al., 1999). While the alkyl chain of fluorotelomer carboxylic acids demonstrate tremendous stability, the partially fluorinated compounds are subject to degradation in the environment, breaking down at the ethanoic functional group (Lehmler, 2005).

While data are not available regarding the specific physical and chemical properties of the compounds, several observations have been made by researchers. Based on their structure, fluorotelomer carboxylates demonstrate increased water solubility and lower vapour pressure compared to their precursors, the FTOHs, potentially resulting in the deposition of these compounds in surface waters (Loewen et al., 2005). Also, it has been observed that generally the solubility of fluorotelomer carboxylic acids decreases with increasing carbon chain length, and the saturated acids tend to be more soluble than the corresponding unsaturated compounds (MacDonald, 2005).

Research has also indicated that significant intra-molecular hydrogen bonding occurs between the hydrocarbon and fluorocarbon portions of FTOHs in a cyclic manner, thus resulting in high vapour pressures of the FTOHs (Ellis and Mabury, 2003; Stock et al., 2004a). It has been proposed that this type of hydrogen bonding may also occur in the FTCAs and FTUCAs (Ellis and Mabury, 2003).

1.2.2.3 *Fate and Environmental Concentrations*

Although fluorotelomer carboxylic acids, including the 10:2 FTCA (Table 1.2), have not been widely detected in the environment, it is expected that they may be present in waters (Loewen et al., 2005; Phillips et al., 2007). With the ubiquitous detection of FTOHs in the atmosphere (Martin et al., 2002; Stock et al., 2004b) and the widespread occurrence of PFCAs in the aquatic environment (Hansen et al., 2002; Boulanger, 2004), it is suggested that the intermediary breakdown products of the atmospheric oxidation of FTOHs, the fluorotelomer carboxylic acids, may subsequently be deposited in the aquatic environment. This is especially probable in relatively remote and rural locations where atmospheric NO_x concentrations are low (Phillips et al., 2007). Detection of FTCAs and FTUCAs in precipitation samples from across North America support this hypothesis (Loewen et al., 2005; Scott et al., 2006). It is also suggested that fluorotelomer carboxylic acids may potentially reach surface waters through the breakdown of polyfluorinated polymers and release of incorporated FTOHs in sewage treatment plants, and the subsequent microbial degradation of the alcohols (Dinglasan et al., 2004), although this occurrence has yet to be investigated.

As mentioned, there are few reports in the literature of the presence of fluorotelomer carboxylic acids in the environment. The acids were recently detected in a

study assessing the presence of a suite of polyfluoroalkyl compounds in bottlenose dolphin from the Gulf of Mexico and the Atlantic Ocean. Plasma samples from the organisms were found to contain ~ 1 ngg^{-1} wet weight levels of 8:2 and 10:2 FTUCAs, while FTCA concentrations were not detected. In another recent investigation, researchers detected the 8:2 FTCA, the 8:2 FTUCA, the 10:2 FTCA, and the 10:2 FTUCA in the liver samples from two ringed seal population in the Canadian Arctic, Arviat (Western Hudson Bay) and Resolute Bay (Lancaster Sound) (Butt et al., 2007). However, the concentrations of the 8:2 FTCA and FTUCA were below the method detection limits, and quantification problems with the 10:2 FTCA in samples prevented analysis of the levels in the tissue. The 10:2 FTUCA was found to range from<0.75 to 9.6 ng/g weight wet in Arviat samples, and from <0.75 to 1.3 ng/g wet weight in Resolute Bay samples.

Loewen et al. (2005) present the first report of the 10:2 FTCA and the 10:2 FTUCA in the environment, detected in rainwater collected from a single rainfall event in Winnipeg, Manitoba, Canada, an urban centre. Analysis of the samples determined concentrations of acids to be 0.30 ± 0.04 ng/L (n = 3) of 10:2 FTCA and 0.12 ± 0.01 ng/L of 10:2 FTUCA. They also detected concentrations of the 8:2 FTCA and FTUCA of 1.00 ± 0.08 ng/L (n = 3) and 0.12 ± 0.02 ng/L (n = 3), respectively. The researchers hypothesize that both the FTCA and FTUCA compounds are produced abiotically, however, the differences in concentrations of the 8:2 and 10:2 FTCA compared to the equal concentrations observed across the FTUCAs suggest that the transformation rates of the 8:2 and 10:2 FTCA to corresponding FTUCAs may differ. The concentrations of FTCAs and FTUCAs were also determined in an investigation conducted using wet only

precipitation samples from nine sites in North America (Scott et al., 2006). Fluorotelomer acids, including the 8:2 FTCA , 8:2 FTUCA, 10:2 FTCA and 10:2 FTUCA were detected at all sample sites at concentration ranging from <0.07 – 8.6 ng/L, the 10:2 FTUCA infrequently detected above the minimum detection limit (0.07 ng/L). These are the only reports of fluorotelomer carboxylic acids in the environment, likely due in part to the difficulty in developing an appropriate methods for detection (Loewen et al., 2005).

1.2.2.4 Toxicity

The toxicity of fluorotelomer carboxylic acids is largely unexamined, with a single investigation available reporting toxicity data on these compounds to aquatic organisms. This laboratory-based study assessed the effects of the 4:2, 6:2, 8:2, and 10:2 FTCAs and FTUCAs on three freshwater organisms: the pelagic microcrustacean *Daphnia magna*, the benthic macroinvertebrate *Chironomus tentans*, and the floating macrophyte *Lemna gibba*, using standard test methods and a static renewal dosing regime when appropriate (MacDonald, 2005; Phillips et al., 2007). The researchers employed a tiered approach, first using each of the eight test compounds in initial acute range-find assays in order to determine the compounds and concentrations most suitable for further acute definitive tests. Endpoints evaluated in the acute tests included growth, and mortality, in the case of animal tests, and the results from these assays were then used to select the compounds and concentrations for the chronic life cycle assays. The life cycle tests conducted on *C. tentans* and *D. magna* included reproductive endpoints, as well as emergence for *C. tentans*.

Generally, they found that toxicity is species-dependent and is influenced by the fluorinated carbon chain length and the saturation status of the α-β carbon bond, ie.

saturated versus unsaturated molecules. Of the acids with chain lengths ≤ 8 fluorocarbons, *L. gibba* was most sensitive of the three species. However, all of the species were more sensitive to the acids with chain lengths ≥ 8 fluorocarbons, and an increase in toxicity was determined associated with increasing chain length. This trend continued in the case of *D. magna* through chain lengths of 10, although increasing toxicity with increasing chain length was not observed for either *C. tentans* or *L. gibba* beyond the 8:2 FTCAs (Phillips et al., 2007). Of the three species, *D. magna* was most sensitive to fluorotelomer carboxylic acids with chain lengths > 8. Toxicity tests conducted using the 10:2 FTCA specifically, determined that *D. magna* were the most sensitive to the compound, with 48-hour LC_{50} and EC_{50} (immobility) values of 0.06 (0.04, 0.11) mg/L and 0.03 (0.02, 0.03) mg/L, respectively, with values in parentheses representing the 95% confidence intervals for each point estimate. The 10:2 FTCA proved least toxic to *C. tentans*, with 10-day LC_{50} and EC_{50} (growth) values greater than 16.27 mg/L. The 7-day EC_{50} value (frond number) for exposure of 10:2 FTCA to *L. gibba* was greater than 4.30 mg/L. Phillips et al. (2007) also found that saturated fluorotelomer carboxylic acids were typically more toxic than their unsaturated counterparts, except in the case of the 8:2 FTCA for *L. gibba* and the 10:2 FTCA for *C. tentans* and *L. gibba*.

A relationship between perfluorinated carbon chain length and relative toxicity is observed in several other studies conducted using PFCAs (Upham et al., 1998; Boudreau, 2002). In an investigation of the toxicity of PFCAs to aquatic organisms, no toxic effects were observed for any test species for the 4 - 7-C PFCAs at concentrations up to 1 g/L, while significant decreases in growth with exposure of *Chlorella vulgaris*, *Selenastrum*

capricornutum and *L. gibba* to the 3-C and 8 - 10-C PFCAs were reported (Boudreau, 2002; MacDonald, 2005). Also worth noting, Phillips et al. (2007) established that the fluorotelomer carboxylic acids are 1 - 4 orders of magnitude more toxic than the PFCAs, although PFCAs are the focus of a great deal of research in the field of fluorinated organics. This demonstrates that further investigation of the environmental fate, concentrations and toxicity of these compounds is of importance.

1.2.3 Atrazine

The Geigy Chemical Company of Switzerland first patented atrazine (Table 1.2) in 1958 (USEPA, 1994). Since its registration in the United States in 1959, atrazine has become one of the most widely used herbicides in North America (Lytle and Lytle, 2005). The triazine herbicide is principally applied pre-emergence for crop protection against selected broadleaf and grassy weeds in corn, sorghum, and sugarcane production, but may also be applied preplant and postemergence and in other crop and landscape systems (WSSA, 1989). Atrazine is a photosynthetic inhibitor that acts by binding to the QB-binding niche on the D1 protein of the photosystem II complex, thus interfering with electron transport under light conditions, and ultimately terminating the Calvin cycle (Fuerst and Norman, 1991; Fairchild et al., 1998). Generally, the compound is more toxic towards plants than animals because it acts on the photosynthetic metabolic pathway (Giddings et al., 2005).

As a consequence of the high agricultural use of the compound, atrazine is widely detected as a contaminant of both surface and groundwater systems, with approximately 0.1 to 3.0 % of atrazine sprayed on fields lost into aquatic systems (Jones et al., 1982; Gaynor et al., 1995). Contamination is of greatest concern in mid-western corn-growing

regions of the United States and to lesser extent, Ontario, Canada (Giddings et al., 2005). The compound is detected at concentrations most frequently ranging from 5 μg/L to 20 μg/L (Solomon et al., 1996), but at times may reach as high as 1 mg/L in reservoirs adjacent to treated fields (Kadoum and Mock, 1978). The known physical and chemical properties of the compound are presented in Table 1.5. Generally, atrazine is considered moderately mobile and moderately persistent in the environment. The low Henry's law constant and vapour pressure of the compound indicate that is is unlikely to be transported from water or soil via volatilization. Atrazine does not degrade rapidly in soils, with a half-life of ranging between 37 days and 3 to 5 years in agricultural systems (Armstrong et al., 1967). The compound enters surface waters primarily through surface runoff (Glotfelty et al., 1984), with propensity for movement in the dissolved state from treated soils, and is unlikely to adsorb strongly to sediments (Solomon et al., 1996). Its half-life in water is estimated to range from 3 to 90 plus days (Giddings et al., 2005).

In November 1994, the United States EPA put forth a request for special review of atrazine and other registered triazine herbicides, under provisions of the United States Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (USEPA, 1994). While the EPA presented the request based on human health concerns, they also noted concern over, "the potential ecological impacts of ground and surface water contamination resulting from the use of products containing triazines." In response, the main manufacturer of atrazine, Ciba Crop Protection, requested that ECORISK, Inc. of Ferndale, Washington, U.S, assemble a panel consisting of independent and multidisciplinary members to conduct an aquatic ecological risk assessment of atrazine. The assessment was conducted using established methods (USEPA, 1992; SETAC, 1994)

and a report was submitted to the EPA in 1995 (Ciba-Geigy, 1995). Additionally, the panel published their results in the peer-reviewed literature (Solomon et al., 1996). The Atrazine Ecological Risk Assessment Panel was reconvened in 1999 to update the risk assessment to reflect new data and recognized assessment methods (ECOFRAM, 1999). The results were again published (Giddings et al., 2005). Due to the extensive body of existing toxicity and environmental fate data for atrazine, and the prior existing environmental risk assessments (Eisler, 1989; Huber 1993), the assessments conducted by the panel represent a comprehensive effort, with several noted uncertainties associated with the process. Both the consideration of responses mediated through endocrine and developmental mechanisms, and effects on amphibians were not evaluated in detail (Giddings, et al., 2005).

As discussed in the most recent ecological risk assessment, freshwater macrophytes, freshwater phytoplankton, and saltwater phytoplankton demonstrate comparable sensitivity to atrazine; the geometric means for acute toxicity of each group range between 85 and 123 μg/L (Giddings et al., 2005). A summary of the available laboratory-based toxicity data for ten freshwaters species is presented in Table 1.6. While the mean of the data for freshwater macrophytes falls within the range of other plant groups, the data also demonstrate differing sensitivity to atrazine within freshwater macrophytes associated with varying species and monitored endpoints. *E. canadensis* is found to be the most sensitive freshwater plant based on the acute laboratory-derived estimate of a 14-day EC_{50} (wet weight) of 21 μg/L (Fairchild et al., 1998), while *M. spicatum* was determined to be significantly less sensitive. About 3.7 mg/L of atrazine is required to produce a 50% reduction in the number of branches produced by *M. spicatum*

after 5 days (Bird, 1993), and about 91 μg/L to inhibit stem dry weight by 50% over 28 days (Kemp et al., 1985).

To investigate the effects of atrazine on aquatic communities, over 30 simulated field studies have been conducted (Giddings et al., 2005). The data from these microcosm and mesocosm studies were incorporated into the effects characterization stages of the ecological risk assessments in order to provide measurement endpoints that more closely reflect community-level responses, such as primary productivity and community structure (Solomon et al., 1996; Giddings et al., 2005). These types of studies also help to put laboratory toxicity data into perspective. In general, the literature indicates that exposure to 20 μg/L atrazine or less, rarely results in an aquatic community level response, and that recovery from effects often occurs at exposures of up to 50 μg/L atrazine (Giddings et al., 2005). These simulated field-level assessments have focused largely on investigating the response of phytoplankton and periphyton to atrazine, while far fewer studies specificially examine the toxicity of atrazine to freshwater macrophytes (Table 1.7). The results presented in Table 1.7 correspond to those studies that investigate the response of freshwater macrophyte communities to atrazine exposure under model ecosystem conditions, and that were deemed appropriate for use in the ecological risk assessment process (Giddings et al., 2005). Several other microcosm and mesocosm studies are not included due to a lack or replication in the study designs, inadequate description of methods or results, or other issues rendering them difficult to analyze and interpret. Because of differences in study systems, community composition, and monitored endpoints, it is not easy to generalize about the response of macrophyte communities to atrazine, especially considering several papers present seemingly

conflicting results. Under pond microcosm conditions, Carney (1983), deNoyelles et al. (1989), and deNoyelles et al. (1994) report that macrophyte biomass and species distributions were unaffected at an exposure concentration of 20 μg/L atrazine. Correspondingly, in a simulated-pond study conducted by Fairchild et al. (1994), they found that a higher concentration of atrazine (50 μg/L) produced no effect on total macrophyte biomass, although a shift from *Najas* sp. to macroalga *Chara* was observed. Examined under a similar study design, Kettle (1982), and Kettle et al. (1989) report reduced macrophyte densities at concentrations as low as 20 μg/L atrazine, although these results may have been influenced by the presence of grass carp in the test units. Under semi-field conditions, all of the examined macrophyte communities were effected by concentrations ≥ 100 μg/L atrazine.

The use of microcosm and mesocosm testing also allows for the observation of community level recovery following exposure to a toxicant. In the examination of atrazine effects on a wetland microcosm communitiy, gross primary production significantly decreased upon exposure to concentrations of 10, 100, and 1000 μg/L (Johnson, 1986). At the two lowest treatment levels however, recovery of primary production was observed after only 7 days. These results are not unexpected, as atrazine binding is found to be reversible in individual plants (Jensen et al., 1977), potentially linked to enzymatic detoxification of the compound, vacuolization within the plant, or release of previously bound atrazine back into the water (Kemp et al., 1985; Solomon et al., 1996). In another study, following short-term exposure (3 hours) of *Potamogeton perfoliatus* to atrazine and subsequent washing with uncontaminated medium, the plants demonstrated complete photosynthetic recovery (Jones et al., 1983). Photosynthetic

recovery of macrophytes exposed to ≤ 100 μg/L atrazine over a 4 week duration has also been observed in the laboratory (Kemp et al., 1985). Despite the fact that direct toxic effects of atrazine on aquatic plants may occur under certain circumstances, from repeated inputs of high concentrations into a reservoir for example, the risk assessments presented to the EPA concluded that atrazine does not pose an ecologically significant risk to most aquatic environments in North America (Solomon et al., 1996; Giddings et al., 2005). This evaluation took into account the fact that inhibitory effects on aquatic plants are often transient and unless exposure is sustained for weeks, recovery of plant community productivity is likely.

Atrazine was utilized in our investigation for several reasons. Laboratory testing and model ecosystem studies have shown that atrazine may cause effects in non-target freshwater plant species, including *E. canadensis* and *M. spicatum*, at environmentally relevant concentrations (Forney and David, 1981; deNoyelles et al., 1989; Fairchild et al., 1998). As a known phytotoxicant, we selected the compound to serve as a model chemical stressor. By using a range of test concentrations that are recognized in the literature to produce an inhibitory effect in these selected species, we were able to investigate several primary research objectives as listed below. Additionally, due to potential unexpected ecological responses (Lydy et al., 2004; Hayes et al., 2006; Trimble and Lydy, 2006), the compound has come under renewed scrutiny and the collection of additional microcosm based data will add to the body of literature and allow for a more extensive analysis of risk to aquatic macrophytes to be undertaken.

1.3 HYPOTHESES

It was hypothesized that environmentally relevant concentrations of monensin and predicted environmental concentrations of 10:2 FTCA would prove phytotoxic to aquatic macrophytes when evaluated in a microcosm-based testing scheme under chronic exposures. If this hypothesis is correct it would indicate that acute laboratory tests are not necessarily predictive of simulated field-level responses. It was also hypothesized that intra- and inter-species plant relations would modify the response of aquatic plants to atrazine, a phytotoxicant, thus the field-level response of individually-grown freshwater macrophytes are not expected to reflect the responses of plants grown in pure and mixed assemblages. This would indicate that individual-based toxicity testing systems may over- or underestimate toxicity in simulated field studies. Finally, it was hypothesized that those plants and planting systems with higher relative growth rates would be more sensitive to environmental toxicants.

1.4 RESEARCH OBJECTIVES

The lack of data available investigating the response of aquatic macrophytes when exposed to monensin and 10:2 FTCA and the suggested likelihood that these contaminants are present in the aquatic environment establishes the need for evaluation of the compounds' phytotoxicity. Also, the common use of individually grown aquatic plants in the evaluation of contaminant toxicity lends to the question of whether this practice is appropriate and if observed responses are reflective of those expected in higher levels of biological organization. Four research objectives were developed with these considerations in mind:

1) Evaluate the toxicity of monensin, and 10:2 FTCA to aquatic plants (*Lemna gibba, Myriophyllum spicatum*, *Elodea canadensis*, and *Egeria densa*) under microcosm conditions.

2) Assess the ability of the standard laboratory-based plant assays (utilizing *Lemna gibba*) to predict the field level response of aquatic macrophytes to the stressor.

3) Investigate how well macrophyte responses observed in the individual-plant based microcosm test reflect responses observed in model populations and two-species communities using biomass, relative growth rate measures, and effective concentrations as mechanisms for comparison, and monensin, 10:2 FTCA and atrazine as chemical stressors.

4) Investigate how intra- and inter-species plant relations may modify plant response to a toxicant by conducting testing on plants grown in monoculture and mixed cultures and at varying planting densities.

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Table 1.2 Chemical names and structures for test compounds.

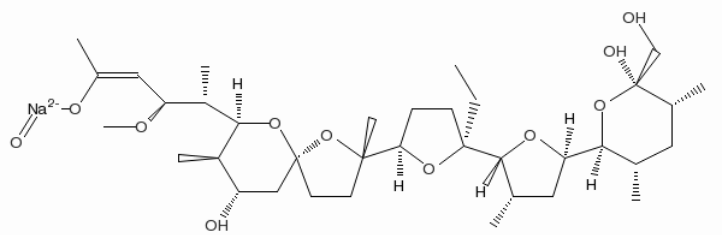
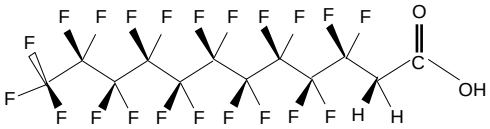
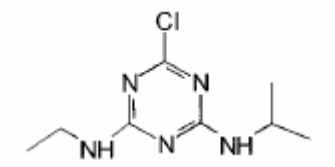
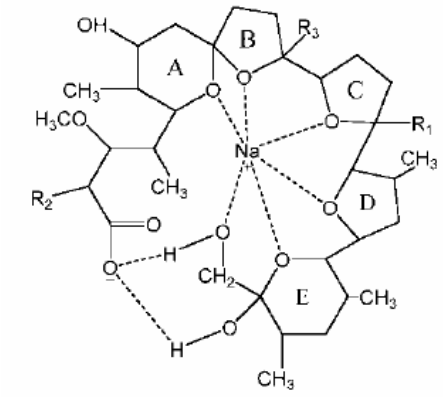
Compound	Chemical Name and Structure
Monensin	2-(2-ethyloctahydro-3'-methyl-5'-tetrahydro-6-hydroxy-6-(hydroxymethyl)-3,5-dimethyl-2H-pyran-2-yl][2,2'-bifuran]-5-y1]-9-hydroxy-~methoxy-or,y'2,8-tetramethyl-1,6-dioxaspiro(4,S]decane-7-butanoic acid 
10:2 saturated fluorotelomer carboxylic acid	2H,2H-perfluorododecanoic acid 
Atrazine	2-chloro-4-ethylamino-6-isopropylamino-1-*s*-triazine 

Table 1.3 Structures for monensin factors A, B, C, and D (adapted from Carlson and Mabury, 2006).



Factor	R_1	R_2	R_3
MON A	CH_2CH_3	CH_3	CH_3
MON B	CH_3	CH_3	CH_3
MON C	CH_3	CH_2CH_3	CH_3
MON D	CH_3	CH_3	CH_2CH_3

Table 1.4 Chemical and physical properties of monensin.

CAS number	22373-78-0	
Molecular weight	670 (acid), 692.85 (salt)	
Molecular formula	$C_{36}H_{62}O_{11}$ (acid), $C_{36}H_{61}O_{11}Na$ (salt)	(Elanco, 1989)
Melting point	103-105°C (acid) 267-269°C (sodium salt)	(Elanco, 1989)
Water solubility	4.8–8.9 mg/L	(Lissemore et al., 2006)
Vapor pressure	low, non-volatile	(Elanco, 1989)
Henry's law constant	not available	
Log K_{OW}	2.8 – 4.2	(Elanco, 1989)
pK_a	6.65	(Lissemore et al., 2006)
Log K_{OC}	2.1 – 3.8	(Sassman and Lee, 2007)
Hydrolysis	stable for 30 d at pH 5-9 at 25ºC	(Elanco, 1989)
Aqueous photolysis	*t*½ 44 d at pH 7	(Elanco, 1989; Lissemore et al., 2006)
Aerobic soil metabolism	*t*½ 1.6 - 3.3 d, manure-amended *t*½ 1.2 - 3.8 d, manure-free	(Sassman and Lee, 2007; Carlson and Mabury, 2006)
Anaerobic soil metabolism	*t*½ 28 d, manure pile	(Donoho, 1984)

Table 1.5 Chemical and physical properties of atrazine (adapted from Solomon et al., 1996).

CAS number	1912-24-9
Molecular weight	215.70 g/mol
Molecular formula	$C_8H_{14}N_5Cl$
Melting point	175–177ºC
Water solubility	33 μg/mL at 22ºC
Vapor pressure	2.89 x 10^{-7} mm Hg at 25ºC
Henry's law constant	2.48 x 10^{-9} atm m^3 mol^{-1}
Log K_{ow}	2.68 at 25ºC
Hydrolysis	stable for 30 d at pH 5-9 at 25ºC
Aqueous photolysis	natural light: *t*½ 335 d at pH 7 mercury lamp: *t*½ 17.5 h at pH 7
Soil photolysis	natural light: *t*½ 12 d mercury lamp: *t*½ 5 d xenon lamp: *t*½ 45 d
Aerobic soil metabolism	*t*½ 146 d, CA loam
Anaerobic soil metabolism	*t*½ 77 d, CA sandy loam *t*½ 159 d, CA loam
Anaerobic aqueous metabolism	*t*½ 608 d, GA sandy clay

Table 1.6 A summary of the effects of atrazine on the growth and development of freshwater aquatic macrophytes.

Macrophyte Species	Test Type	Endpoint	Exposure Duration	EC_{50}/ IC_{50} (μg/L)	Reference
EIodea canadensis	Laboratory	Length	21 days	109	Forney and Davis (1981)
	Laboratory	Length	28 days	80	Forney and Davis (1981)
	Laboratory	Wet weight	14 days	21	Fairchild et al. (1998)
Ceratophyllum demersum	Laboratory	Wet weight	14 days	22	Fairchild et al. (1998)
Lemna gibba	Laboratory	Frond number	5 days	170	Hughes et al. (1988)
Lemna minor	Laboratory	Frond number	10 days	56	Kirby and Sheahan (1994)
	Laboratory	Frond weight	10 days	60	Kirby and Sheahan (1994)
	Laboratory	Total chlorophyll	10 days	62	Kirby and Sheahan (1994)
	Laboratory	Frond number	96 hours	92	Fairchild et al. (1998)
Lemna paucicostata	Laboratory	Leaf surface area	10 days	69	Retzlaff (1992)
Myriophyllum heterphyllum	Laboratory	Frond number	14 days	132	Fairchild et al. (1998)
Myriophyllurn sibiricum	Laboratory	Root length	14 days	1130	Roshon (1997)
	Laboratory	Dissolved O_2	14 days	1999	Roshon (1997)
	Laboratory	Nodes per cm	14 days	2066	Roshon (1997)
Myriophyllum spicatum	Laboratory	Length	28 days	1104	Forney and Davis (1981)
	Laboratory	Photosynthesis	24 hours	104	Jones and Winchell (1984)
	Indoor microcosm	Stem dry weight	28 days	91	Kemp et al. (1985)
	Indoor microcosm	Oxygen Production	28 days	117	Kemp et al. (1985)
	Laboratory	Branch number	5 days	3700	Bird (1993)
Najas sp.	Laboratory	Frond number	14 days	24	Fairchild et al. (1998)
Potamogeton perfoliatus	Laboratory	Length	21 days	474	Forney and Davis (1981)
	Laboratory	Dry Weight	21 days	907	Forney and Davis (1981)
	Laboratory	Photosynthesis	24 hours	77	Jones and Winchell (1984)
	Laboratory	Photosynthesis	4 hours	80	Jones et al. (1986)
	Indoor microcosm	Stem dry weight	28 days	30	Kemp et al. (1985)
	Indoor microcosm	Oxygen Production	28 days	55	Kemp et al. (1985)

Table 1.7 A summary of the effects of atrazine on freshwater plant communities in microcosms and mesocosms studies (adapted from Giddings et al., 2005).

Conc (μg/L)	Experiment System & Species	Measurment endpoint	Study conditions	Response	Reference
10	Static wetland microcoms	Biomass, productivity	30 day duration; single addition (slurry)	No effect on biomass; productivity slightly reduced; recovery after 7 days	Johnson (1986)
20	Pond mesocosms	Biomass, abundance	1 year duration; single addition	Biomass reduced; *Chara* replaced *Potamogeton;* recovery > 1 year	Kettle (1982); Kettle et al. (1987)
20	Pond mesocosms	Biomass, abundance	1 year duration; single addition	Biomass and species distribution unaffected	Carney (1983); deNoyelles et al. (1989); deNoyelles et al. (1994)
50	Static pond mesocosms	Biomass, species abundance, community productivity	15 weeks duration; single addition (slurry)	No effect on total macrophyte biomass; *Chara* replaced *Najas*; productivity decreased slightly; recovery > 15 weeks	Fairchild et al. (1994)
100	Static wetland microcoms	Biomass, productivity	30 day duration; single addition (slurry);	No effect on biomass; productivity slightly reduced; recovery after 7 days	Johnson (1986)
100	Pond mesocosms	Abundance	1 year duration; single addition	Macrophyte density unaffected; species distribution changed; recovery > 1 year	deNoyelles et al. (1982); Carney (1983); deNoyelles et al. (1989)
500	Pond mesocosms	Abundance	1 year duration; single addition;	Macrophyte cover decreased; recovery > 1 year	deNoyelles et al. (1982); Kettle (1982); Kettle et al. (1987); deNoyelles et al. (1989)
1000	Static wetland microcoms	Biomass, productivity	30 day duration; single addition (slurry)	Biomass and productivity decreased; recovery > 30 days	Johnson (1986)

Figure 1.1 (A) The aquatic microcosms used in these studies to examine the effects of environmental contaminants on macrophytes. Each microcosm is approximately 3.9 m in diameter with a height of 1 m to the top of the standpipe, holding approximately 12 000 L. (B) An empty microcosm filled with sediment trays. (C) The floating wooden corrals (38 x 14 cm), subdivided into 3 sections, used as test containers for *L. gibba* plants. (D) The planting trays that hold "cone-tainers". (E) The plastic pots in which mixed and mono-cultures of submergents were grown, used in the monensin and 10:2 FTCA studies. (F) The plastic sterilite containers (34.9 x 20.6 x 32.1 cm deep) in which mixed and mono-cultures of submergents were grown in the atrazine investigation (Sterilite Corporation, Townsend, MA). (G) The "cone-tainers" (Stuewe & Sons, Corvallis, OR) in which individually grown submergent macrophytes were grown, 115 mL (left) and 164 mL (right).

2 MONENSIN IS NOT TOXIC TO AQUATIC MACROPHYTES AT ENVIRONMENTALLY RELEVANT CONCENTRATIONS

2.1 ABSTRACT

Monensin, a common livestock feed additive, has been detected in surface waters around areas of intensive agriculture. The effect of this ionophore antibiotic on floating (*Lemna gibba*) and submersed (*Myriophyllum spicatum, Elodea canadensis*, *Egeria densa*) freshwater macrophytes was investigated under seminatural field conditions using 12,000 L of outdoor microcosms. Exposure concentrations of 0, 12, 25, 50, and 100 μg/l (n = 3) were evaluated over a 35-day period. Submersed plants were grown individually in 115-ml plastic "cone-tainers" and assessed for various growth and pigment endpoints. *E. canadensis* and *M. spicatum* also were grown in assemblages to represent model populations and two-species communities. Few statistically significant differences from control organisms were observed for any of the monitored endpoints. Overall, monensin is deemed unlikely to cause toxicity in freshwater macrophytes at current environmental concentrations. However, the ability to characterize toxicity in macrophytes is based partially on the relative growth rates (RGRs) of the plants. The greater the RGR, the more sensitive the assay may be to contaminants. The RGRs of *E. canadensis* and *M. spicatum* grown in model populations and communities were found to be significantly higher than the RGRs of plants grown individually. This implies that the "cone-tainer" method, although simple and easy to perform, may underestimate toxicity in simulated field studies.

2.2 INTRODUCTION

Veterinary pharmaceuticals are commonly used in agriculture for preventive, therapeutic, and growth promotion purposes. Although these compounds have been in use for many years, only recently have they been identified as an important class of environmental contaminants (Halling-Sorensen et al., 1998; Daughton and Ternes, 1999; Boxall et al., 2003). Research has documented widespread occurrences of these chemicals in environmental matrices, including soils (Christian et al., 2003) and surface waters (Kolpin et al., 2002; Richardson, 2003), due to the development of new detection methods.

Monensin, an ionophore antibiotic, is used exclusively in veterinary applications. Currently, in North America it is commonly administered as both a coccidiostat for poultry and as a feed additive for growth promotion in livestock production. Studies have indicated that orally administered monensin is partly absorbed by cattle and poultry, rapidly and extensively metabolized, excreted in the bile, and almost exclusively eliminated in the feces (Herber and Van Duyn, 1969; Herberg et al., 1978; Donoho 1984). Approximately 50% of the oral dose administered to cattle is excreted as monensin metabolites, and the remainder as parent molecule (Donoho et al., 1978). Subsequently, the land application of manure containing the unchanged compound likely serves as the most significant source of monensin to the environment (Halling-Sorenson et al., 1998). To a lesser extent, the compound may enter the environment through leakage or overflow of animal waste storage structures (Kolpin et al., 2002) and through direct input of animal waste to surface waters (Lissemore et al., 2006).

Once in agricultural soil, the degradation of monensin is fairly rapid, with a dissipation half-life estimated to range from 1.2 to 3.8 days depending on soil properties (Carlson and Mabury, 2006; Sassman and Lee, 2007). The compound also shows a propensity for sorption to soils and sediments (Carlson and Mabury, 2006; Kim and Carlson, 2006), with a log K_{OW} of 2.8 to 4.2 (Elanco Products, 1989) and a reported organic carbon normalized sorption coefficient (log K_{oc}) of 2.1 to 3.8, as determined through investigation using various agricultural soils (Sassman and Lee, 2007).

Despite the potential for breakdown and sorption of monensin in soils, the compound frequently has been detected in surface waters and sediments around areas of intense agricultural activity (Hao et al., 2006; Kim and Carlson, 2006; Lissemore et al., 2006). In an agricultural watershed in Ontario, Canada, surface water concentrations have been detected ranging from 6.2 to 1172 ng/L and varying temporally according to manure and biosolid application times (Lissemore et al., 2006). This shows that the compound is moving into the aquatic environment, likely transported bound to particulate matter during surface runoff events after rainfall or irrigation (Lissemore et al., 2006). Because the compound is fairly stable and not readily degraded in surface waters under seminatural field conditions (Lissemore, 2005), chronic exposure to aquatic organisms is a possibility. Additionally, monensin may move readily in and out of solution, potentially desorbing from sediments and soils into surface waters depending on existing conditions (Lissemore, 2005) and thus facilitating semicontinuous release of the compound.

Monensin, a monocarboxylic polyether produced by the fermentation of *Streptomyces cinnamonensis*, is sold commercially as a sodium salt. It is defined by its

ability to induce ion passage selectively across biologic membranes through the formation of dynamically reversible cation complexes (Pressman, 1976; Pressman and Fahim, 1982). This ability imparts toxic function against target microbes. The disruption of cellular membranes and the alternation of ionic balance also result in nontarget toxicity to various plant and animal species (Davidonis, 1993; Spinosa et al., 1999). Findings show that monensin is selectively phytotoxic to several terrestrial plant species when exposed to foliar and root applications, significantly affecting plant growth rates (Mollenhauer et al., 1986; Hoagland, 1996).

Brain et al. (2004), after conducting a 7-day static renewal laboratory test assessing the toxicity of monensin toward *Lemna gibba*, an aquatic macrophyte, reported an EC_{50} of 998 µg/L for growth. These authors also reported visible injury, including beige/white banding, frond separation, and misshapen and underdeveloped fronds with the 1,000-µg/L treatment, and to a lesser extent with the 300-µg/L treatment.

A recent study using microcosms to assess the changes in zooplankton assemblages when exposed to monensin found by visual observation that plant material, including algae and *Myriophyllum spicatum spicatum*, is affected by monensin concentrations of 50 and 500 µg/L (Lissemore, 2005; Hillis et al., 2007). Significant changes observed in the zooplankton populations were deemed likely due to indirect effects of monensin on the algal community. The additional assessment of monensin phytotoxicity to submersed rooted plants is warranted because these plants may be exposed to the compound via water and sediment concentrations.

Toxicity tests assessing the effects of environmental contaminants on nontarget plants commonly rely on the evaluation of responses at an individual level to predict

outcomes at higher levels of biologic organization. This is largely the practice in ecotoxicology as a whole, with a focus on physiologic mechanisms of toxicity and measurement of organism-level responses such as survival and growth (Forbes and Calow, 1999; Preston, 2002). Because relationships between lower-level responses and higher-level effects are not always direct or clear, the consideration of only organism-level endpoints or low-density populations may result in the over- or underestimation of contaminant effects to the larger community (Kramarz et al., 2005; Raimondo and McKenney Jr, 2006).

Many factors may influence the response of a biologic community to a toxicant (Calow and Forbes, 2003), including population density effects (Simkiss et al., 1993; Sibly et al., 2000) and interspecies interactions (Preston, 2002). Laboratory-based studies on submersed macrophytes that assess the potential phytotoxic effects of a contaminant, including the standard assay conducted on *Myriophyllum sibiricum* (ASTM, 1999a), use the individual approach. The results of these single-species tests are used in the lower tiers of ecologic risk assessment. Higher-tier studies investigating effects of contaminants on aquatic macrophytes often follow the same pattern. Aquatic plants are grown as part of a larger model ecosystem, and the responses of a few individuals grown in independent test units are sometimes used to predict the responses of entire populations or communities of plants (Stanley, 1974; Watkins and Hammerschlag, 1984; Hanson et al., 2001; Davies et al., 2003).

These individual-based test conditions are not truly representative of freshwater ecosystems, in which various types of interactions may occur among individuals of the same species or among neighboring plants of differing species. Significant reductions in

biomass production (Driever et al., 2005) and growth rates (Wang et al., 2005) have been observed in response to high densities and crowding within populations of plants. Interrelationships between species such as shading, allelopathy, and competition for resources (Agami and Waisel, 2002) also may significantly modify plant biomass and growth rates (Wu and Yu, 2004), with potentially larger implications for plant community structure and function.

Further investigation is required for a better understanding of how these complex population and community interactions may alter the response of aquatic plants to a toxicant. In general, the literature indicates that the faster the relative growth rate (RGR) and the longer the test duration, the lower the concentration of toxicant needed for an impact on a specific growth rate and the more sensitive a toxicity test conducted on aquatic macrophytes may be to a chemical stressor (Huebert and Shay, 1993; Cedergreen et al., 2004). This indicates that the RGR measures of plants grown under various test systems may serve as an indirect indicator of the potential toxicologic sensitivity of plants grown using various toxicity assessment methods.

The two main objectives of this study were (1) to evaluate the phytotoxicity of monensin to four common aquatic macrophytes, *Myriophyllum spicatum* (Eurasian watermilfoil), *Elodea canadensis* (Canadian waterweed), *Egeria densa* (Brazilian waterweed), and *Lemna gibba* (duckweed) under seminatural field conditions using microcosms, and (2) to investigate whether macrophyte responses to a toxicant observed in an individual plant–based test are reflective of responses observed in model populations and two-species communities using biomass and RGRs as mechanisms for comparison.

2.3 METHODS AND MATERIALS

2.3.1 Test facility

The 15 outdoor microcosms used in this study are located at the University of Guelph Microcosm Facility at the Guelph Turfgrass Institute, Ontario, Canada (Figure 1.1A). Each of the facility's 30 artificial ponds are approximately 1.2 m deep, with a water depth of 1 m, a surface area of 11.95 m^2, and a diameter of 3.9 m. The microcosms, sunk into the ground and lined with black, food-grade polyvinyl chloride, have tops flush with the surface (Fox Pools Canada, Burlington, ON, Canada). They each hold approximately 12,000 L of water, which is supplied from an adjacent spring-fed irrigation pond (62 × 62 × 4 m deep).

To establish a model freshwater ecosystem, 45 plastic propagation trays (52 × 25 × 7 cm; Canadian HydroGardens, Ancaster, ON, Canada) containing an amended sediment mix (Waterdown Garden Supply, Troy, ON, Canada) were added to the bottom of each microcosm (Figure 1.1B). The sediment comprised a three-way mixture of equal parts sand, loam, and organic matter by volume and covered about 49% of each microcosm floor.

Water was circulated between the microcosms and the irrigation pond at a rate of 12,000 L/day for 3 weeks before treatment with monensin. Circulation was undertaken to reduce variability within and between microcosms, and thus to provide consistent physiochemical properties and biologic communities/assemblages. During circulation, four pots of macrophytes (*M. spicatum*) were placed in each microcosm. These plants, obtained from several on-site untreated microcosms, were not used in the assessment of

effects. Circulation was terminated 2 days before treatment (July 5, 2005) for the creation of self-contained systems.

2.3.2 Treatment

Exposure concentrations of 0, 12, 25, 50, and 100 µg/L (n = 3) were selected for evaluation. Treatment of the microcosms took place on July 7, 2005, with each concentration randomly applied to three separate microcosms. Monensin sodium, supplied by Elanco Inc. (Greenfield, IN, USA), was weighed out on the day of dosing and dissolved in 100 mL of acetone. The treatments were mixed in amber bottles, shaken, and added to the microcosms while microcosm water was simultaneously agitated with a stirrer to promote mixing. The same volume of acetone was added to the untreated ponds, rendering them solvent controls. Nominal concentrations are used throughout this report because the fate of monensin in the microcosm was not analyzed.

2.3.3 Water chemistry

Measures of temperature, dissolved oxygen (DO), pH, alkalinity, hardness, and conductivity were taken regularly over the course of the study. Maximum and minimum temperatures were measured daily, Monday through Friday, at a water depth of 20 cm. Point temperatures and dissolved oxygen readings also were taken daily at a depth of 50 cm using a YSI Model 55 meter (YSI, Yellow Springs, OH, USA). Water samples were collected for chemical determinations including water hardness, alkalinity, conductivity, and pH measures on days 1, 8, 14, and 35 using a metal depth-integrated water column sampler (Solomon et al., 1982). Samples were collected in treatment order, and integrated subsamples were taken from a minimum of four randomly selected locations in

the microcosm to a volume of approximately 4 L. A 500-mL aliquot was taken, stored in a plastic bottle, and kept at 4°C for 2 to 3 days before processing. Standard methods and kits by Hach (Hach Company, Loveland, CO, USA) were used to determine hardness and alkalinity. An Accumet Research AR20 pH/conductivity meter (Fisher Scientific, Whitby, ON, Canada) was used to measure pH and conductivity.

2.3.4 *L. gibba* experimental design

Duckweed (*L. gibba* L.) (G-3), was originally obtained from a laboratory colony cultured at the University of Waterloo, Waterloo, ON, Canada, and maintained in 250-mL flasks according to established methods (Marwood et al., 2001). Test cultures were transferred to 1,000 mL of sucrose-free growth media contained in 2,800-mL flasks and photoautotrophically maintained in a growth chamber for 7 days before exposure. The chamber, set at 25°C, contained cool fluorescent lights at 6,800 lux.

The plants were transferred to the microcosms immediately after dosing, on July 7, 2005, for a 7-day exposure. The *L. gibba* were contained in floating wooden corrals (38 × 14 cm) subdivided into three sections (Figure 1.1C). The tops and bottoms of the trays were covered with a black plastic mesh to ensure containment of the *L. gibba*, while allowing water movement and exposure to sunlight (Hanson et al., 2001). Two plants with four fronds each were introduced into each of the three sections per corral. The endpoints monitored included frond number, plant number, growth rate, wet and dry mass, chlorophyll-*a*, chlorophyll-*b*, and carotenoid content.

The plants were sampled on July 14, 2005. They were removed from the wooden corrals, transported back to the laboratory in their respective microcosm water, and immediately evaluated. On the average, three subsections of each wooden corral were

taken. Chlorophyll and carotenoid concentrations were determined simultaneously by extraction in 80% ethanol (Commercial Alcohols Inc, Toronto, ON) and measurement on a fresh-weight basis according to standard methods (ASTM, 1999b) on an Ultrospec 3100 *pro* UV/Visible Spectrophotometer (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA).

2.3.5 *M. spicatum, E. densa,* and *E. canadensis* experimental design

Myriophyllum spicatum L. (Haloragaceae) and *Elodea canadensis* Michx. (Hydrocharitaceae) used in the field study were harvested from a single untreated on-site microcosm obtained originally from a local reservoir (Guelph Correctional Facility, Guelph, ON, Canada). *Egeria densa* (Hydrocharitaceae) was ordered from Carolina Biological Supply (Burlington, NC, USA). The plants were grown in 115-mL "cone-tainers" or plastic planting tubes in 96-well plant trays (Figure 1.1D) (Steuwe and Sons, Corvallis, OR, USA) for assessment of the response of individual plants. The "cone-tainers" were 14 cm long with an internal diameter of 3.8 cm (Figure 1.1G). The tubes were filled with amended sediment and soaked overnight in the irrigation pond to allow the soil to settle. Apical shoots of each species, without any side roots, were cut to 5 cm and planted in the "cone-tainers." Each shoot was planted 2 cm into the soil and surrounded by approximately 0.5 to 1 cm of Turface (Applied Industrial Materials, Buffalo Grove, IL, USA) to secure it in the sediment.

A total of six plants per species were evenly spaced across each planting tray and added to the microcosms 1 day before treatment (July 6, 2005). The trays were placed in the center of each pond to provide maximum sunlight and reduce edge effects. Each species was sampled the day before treatment with monensin, then 14, 28, and 35 days

after treatment. The endpoints evaluated included plant length, biomass (wet mass/dry mass), primary root lengths, RGRs, and pigment contents including chlorophyll-*a*, chlorophyll-*b*, and carotenoid. Pigments were measured as described for *L. gibba*. The RGRs were calculated according to Hunt (1990). On the first day, 10 plants of both species were evaluated as 5-cm apical shoots to obtain their baseline condition. At each other sampling point, two plants of each species were removed randomly from the microcosms, transported back to the laboratory in their respective microcosm water, and immediately evaluated.

2.3.6 Population and community experimental design

Assemblages of *M. spicatum* and *E. canadensis* also were grown in larger planting pots to model small populations and two-species communities (Figure 1.1E). The population pots were plastic, cylindrical, and about 16.5 cm in diameter. The arrangement of the plants consisted of either five *M. spicatum* or five *E. canadensis* plants per pot, with the 5-cm apical shoots planted in a circle configuration (Figure 2.1). To represent a two-species community, *M. spicatum* and *E. canadensis* were grown together in plastic pots approximately 21.5 cm in diameter.

The plants were arranged in four rows of two or three plants each. The species type was alternated by row (Figure 2.1). Five plants per species were positioned in each pot, for a total of 10 plants. Both the population and community pots were filled with sediment and prepared for planting using the same method as that used for the "cone-tainers." The density of the plants in each population and community pot approximated one plant per 40 cm^2 according to a simple replacement series design (de Wit, 1960).

A total of three pots were added to each microcosm and evenly spaced around the planting trays in the center of the ponds. The model populations and communities were sampled the day before treatment and 35 days after treatment. The endpoints monitored were plant number, biomass (wet mass/dry mass), and RGRs. The RGRs were calculated as described for individually grown plants. The final day of the field study was August 12, 2005 (day 35).

2.3.7 Statistical analyses

The effect of monensin concentration on each endpoint at specific time points was evaluated in a one-way analysis of variance (ANOVA) design. The response data of individually grown *M. spicatum*, *E. canadensis*, and *E. densa* plants, as well as *L. gibba* grown in floating trays, were analyzed using SigmaStat 3.5 (Systat Software 2006, Jandel, San Rafael, CA, USA). Nominal concentrations of monensin were used to conduct statistical evaluations. A Kolmogorov-Smirnov test was used to assess ANOVA assumptions of residual normality, and a Levene median test was used to assess equal variance. Any data that did not meet the assumptions were natural ln or square root transformed. Data that did not meet the assumptions after transformation were compared using a nonparametric test, the Kruskis-Wallis one-way ANOVA on ranks in SigmaStat 3.5.

When significance ($p < 0.05$) was found, the means were compared with the control using Dunnett's test, from which a no-observed-effect concentration (NOEC) for that endpoint was determined ($\alpha = 0.05$). The data at each time point also were evaluated using nonlinear regression techniques according to the procedure for plant toxicity outlined in Stephenson et al. (2000). Data modeling was performed in SigmaPlot 2000

(SPSS Inc. Chicago, IL, USA) using a linear or logistic equation (Hanson et al., 2006). The model was selected based on the corrected coefficient of determination and graphic interpretation of the model's fit. For those endpoints showing a significant concentration response ($p < 0.05$), proc NLIN of SAS v9.1 (SAS Institute, Cary, NC, USA) was used to calculate the EC_{10}, EC_{25}, and EC_{50} plus confidence intervals. Before regression analyses, day 1 mean values for shoot growth, wet mass, dry mass, and node number were subtracted from all later respective time point measurements so that only new growth data were used for assessment of effects.

Response data of *M. spicatum* and *E. canadensis* grown in model populations and two-species communities were evaluated in a similar fashion. For each endpoint tested, the data were first standardized to a per plant measure. The effect of monensin concentration on biomass and RGRs at day 35 was evaluated in a one-way ANOVA, and the means were compared with the control using Dunnett's test when significance was found ($p < 0.05$). The data then were evaluated using the nonlinear regression techniques outlined earlier to calculate EC_x values.

Because preliminary data analyses indicated that there was no clear pattern of toxicity (i.e., a statistically significant concentration response for most of the monitored endpoints), day 35 *M. spicatum* and *E. canadensis* biomass and RGR data were averaged across all the concentration levels. This allowed for comparison of biomass and RGRs of individually grown plants with plants grown in model populations and communities using a one-way ANOVA ($\alpha = 0.05$). The ANOVA assumptions of residual normality and homogeneous variance were assessed, and data that did not meet assumptions were natural ln or square root transformed. Any data that did not meet assumptions after

transformation were compared with a nonparametric test, the Kruskis-Wallis one-way ANOVA on ranks in SigmaStat 3.5. When significance ($p < 0.05$) was found, Dunn's test was used for a pairwise comparison between means.

2.4 RESULTS

2.4.1 General parameters

Temperature, dissolved oxygen, pH, alkalinity, hardness, and conductivity data of the microcosms over the course of the study are provided in Table 2.1. The results presented represent the mean of all measurements taken for each specified parameter at a given monensin concentration averaged over all sampling times. There was little variation in these parameters over the 35-day exposure period. There were no significant differences between treatments with regard to any of the aforementioned parameters.

2.4.2 Fate of monensin

The environmental fate of monensin was investigated in an independent study conducted with the University of Guelph microcosms over the summer of 2004 (Lissemore, 2005). Concentrations of monensin in the water column and sediments were monitored over 35 days under a pond setup and dosing regimen similar to that used in our investigation. The researchers found that monensin is fairly stable under seminatural field conditions, observing an average reduction in surface water concentration of about 70% over 35 days, with similar rates of decline across all treatment levels. Using their experimental data, they calculated the half-life of monensin in the water column to be about 20 ± 5 days. Measurements of the compound in the sediment conducted on days 8 and 21 found that concentrations were an order of magnitude higher than surface water

concentrations, indicating that sorption did occur within the microcosms.

The researchers also found that an increase in water column concentrations observed on day 22 likely was associated with desorption of monensin from sediments due to increased water temperatures, indicating that sediment-bound compound should not be considered entirely unavailable to aquatic organisms. Although the fate of monensin in the microcosm was not analyzed in our study, it is reasonable to expect that substantial compound was present in the microcosms over the exposure duration and that it behaved much the same as in the previous investigation because both were conducted over the same test duration, with similar concentration ranges under comparable environmental conditions.

2.4.3 Macrophyte toxicity

Analysis of variance for individually grown *E. canadensis* and *E. densa* for each sampling time of each endpoint found few significant differences ($\alpha = 0.05$) as compared with control values (Table 2.2). Root length, root number, wet mass of stem and roots, dry mass of roots, number of nodes, RGR_{wet}, and pigment endpoints of *E. canadensis* and *E. densa* showed no statistically significant differences between control and monensin-exposed plants. For all endpoints at each sampling time of individually grown *M. spicatum*, there were no significant differences found between control and treatment subjects. Similarly, *L. gibba* showed no discernable toxicity when evaluated 7 days after exposure to monensin. The ANOVA evaluation of *M. spicatum* and *E. canadensis* model population and community data conducted using Dunnett's test for multiple comparisons found no statistically significant effects ($\alpha = 0.05$) between control and replicated treatment subjects for any endpoints on day 35.

Regression analysis techniques conducted for all endpoints at all sampling times for the four test species identified several endpoints displaying a significant concentration response. Effective concentrations required to cause a 10%, 25%, or 50% change in the endpoint of interest were calculated using the best-fitting model, and the results for *M. spicatum* and *E. canadensis* are presented in Table 2.3. The coefficient of determination were found to be weak, generally below 0.50. No significant concentration responses were found for either *L. gibba* or *E. densa*.

2.4.4 Comparison of individual growth to model population and community growth

The biomasses and RGRs of *E. canadensis* and *M. spicatum* grown in model populations and communities were significantly higher ($p < 0.05$) than those of plants grown as individuals in "cone-tainers" Table 2.4. No significant differences were found between RGRs or biomass measures of plants grown in model populations and those of plants grown in two-species communities (Figure 2.2).

2.5 DISCUSSION

No consistent toxicity appeared to be exhibited by *L. gibba*, *E. densa*, *E. canadensis*, or *M. spicatum* at exposure to monensin in the microcosms. Nonlinear regression modeling provided little evidence of a toxic response of macrophytes to monensin. Low coefficients of determination and large confidence intervals around calculated effective concentrations indicate that the few statistically significant concentration–response relationships observed were weak and not particularly predictive. Also, there were few significant differences ($p < 0.05$) between the endpoints of the

control plants and the exposure concentrations as determined by ANOVA, most of which did not demonstrate corresponding significant concentration–response relationships when modeled using nonlinear regression techniques. These observed statistical results likely were due to random variation among the test systems. The stem dry mass of *E. canadensis* after a 14-day exposure was the only endpoint that demonstrated a consistent concentration response as indicated by both ANOVA and regression analysis. However, after 21 days, this relationship was no longer evident, and therefore unlikely to be of biologic significance.

By conducting a basic hazard quotient risk assessment, in which the hazard quotient is equal to the predicted or measured environmental concentration divided by the toxicologic benchmark concentration (Suter, 1995), a hazard quotient of 0.012 is obtained. Because our study found that consistent toxicity was not observed at 100 µg/L, the highest test concentration of monensin, this was set as the NOEC under seminatural field conditions and used as the toxicologic benchmark concentration. Lissemore et al. (2006) reported concentrations of monensin in surface waters as high as 1.172 µg/L. This value is used as the measured environmental concentration because it is the highest quantified level of the compound in the environment to date. The hazard quotient estimate is smaller than 1, indicating that current environmental concentrations of monensin do not pose a risk of toxicity to these aquatic macrophytes or, by extension, to other freshwater aquatic macrophytes. Findings of a 7-day laboratory test performed on *L. gibba* (Brain et al., 2004) are consistent with the toxicity results of this field-level assessment conducted with additional test species and extended durations of exposure. In

this case, the single-species laboratory-based assay proved to be protective of freshwater macrophytes.

Due to the lack of consistent effects observed at the exposure concentrations, we were unable to investigate directly how the use of various test system designs (i.e., individual, population, community tests) influenced the toxicologic sensitivity (EC_xs) of macrophytes to a chemical contaminant, which was the original objective of the study. However, the statistically significant differences observed between RGRs of *E. canadensis* and *M. spicatum* plants grown as individuals and those grown in population and community pots indirectly indicate the sensitivity of the various toxicity assessment methods. Therefore, the results indicate that the individual plant test system or "cone-tainer" method may underestimate toxicity because these plants demonstrate significantly slower growth.

Several factors may have influenced the rate of plant growth under the various bioassay methods, including the test containers themselves. Although the "cone-tainers" allotted slightly more than 11 cm^2 of growth space per plant, the model population and community pots provided approximately 40 cm^2 of available space per plant, representing more resources for growth. Hindrance of plant growth due to the small size of test containers has been observed in other experiments (Agami and Reddy, 1990). In a study investigating nutrient competition among three submersed plant species, root size, depth distribution, and volume of soil occupied by roots were found to adjust when grown in mixed cultures. This was thought to occur in response to competition for sediment-based nutrients (Spencer and Ksander, 2005). In our study, the higher RGRs observed in model populations and communities may have occurred in response to intra- and interspecific

pressures for resource acquisition not occurring in the "cone-tainer"–grown plants.

The results of the one-way ANOVA conducted with *E. canadensis* and *M. spicatum* populations and communities are not consistent with plant relation data found in the literature. A study conducted by Abernethy et al. (1996) investigated interspecific interactions between the two plant species. They found that *M. spicatum* is less competitive than *E. canadensis*, demonstrating a significant loss in biomass when grown in mixed cultures compared with monoculture control plants. The current study found that there were no significant differences between a biomass of plants grown in model populations and that of plants grown in two-species communities. Differences in planting densities and experimental design may explain this contradiction. Abernethy et al. (1996) used an additive design to investigate competition (Martin and Snaydon, 1982), with initial densities of one plant per 31.7 cm^2 and one plant per 15.8 cm^2.

Our study used a lower density of about one plant per 40 cm^2. Although this falls within the range of biomass density found in the field (Lillie et al., 1997), it may have been too low for observation of interspecies competition. Also, the use of a replacement series design with only one density may have prevented the effects of interspecific competition from being isolated from intraspecific effects (Valley and Newman, 1998). This illustrates the importance of selecting a range of test densities for future evaluations.

Exposure to monensin was observed to cause few significant effects for any of the monitored endpoints both when aquatic plants were grown individually and when they were grown in model populations and communities. This indicates that monensin is unlikely to cause toxicity in freshwater macrophytes at the tested concentrations, which were well above current environmental concentrations (6.2–1,172 ng/L). In this case, the

individual plant–based microcosm test appears to reflect results obtained when testing of model populations and two-species plant communities is used. However, the statisitically significant differences in RGRs and plant biomass measures observed in this study indicate that the individual-based test may underestimate toxicity to the larger plant community, especially when used to assess more phytotoxic agents such as herbicides. This speaks of the need for further investigation of how plant interactions, including density effects, may alter the response of aquatic plant communities to an environmental contaminant.

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Table 2.1 Chemical and physical parameters of the microcosms averaged over the 35-day monensin antibiotic study plus pretreatment measurements[a].

Treatment (μg/L)	Minimum temperature (°C) (n=22)	Maximum temperature (°C) (n=22)	DO (mg/L) (n=22)	pH (n=4)	Alkalinity[c] (mg/L) (n=4)	Hardness[b] (mg/L) (n=4)	Conductivity (μs/cm) (n=4)
Control	22.2 ± 1.7	26.2 ± 1.7	11.0 ± 1.8	8.4 ± 0.4	223 ± 16	357 ± 32	721 ± 51
12	22.7 ± 1.2	27.1 ± 1.8	13.1 ± 3.2	8.7 ± 0.5	206 ± 10	348 ± 24	684 ± 68
25	22.0 ± 1.1	27.1 ± 1.7	11.8 ± 2.1	8.5 ± 0.5	215 ± 3	362 ± 20	707 ± 56
50	23.0 ± 1.5	26.6 ± 1.6	13.0 ± 3.3	8.7 ± 0.5	203 ± 7	342 ± 23	684 ± 72
100	22.6 ± 1.5	26.7 ± 1.6	12.5 ± 2.6	8.6 ± 0.5	213 ± 9	351 ± 24	695 ± 63

DO, dissolved oxygen

[a] The values presented are the means and standard deviations of measurements taken for three replicates at each exposure concentration for each measurement event. These means were then averaged for all the measurement events taken at that concentration over the 35 day period.

[b] Measured as mg/L of $CaCO_3$

Table 2.2 Statistically significant differences ($p < 0.05$) of individually grown plants as determined by analysis of variance using Dunnett's test for *Elodea canadensis* and *Egeria densa* exposed to monensin over 35 days[a].

Plant species	Endpoint	Day	Monensin (µg/L)	Control	Exposed	p-value
E. canadensis	Dry mass stem (g)	14	25	0.118 ± 0.024	0.077 ± 0.016	0.03
E. canadensis	Dry mass stem (g)	14	50	0.118 ± 0.024	0.078 ± 0.022	0.03
E. canadensis	Dry mass stem (g)	14	100	0.118 ± 0.024	0.064 ± 0.013	0.03
E. canadensis	RGR_{dry} ($gg^{-1}day^{-1}$)	14	25	0.161 ± 0.013	0.127 ± 0.014	0.04
E. canadensis	RGR_{dry} ($gg^{-1}day^{-1}$)	14	100	0.161 ± 0.013	0.120 ± 0.015	0.04
E. densa	Stem length (cm)	28	25	11.617 ± 2.259	16.6 ± 3.0	0.03
E. canadensis	Stem length (cm)	35	25	26.700 ± 0.954	17.8 ± 3.7	0.04

E. canadensis, *Elodea canadensis*; *E. densa*, *Egeria densa*

[a]The values shown are the mean of three microcosms ± standard deviation). No statistically significant differences were found for any monitored endpoints of *Myriophyllum spicatum* or *Lemna gibba*.

Table 2.3 Effective concentrations (µg/L) required to cause a decrease in the endpoints of interest by 10%, 25%, and 50% (EC_{10}, EC_{25}, and EC_{50}) with associated 95% confidence intervals, as calculated using linear and non-linear modeling techniques using SAS v9.1 (SAS Institute, Cary, NC, USA), for endpoints with statistically significant responses in Myriophyllum spicatum and Elodea canadensis exposed to monensin in aquatic microcosms[a].

Endpoint	Day	EC_{10} (95% CI)	EC_{25} (95% CI)	EC_{50} (95% CI)	Model[d]	Parameters	Corrected r^2
M. spicatum							
Chlorophyll-*a* (µg/mg)[b]	35	32.2 (3.7, 60.6)	80.4 (9.2, 151.6)	160.7 (18.3, 303.1)	Linear	$b = 0.375$; $x = 160.7$	0.23
RGR_{wet} ($gg^{-1}day^{-1}$)[c]	35	39.46 (8.40, 70.53)	98.66 (20.99, 176.3)	197.3 (41.98, 352.7)	Linear	$b = 0.1128.$; $x = 197.3$	0.27
E. canadensis							
Wet mass stem (g)[b]	14	0.0023 (0, 1019)	4.197 (0, 53.1)	7680.2 (0, 188670)	Logistic	$b = 0.146$; $t = 0.688$; $x = 7680.2$	0.31
Dry mass stem (g)[b]	14	0.381 (0, 2.978)	6.032 (0, 25.54)	95.493 (0, 274.2)	Logistic	$b = 0.398$; $t = 0.103$; $x = 95.493$	0.55
RGR_{dry} ($gg^{-1}day^{-1}$)[b]	14	3.467 (0, 20.861)	123.8 (0, 414.50)	4419.9 (0, 35590)	Logistic	$b = 0.307$; $t = 0.161$; $x = 4419.9$	0.46

CI, confidence interval; *M. spicatum*, *Myriophyllum spicatum*; *E. canadensis*, *Elodea canadensis*; RGR, relative growth rate

[a] No significant concentration-responses were found for either *Lemna gibba* or *Egeria densa*.
[b] Plants are grown as individuals. [c] Plants are grown in model populations.
[d] The reparameterized equations used to fit the concentration-responses of monensin exposed *M. spicatum*, and *E. canadensis*: Linear $y = ((-b \times 0.5)/x)x_o + b$; Logistic $y = t/[1 + (x_o/x)^b]$. The variable x is the calculated EC_{50} for the concentration-response relationship modeled, x_o is the actual concentration (µg/L) being evaluated, y is the response or change from control of the endpoint modeled, and b, and t are constants.

Table 2.4 Statistically significant differences ($p < 0.05$) in biomass and relative growth rates (RGRs) of individually grown *Myriophyllum spicatum* and *Elodea canadensis* and the plants grown in model populations and communities over 35 days in outdoor microcosms as determined by analysis of variance using Dunn's test.

Endpoint	Individual	Population	Community	p-value
M. spicatum				
Total wet mass (g)	2.818 ± 0.588[a]	7.401 ± 3.244[b, c]	9.001 ± 3.779[a, c]	<0.001
Total dry mass (g)	0.436 ± 0.167[a]	0.904 ± 0.484[b, c]	1.103 ± 0.611[a, c]	0.001
RGR_{wet} ($gg^{-1}day^{-1}$)	0.078 ± 0.007[a]	0.102 ± 0.018[b, c]	0.109 ± 0.015[a, c]	<0.001
RGR_{dry} ($gg^{-1}day^{-1}$)	0.066 ± 0.011[a]	0.084 ± 0.020[b, c]	0.090 ± 0.019[a, c]	0.001
E. canadensis				
Total wet mass (g)	1.327 ± 0.535[a]	6.256 ± 1.703[a, c]	5.312 ± 2.587[b, c]	<0.001
Total dry mass (g)	0.275 ± 0.108[a]	0.697 ± 0.219[a, c]	0.580 ± 0.316[b, c]	<0.001
RGR_{wet} ($gg^{-1}day^{-1}$)	0.077 ± 0.012[a]	0.122 ± 0.009[a, c]	0.115 ± 0.014[b, c]	<0.001
RGR_{dry} ($gg^{-1}day^{-1}$)	0.083 ± 0.011[a]	0.110 ± 0.009[a, c]	0.102 ± 0.016[b, c]	<0.001

M. spicatum, *Myriophyllum spicatum; E. canadensis*, *Elodea canadensis*;
RGR, relative growth rate

[a] Values are the mean (n=14) ± standard deviation.
[b] Values are the mean (n=15) ± standard deviation.
[c] Statistically significant difference from individually grown plants.

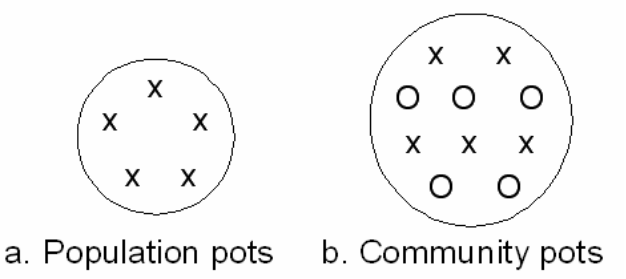

Figure 2.1 Planting arrangement used for model populations and communities of *Myriophyllum spicatum* and *Elodea canadensis*. The density of plants in each population and community pot approximated 1 plant per 40 cm^2. (a) *M. spicatum*: *E. canadensis* planting density was 5:0 or 0:5 (monoculture). (b) *M. spicatum* : *E. canadensis* planting density was 5:5 (mixed culture).

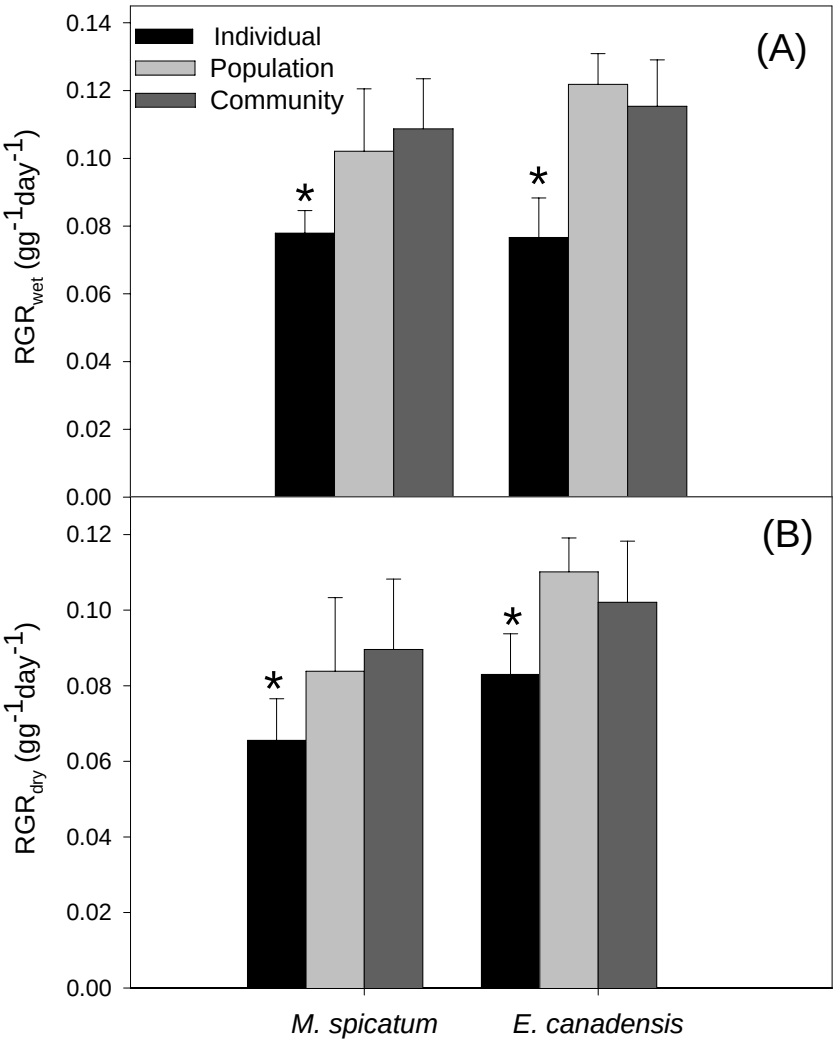

Figure 2.2 Relative growth rates (RGR) ($gg^{-1}day^{-1}$) of *Myriophyllum spicatum* and *Elodea canadensis* grown as individuals and in model populations and communities over 35 days in 12,000-L outdoor microcosms. Error bars represent the standard deviation about the mean. An asterisk (*) denotes a statistically significant difference as detected using a Dunn's test ($p < 0.05$). (A) RGR calculated from total wet biomass. (B) RGR calculated from total dry biomass.

3 FRESHWATER MICROCOSM ASSESSMENT OF THE TOXICITY OF 10:2 SATURATED FLUOROTELOMER CARBOXYLIC ACID TO AQUATIC MACROPHYTES

3.1 ABSTRACT

The 10:2 saturated fluorotelomer carboxylic acid (10:2 FTCA) is a stable intermediary breakdown product in the degradation of fluorotelomer alcohols to perfluorinated carboxylic acids (PFCAs). It is expected that the compound will be found in surface waters, as suggested by the high frequency of occurrence of PFCAs in the aquatic environment and the likelihood that fluorotelomer alcohol degradation is a significant source of PFCAs. The effect of the 10:2 FTCA on floating (*Lemna gibba*) and submersed (*Myriophyllum spicatum, Elodea canadensis* and *Egeria densa*) freshwater macrophytes was investigated under semi-natural field conditions using 12 000 L outdoor microcosms. Exposure concentrations of 0.1, 0.5, 1, 10, 50, 100, 250, 750, 1000, 2000 µg/L (n=1) plus controls (n=3) were evaluated in a regression design. The response of *L. gibba* was assessed after 7 day and 14 day exposure durations. Individual rooted plants were grown in 115 mL plastic "cone-tainers" and assessed for various growth and pigment endpoints over an 84 day exposure period. *E. canadensis* and *M. spicatum* were also grown in assemblages to represent model populations and two-species communities. Few significant effects were observed for any of the monitored endpoints, indicating that the 10:2 FTCA is unlikely to cause toxicity in freshwater macrophytes at the tested concentrations. The relative growth rates (RGRs) of *E. canadensis* and *M. spicatum* grown in model populations and communities were found to be significantly greater than RGRs of plants grown as individuals at day 42 and 84. As the ability to characterize toxicity in macrophytes is partially based on the RGR, the greater the RGR the more

sensitive the assay can be to contaminants, this demonstrates that the "cone-tainer" method may underestimate toxicity in simulated field-studies.

3.2 INTRODUCTION

In recent years, long-chain perfluorinated carboxylic acids (PFCAs) have been recognized as persistent and bioaccumulative environmental contaminants (Martin et al., 2003; Martin et al., 2004a). These compounds have been widely detected in biological systems including surface waters (Hansen et al., 2002; Boulanger, 2004), biota (Moody et al., 2002; De Silva and Mabury, 2004; Martin et al., 2004b), and human serum (Kannan et al., 2004). They are often found in regions where no direct sources of PFCAs are evident such as the Canadian Arctic and open-ocean waters (Martin et al., 2004b; Yamashita et al., 2004).

It is suggested that fluorotelomer alcohols (FTOHs) are an environmentally relevant source of the acids (Lange, 2002; Dinglasan et al., 2004; Ellis et al., 2004; Gauthier and Mabury, 2005; Wallington et al., 2006; Wang et al., 2005b). As transport of PFCAs in the atmosphere is unlikely due to their low volatility and removal by wet and dry deposition (Hurley et al., 2004), FTOHs may serve as volatile precursors that facilitate movement (Ellis et al., 2004). FTOHs are detected in the North American troposphere at concentrations ranging from 11 to 165 pg/m^3 (Stock et al., 2004b), and the atmospheric lifetime of the compounds is estimated to be 20 days, in which time they may be transported to remote regions (Ellis et al., 2003). A recent investigation confirmed the occurrence of the 6:2, 8:2, and 10:2 FTOH in the arctic atmosphere, with the mean concentration of the 8:2 FTOH only a factor of 3 lower than samples collected from an urban centre (Shoeib et al., 2006).

Several studies investigating the degradation of FTOHs by atmospheric oxidation (Ellis et al., 2004), microbial-based transformation (Dinglasan et al., 2004; Wang et al., 2005b) and indirect aqueous photolysis processes (Gauthier and Mabury, 2005) have identified saturated fluorotelomer carboxylic acids (FTCAs: $CF_3(CF_2)_nCH_2CO_2H$ n = 3, 5, 7, ...) and unsaturated fluorotelomer carboxylic acids (FTUCAs: $CF_3(CF_2)_{n-1}CF{=}CHCO_2H$ n = 3, 5, 7, …) as metabolites. The metabolic biotransformation of FTOHs in biota also yields these acids. A study conducted by Hagen et al. (1981) detected the production of 8:2 FTCA when a single oral dose of the 8:2 FTOH was administered to adult male rats. Saturated and unsaturated fluorotelomer acids were also formed as metabolic products by isolated rat hepatocytes (Martin et al., 2005) and detected in plasma samples in additional rodent FTOH-exposure studies (Kudo et al., 2005; Fasano et al., 2006). Research has focused mainly on investigating transformation pathways of the 8:2 FTOH, and while it is expected that the 10:2 FTOH will degrade similarly, producing the 10:2 fluorotelomer acids and the corresponding chain length perfluorocarboxylic acid, perfluorodecanoic acid (PFDA) (Dinglasan et al., 2004), this is yet to be confirmed.

The occurrence of saturated fluorotelomer carboxylic acids (FTCAs) has been confirmed, but there is minimal literature that examines the environmental fate or toxicity of these intermediate compounds. Loewen et al. (2005) present the first report of the 10:2 FTCA and the 10:2 FTUCA in the environment, detected in rainwater collected in Winnipeg, Manitoba, Canada. Analysis of the samples determined concentrations of acids to be 0.30 ± 0.04 ngL^{-1} (n = 3) of 10:2 FTCA and 0.12 ± 0.01 ngL^{-1} of 10:2 FTUCA. The acids were also detected in a larger investigation of North American precipitation, present in samples collected from 4 sites in the Northeastern United States,

and 2 urban Canadian locations (Scott et al., 2006). The concentrations of the 10:2 FTCA and 10:2 FTUCA ranged from <0.07 – 1.3 ng/L and <0.07– 0.8 ng/L, respectively, although the 10:2 FTUCA was infrequently detected above the minimum detection limit (0.07 ng/L), and 10:2 FTCA concentrations were significantly correlated with PFDA (Scott et al., 2006). Fluorotelomer acids have also been detected in biota in the Gulf of Mexico and the Atlantic Ocean (Houde et al., 2005). Plasma of dolphins was found to contain low ngg^{-1} wet weight levels of 8:2 and 10:2 FTUCAs, while FTCA concentrations were not detected. In another investigation, researchers detected the 8:2 FTCA, the 8:2 FTUCA, the 10:2 FTCA, and the 10:2 FTUCA in the liver samples from two ringed seal population in the Canadian Arctic, Arviat (Western Hudson Bay) and Resolute Bay (Lancaster Sound) (Butt et al., 2007). However, the concentrations of the 8:2 FTCA and FTUCA were below the method detection limits, and quantification problems with the 10:2 FTCA in samples prevented analysis of the levels in the tissue. The 10:2 FTUCA was found to range from<0.75 to 9.6 ng/g weight wet in Arviat samples, and from <0.75 to 1.3 ng/g wet weight in Resolute Bay samples.

Greater water solubility and lower volatility of the acids compared with corresponding FTOHs is expected (Loewen et al., 2005), suggesting that fluorotelomer acids produced through atmosphere oxidation of FTOHs are deposited in surface waters through wet deposition (Phillips et al., 2007). Potentially, the breakdown of materials incorporating FTOHs and the subsequent microbial degradation of those FTOHs in wastewater treatment plants could also result in the release of FTCAs and FTUCAs to surface waters (Dinglasan et al., 2004). Although these compounds have yet to be

quantified in the environment, it is reasonable to assume that aquatic organisms are exposed to fluorotelomer acids.

Laboratory testing assessing the toxicity of the 4:2, 6:2, 8:2 and 10:2 FTCAs and FTUCAs to the water flea *Daphnia magna*, the midge *Chironomus tentans*, and duckweed plant *Lemna gibba*, has been performed by MacDonald (2005). Standard acute and chronic bioassays conducted on the three aquatic species indicated that as fluorocarbon chain length increases so does toxicity. The relationship was most noted when chain lengths are ≥ 8 fluorocarbons. As well, FTCAs were found to be generally more toxic than corresponding FTUCAs, except in the case of the 8:2 FTCA for *L. gibba* and the 10:2 FTCA for *C. tentans* and *L. gibba*. In both acute and chronic testing, *D. magna* were found to be most sensitive to the 10:2 FTCA. The 48-hour LC_{50} and EC_{50} (immobility) values were 0.06 (0.04, 0.11) mgL^{-1} and 0.03 (0.02, 0.03) mgL^{-1}, respectively. Of the three species, the 10:2 FTCA proved least toxic to *C. tentans*, with 10-day LC_{50} and EC_{50} (growth) values greater than 16.27 mgL^{-1}. The 7-day EC_{50} value (frond number) for exposure of 10:2 FTCA to *L. gibba* was greater than 4.30 mgL^{-1}.

Laboratory tests were also conducted on *C. tentans* in order to assess the acute toxicity of perfluorooctanoic acid (PFOA), the eight-carbon length perfluorinated carboxylic acid (PFCA) (MacDonald et al., 2004). No significant impacts on survival or growth of the organisms was observed at concentrations up to 100 mgL^{-1}, while the LC_{50} estimate for survival of *C. tentans* was 12.4 mgL^{-1} of the 8:2 FTCA (Phillips, 2007). This illustrates that although PFCAs are the current focus of scientific study, FTCAs may be more toxic to aquatic organisms than corresponding PFCAs, thereby warranting further investigation of these intermediate breakdown compounds. The current study was

conducted, therefore, to evaluate the toxicity of the 10:2 FTCA to four common aquatic macrophytes, *Lemna gibba, Myriophyllum spicatum*, *Elodea canadensis* and *Egeria densa*, in an outdoor microcosm system. To this end, we examined a suite of endpoints in the macrophytes exposed to 10:2 FTCA for varying durations and at multiple concentrations.

An ancillary goal in this study was to examine whether toxicity observed in individually grown plants is consistent with the response of plants grown in model populations and two-species assemblages. Toxicity tests assessing the effects of environmental contaminants on non-target plants commonly rely on the evaluation of responses at an individual level to predict outcomes at higher levels of biological organization. This is largely the practice in ecotoxicology as a whole, with a focus on physiological mechanisms of toxicity and measurement of organism-level responses, such as survival and growth (Forbes and Calow; 1999; Preston, 2004). As relationships between lower-level responses and higher-level effects are not always direct or clear, the consideration of only organism-level endpoints or low density populations may result in the over- or under-estimation of contaminant effects to the larger community (Kramarz et al., 2005; Raimondo and McKenney Jr., 2006). Many factors may influence the response of a biological community to a toxicant (Calow and Forbes, 2003), including population density effects (Simkiss et al., 1993; Sibly et al., 2000), and inter-species interactions (Preston, 2004). Laboratory-based studies that assess the potential phytotoxic effects of a contaminant, including the standard assay conducted on *Myriophyllum sibiricum* (ASTM, 1999), use the individual approach. The results of these single-species tests are used in the lower tiers of ecological risk assessment. Higher-tier studies investigating effects of

contaminants to aquatic macrophytes often follow the same pattern. Aquatic plants are grown as part of a larger model ecosystem and the responses of a few individuals grown in independent test units are sometimes used to predict responses of entire populations or communities of plants (Stanley, 1979; Watkins and Hammerschlag, 1984; Hanson et al., 2001; Davies et al., 2003). These individual-based test conditions may not be representative of freshwater ecosystems, in which various types of interactions may occur among individuals of the same species or among neighbouring plants of differing species. Significant reductions in biomass production (Driever et al., 2005) and growth rates (Wang et al., 2005a) have been observed in response to high densities and crowding within populations of plants. Interrelationships between species, such as shading, allelopathy, and competition for resources may also significantly modify plant biomass and growth rates (Agami and Waisel, 2002; Wu and Yu, 2004), with potentially larger implications on plant community structure and function. Further investigation is required to understand how these complex intra- and inter-species interactions may alter the response of aquatic plants to a toxicant.

In general, the literature indicates that the faster the relative growth rate (RGR) and the longer the test duration, the lower the concentration of toxicant needed to impact a specific growth rate, and the more sensitive a toxicity test conducted on aquatic macrophytes may be to a chemical stressor (Huebert and Shay 1993; Cedargreen et al., 2004). This indicates that the RGR measures of plants grown under various test systems may serve as an indirect indicator of the potential toxicological sensitivity of plants grown using various toxicity assessment methods. A comparison of RGRs of plants grown under individual level conditions in our test systems to those grown in model

populations and two-species assemblages, was therefore conducted in order to examine whether the toxicological sensitivities of plants tested as individuals, monocultures, and mixed assemblages are significantly different.

3.3 METHODS AND MATERIALS

3.3.1 Test facility

The 15 outdoor microcosms utilized in this study are located at the University of Guelph Microcosm Facility at the Guelph Turfgrass Institute, Ontario, Canada (Figure 1.1A). Each of the artificial ponds is approximately 1.2 m deep with a water depth of 1 m, a surface area of 11.95 m^2, and a diameter of 3.9 m. The microcosms are lined with black, food-grade polyvinyl chloride (Fox Pools Canada, Burlington, ON) and are sunken into the ground with the tops flush with the surface. They each hold approximately 12 000 L of water which is supplied from an adjacent deep-well-fed irrigation pond (62 x 62 x 4 m deep).

In order to establish a model freshwater ecosystem, 45 plastic propagation trays (52 x 25 x 7 cm; Canadian HydroGardens, Ancaster, ON) were added to the bottom of each microcosm, containing an amended sediment mix (Waterdown Garden Supply, Troy, ON). The sediment consisted of a 3-way mixture, equal parts sand, loam and organic matter by volume and covered about 49% of each microcosm floor (Figure 1.1B). Water was circulated between the microcosms and the irrigation pond at a rate of 12 000 L/day for four weeks prior to treatment with 10:2 FTCA. Circulation was undertaken in order to reduce variability within and between microcosms, providing consistent physicochemical properties and biological communities. During circulation, eight potted

macrophytes (*M. spicatum*) were placed in each microcosm. These plants were obtained from several on-site untreated microcosms and were not used in the assessment of effects. One day prior to treatment (June 6, 2005) circulation was terminated in order to create self-contained systems.

3.3.2 Treatment and sampling regime

10:2 fluorotelomer saturated acid (10:2 FTCA) was synthesized from 10:2 fluorinated telomer alcohol (10:2 FTOH) according to methods adapted from Achilefu et al. (1995). The 10:2 FTOH was purchased from SynQuest Labs (Alachua, FL) and had a minimum purity of 95%. The treatments were measured out, diluted with microcosm water in 4-L Nalgene bottles, shaken and added to the microcosms, while simultaneously agitating with a stirrer to promote mixing. Exposure concentrations were randomly assigned to microcosms and applied at nominal concentrations of 0.1, 0.5, 1, 10, 50, 100, 250, 750, 1000, 2000 µg/L (n=1), plus controls (n=3). Treatment occurred on June 7, 2005.

Water samples for analysis of 10:2 FTCA were collected on days -1, 0, 2, 7, 14, 28, 49, and 98, using a metal depth-integrated water column sampler (Solomon et al. 1982). Approximately 4-L integrated subsamples were collected from a minimum of four randomly selected locations in the microcosm. A 5-mL aliquot was taken and diluted 1:1 with EMD Omni-Solv grade methanol and stored at 4°C until analysis.

3.3.3 Water chemistry and photosynthetically active radiation measurements

Measures of temperature, dissolved oxygen (DO), pH, alkalinity, hardness, and conductivity were taken regularly over the course of the study. Maximum and minimum

temperatures were measured daily, Monday through Friday, at a water depth of 20 cm. Point temperatures and DO readings were also taken Monday through Friday at a depth of 50 cm using a YSI Model 55 meter (YSI, Yellow Springs, OH). Water hardness, alkalinity, conductivity and pH were measured on days -1, 7, 14, 28, 50, and 71. Water chemistry samples were collected using a metal depth-integrated water column sampler and stored in 500-mL plastic bottles at 4°C for two to three days before processing. Standard methods and kits by Hach (Hach Company, Loveland, CO) were used to determine hardness and alkalinity. An Accumet Research AR20 pH/Conductivity meter (Fisher Scientific, Whitby, ON) was used to measure pH and conductivity.

Photosynthetically active radiation (PAR) was measured three times over the course of the study using a Li-Cor Quantum/Radiometer/Photometer model LI-185A (Li-Cor, Lincoln, NE). Readings were taken on clear sunny days between the hours of 12 and 2 pm. Measures were taken at the surface of each microcosm, at the surface in a glass jar, and at a depth of 60 cm in a glass jar in order to normalize the reading.

3.3.4 Analysis of water samples

Nominal concentrations of the 10:2 FTCA are used throughout this paper due to unexpected analytical complications.

3.3.5 *L. gibba* experimental design

Duckweed, *Lemna gibba* L. (G-3), was originally obtained from a laboratory colony cultured at the University of Waterloo, Waterloo, ON, Canada and maintained in 250-mL flasks according to established methods (Marwood et al., 2001). Test cultures were transferred to 1000-mL of sucrose-free growth media contained in 2800-mL flasks

and photoautotrophically maintained in a growth chamber for 7 days prior to exposure. The chamber was set to 25^{o}C and contained cool fluorescent lights at 6800 lux. The plants were transferred to the microcosms immediately after dosing for a 7-day exposure duration. The *L. gibba* were contained in floating wooden corrals (38 x 14 cm), subdivided into 3 sections (Figure 1.1C). The tops and bottoms of the trays were covered with a black plastic mesh to ensure containment of the *L. gibba*, while allowing water movement and exposure to sunlight (Hanson et al., 2002). Two plants, with four fronds each, were introduced into each of the three sections per corral. The endpoints monitored included frond number, plant number, growth rate, wet and dry mass, and chlorophyll-*a,* chlorophyll-*b,* and carotenoid contents. Plants were sampled on June 14, 2005. Chlorophyll and carotenoid concentrations were determined simultaneously by extraction in 80% ethanol (Commercial Alcohols Inc, Toronto, ON) using methods outlined in ASTM (1999) on an Ultrospec 3100 *pro* UV/Visible Spectrophotometer (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

Test cultures were prepared as described above and introduced to the microcosms for a second exposure of 14 days starting June 27, 2005. Frond and plant number were enumerated on day 7 and biomass and pigments were taken on day 14.

3.3.6 *M. spicatum, E. densa,* and *E. canadensis* experimental design

Myriophyllum spicatum L. (Haloragaceae) used in the field study was harvested from a single untreated on-site microcosm, originally obtained from a local reservoir (Guelph Correctional Facility, Guelph ON, Canada). *Elodea canadensis* Michx. (Hydrocharitaceae) was obtained from the same local reservoir and *Egeria densa* (Hydrocharitaceae) was purchased from Carolina Biological Supply (Burlington, NC,

USA). The plants were grown in 115 mL "cone-tainers" or plastic planting tubes held in 96-well planting trays (Figure 1.1D) (Steuwe and Sons, Corvallis, OR, USA) for assessment of the response of individual plants. The cone-tainers were 14 cm long with a 3.8 cm internal diameter (Figure 1.1G). The tubes were filled with amended sediment and soaked overnight in the irrigation pond to allow the soil to settle. Apical shoots of each species, without any side roots or shoots, were cut to 5 cm and planted in the cone-tainers. Each shoot was planted 2 cm into the soil and surrounded by approximately 0.5 to 1 cm of Turface (Applied Industrial Materials, Buffalo Grove, IL, USA) to secure the plants in the sediment. A total of 12 plants per species were evenly spaced across two planting trays and added to the microcosms one day prior to treatment (June 6, 2005). The trays were placed into the centre of each pond in order to provide maximum sunlight and reduce edge effects. Each species was sampled the day before treatment and at 14, 28, 42, 56, 70, and 84 days after treatment. On day -1, ten plants of both species were evaluated as 5 cm apical shoots, to obtain their baseline condition. At each other sampling point, two plants of each species were removed randomly from the microcosms, transported back to the laboratory in their respective microcosm water and immediately evaluated. The endpoints evaluated were growth (plant length), biomass (wet mass/dry mass), primary root lengths, relative growth rate (RGR), and chlorophyll-*a,* chlorophyll-*b,* and carotenoid content. Pigments were measured as described for *L. gibba*. RGRs were calculated according to Hunt (1990).

3.3.7 Population and community experimental design

M. spicatum and *E. canadensis* were also grown in larger planting pots in order to model small populations and two-species assemblages (Figure 1.1E). The population

pots were plastic, cylindrical and measured about 16.5 cm in diameter. The arrangement of plants consisted of either 5 *M. spicatum* or 5 *E. canadensis* plants per pot grown in a monoculture, with the 5-cm apical shoots planted in a circular configuration (Figure 3.1). *M. spicatum* and *E. canadensis* were grown together in approximately 21.5 cm diameter plastic pots in order to represent a mixed, two-species community. The plants were arranged in 4 rows with either 2 or 3 plants each. The species type was alternated by row (Figure 1). Five plants per species were positioned in each pot, a total of 10 plants. Both the monoculture and mixed pots were filled with sediment and prepared for planting using the same method as the cone-tainers. The density of plants in each pot approximated 1 plant per 40 cm^2, using a simple replacement series design (de Wit, 1960).

A total of 9 pots, 6 small and 3 large containers, were added to each microcosm and evenly spaced around the planting trays in the centre of the ponds. Each species was sampled the day before treatment as described previously. Populations and communities were also sampled at 14, 42 and 84 days after treatment. The endpoints monitored were plant number, biomass (wet mass/dry mass) and RGR. The final day of the plant sample collection was August 29, 2005 (Day 84).

3.3.8 Statistical analyses

Response data of individually grown *M. spicatum*, *E. canadensis,* and *E. densa* plants from "cone-tainers" and *L. gibba* grown in floating trays were analyzed using non-linear regression techniques according to the procedure for plant toxicity outlined in Stephenson et al. (2000). The effect of 10:2 FTCA concentration on each endpoint at specific time points was initially modeled in SigmaPlot 2000 (SPSS Inc. Chicago, IL,

USA) using a linear and logistic equation. For those endpoints showing a concentration-response ($p<0.05$), proc NLIN of SAS v9.1.3 (SAS Institute, Cary, NC, USA) was used to confirm significance and to calculate the EC_{10}, EC_{25}, and EC_{50}, plus confidence intervals (Hanson et al., 2006). The best-fitting model was selected based on the corrected coefficient of determination and by graphical interpretation of the model's fit. Nominal concentrations of 10:2 FTCA were used to conduct statistical evaluations. Prior to regression analyses, day -1 values for shoot growth, wet mass, dry mass, and node number were subtracted from all later respective time point measurements, so that only new growth data were used for assessment of effects.

Response data of *M. spicatum* and *E. canadensis* grown in model populations and two-species communities were evaluated in a similar fashion. For each endpoint tested, the data were first standardized to a per plant measure. The effect of 10:2 FTCA concentration on biomass and relative growth rates at days 14, 42 and 84 were evaluated using the non-linear regression techniques outlined above.

As preliminary data analyses indicated that there was no clear pattern of toxicity illustrated by a statistically significant concentration-response, for most of the monitored endpoints, *M. spicatum* and *E. canadensis* biomass and RGR data were averaged across the treatment levels, with the exception of the 1000 and 2000 μg/L FTCA data. This allowed for comparison of biomass and RGRs of individually grown plants to plants grown in model populations and communities at days 14, 42 and 84, using a one-way analysis of variance (ANOVA) ($\alpha = 0.05$). ANOVA assumptions of residual normality and homogeneous variance were assessed and data that did not meet assumptions were natural ln or square root transformed. Any data that did not meet assumptions after

transformation were compared with a non-parametric test, Kruskis-Wallis one-way ANOVA on ranks in SigmaStat 3.5 (Systat Software 2006, Jandel, San Rafael, CA, USA). When significance ($p<0.05$) was found, Tukey's test was used to conduct a pair-wise comparison between means.

3.4 RESULTS

3.4.1 General parameters

General trends in point temperature, dissolved oxygen, pH, alkalinity, hardness, and conductivity profiles of the microcosms over the course of the study are provided in Table 3.1. The results presented are the average over time of each specified parameter at a given 10:2 FTCA concentration. There was little variation in these parameters over the 84 day exposure period. There were no significant differences between treatments in regard to any of the above parameters.

3.4.2 Macrophyte toxicity

Regression analysis techniques conducted on all endpoints at all sampling times for the four test species, identified several endpoints of *M. spicatum, E. canadensis* and *L. gibba* that displayed significant linear or logistic relationships. Effective concentrations required to cause 10, 25, and 50% change in the endpoint of interest were calculated using the best fitting model, and the results are presented in Table 3.2. All of the coefficients of determination were below 0.50 and the confidence intervals about the effective concentrations were generally large. No significant regressions were observed for any endpoints at each sampling time of individually grown *E. densa*. Similarly, evaluation of *M. spicatum* and *E. canadensis* grown in model populations and

communities found only one significant concentration-response. A linear relationship was observed at day 14 between increasing concentration of 10:2 FTCA and decreasing RGR_{dry} of *M. spicatum* populations, but the result was not observed at later time points.

3.4.3 Comparison of individual plant, model population, and model community growth

After 14 days, biomass and RGRs of *E. canadensis* grown in model populations and communities were found to be significantly ($p<0.05$) lower than those of plants grown as individuals, while no significant differences were found between biomass or RGRs of *M. spicatum* grown under each condition. Conversely, plants from model populations and communities were found to have significantly ($p<0.05$) higher RGRs and biomass measures than those grown as individuals after 42 and 84 days of growth. Across time, no significant differences were found between RGRs or biomass measures of plants grown in model populations compared to those grown in two-species communities (Table 3.3, Figure 3.2).

3.5 DISCUSSION

There did not appear to be any consistent toxic response exhibited by *L. gibba*, *E. densa*, *E. canadensis*, or *M. spicatum* upon exposure to 10:2 FTCA in the microcosms. Few significant effects were observed for any of the monitored endpoints when aquatic plants were grown individually, or as model populations and communities. Of over three-hundred potential concentration-response relationships only six were found to have a statistically significant linear relationship, under which an increase in 10:2 FTCA concentration is associated with a decrease in the endpoint of interest. In all six cases, the

corrected coefficient of determination was below 0.50 indicating that the linear trends between the concentration and response variables were fairly weak and not particularly predictive. When the response data of plants exposed to the two highest concentrations levels, 1000 and 2000 μgL^{-1}, were removed from the regression analysis, the negative linear concentration-response was no longer observed for any of the six endpoints of interest. These relationships were likely driven largely by the highest concentrations and are not descriptive of the data at lower concentration levels (Anscombe, 1973). Although there was not a strong phytotoxic response across all of the monitored endpoints associated with exposure to the two highest 10:2 FTCA concentrations, the response data from the 1000 and 2000 μg/L FTCA microcosms were not included when *M. spicatum* and *E. canadensis* biomass and RGR data were averaged across concentration levels.

Five of the six linear concentration-response relationships were only evident after 14 days of exposure, and not at later time points across the 84 day period. This may indicate that the macrophytes were affected after 14 days, but were able to recover within the test duration. As implied by the observed degradation of the 8:2 FTCA in waters (Gauthier et al., 2005), the 10:2 saturated acid was expected to breakdown in microcosm water and ultimately produce PFCAs of carbon chain lengths ≤ the chain length of the original 10:2 FTCA (Phillips et al., 2007). Phillips et al. (2007) demonstrated that PFCAs are generally less toxic to aquatic organisms than FTCAs of equal fluorocarbon chain lengths. This suggests that the breakdown of 10:2 FTCA may have produced less phytotoxic metabolites and, in part, may explain the observed plant recovery. However, due to the lack of supportive residue analysis data, the concentration of 10:2 FTCA in the

water over time is unknown and we are not able to determine whether these transient effects were due to breakdown of the compound in water during the 84 day study.

In addition to the six negative linear relationships, two significant logistic concentration-response relationships were evident. The effective concentrations required to cause a decrease in the endpoints of interest by 10% (EC_{10}) were much lower than EC_{10}s calculated from the significant linear concentration-response relationships. Both values were less than 1 μgL^{-1} and in each case seemed to be driven by a single large control response data point, which are likely outlier values. When the single data points were removed from each analysis the trends were no longer statistically significant, illustrating that the two logistic relationships are not very representative of the larger data set. Nine other endpoints monitored in *L. gibba* and *M. spicatum* demonstrated a positive linear relationship between concentration and response variables, in which an increase in the endpoint of interest was observed in response to increasing 10:2 FTCA concentration (Table 2). There does not appear to be a consistent biological relevance of these observed concentration-responses as they are not related across time, with the exception of wet mass of *M. spicatum* roots that demonstrate an increase in mass with concentration and time.

Due to a lack of data quantifying the concentration of 10:2 FTCA in the environment, a range of test concentrations were selected. Some treatments fell in the low ngL^{-1} range in order to correspond to predicted environmental concentrations (MacDonald, 2005). However, considering the findings of the seven-day static renewal laboratory test conducted using *L. gibba* (Phillips et al., 2007), the lack of phytotoxicity observed in our study is expected under acute conditions. While our greatest test

concentrations were in the range of estimated EC_{10} values for dry mass and frond number in *L. gibba* from the study of Phillips et al.(2007), all of our test concentrations were less than EC_{50} values estimated in their laboratory assay. Phillips et al. (2007) estimated the dry weight EC_{50} value for exposure of 10:2 FTCA to *L. gibba* to be 4.3 mg/L, and the EC_{50} value for frond number to be greater than 4.3 mg/L. An unreplicated regression design was used in our investigation in attempt to screen a range of concentrations and capture the no effect concentration (EC_{10}). However, the lack of replication of test concentrations immediately around the predicted no effect concentration resulted in large confidence intervals typically seen in this type of design (Liber et al., 1992). Replication around the predicted no effect concentration would have also allowed us to determine whether the highest test concentrations, 1000 and 2000 µg/L, in fact prove to have a large influence on driving concentration-response relationships. While the results of our study suggest that there is a lack of biologically significant effects associated with exposure of aquatic plants to 10:2 FTCA at tested concentrations, overall, the lack of replication and the absence of supportive water residue analysis data yield uncertainty in our analysis. These study design criticisms coupled with the present uncertainty associated with actual environmental concentrations of 10:2 FTCA and the associated information on whether exposure to the 10:2 FTCA would be chronic or via pulse wise releases, we cannot reasonably assess the risk to aquatic macrophytes. However, as the results of our multiple species semi-field assessment generally correspond with results from the 7-day laboratory assay conducted by Phillips et al. (2007), it may serve to provide support for their findings.

The statistically significant differences observed between RGRs of *E. canadensis* and *M. spicatum* plants grown as individuals and those grown in population and community pots acts as an indicator of the sensitivity of the various toxicity assessment methods. In general, the greater the RGRs of an aquatic plant the more sensitive the plant may be to a toxicant (Huebert and Shay, 1993; Cedergreen et al., 2004). After 14 days, RGRs of *E. canadensis* grown individually were found to be significantly ($p<0.05$) greater than those of plants grown in model populations and communities. However, after 42 and 84 days of growth both individually grown *E. canadensis* and *M. spicatum* plants were found to have significantly ($p<0.05$) smaller RGRs than those grown in assemblages. The significant differences between RGRs indicate that the individual plant test system or "cone-tainer" method may provide an underestimate of toxicity when used over longer test durations. When used over short exposures, the individual assay appears to be just as sensitive as assemblages for *M. spicatum* testing and more sensitive then the model population and community test method when used to evaluate response of *E. canadensis*. These findings are consistent with results from McGregor et al. (2007) that demonstrate after 35 days of growth, *E. canadensis* and *M. spicatum* grown in model populations and communities have significantly higher RGRs when compared to individually grown plants.

During the first 14 days of growth, neither *E. canadensis* nor *M. spicatum* plants grown in cone-tainers demonstrated reduced growth rates, likely because plants were still relatively small and the resource requirements easily met. Over these two weeks, the significantly slower growth of *E. canadensis* plants in mixed and monoculture pots compared to plants grown in individual cone-tainers may have had to do with the

increased demand on the inorganic carbon supplies surrounding plant leaves (James et al., 1999). This pressure could be experienced in plant assemblages due to the presence of neighbouring plants in close growing vicinity, while likely not encountered by plants growing individually. Over the longer growth periods, the size of the test containers may have played a more significant role in influencing the rate of plant growth under the various bioassay methods. The cone-tainers allot just over 11 cm^2 of growth space per plant while the model population and community pots provide approximately 40 cm^2 per plant of available space, representing more sediment resources for growth. Hindrance of plant growth due to the small size of test containers has been observed in other experiments (Agami and Reddy, 1990), and may have been prominent after 42 and 84 days of growth in this case. In general, differences between RGRs of plants grown under various test methods indicates that testing on only individually grown plants may not allow for effective prediction of a phytotoxic response expected in the larger plant population or community. The use of these methods in combination may lend to a more complete assessment of risk to the aquatic plant community.

3.6 REFERENCES

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Table 3.1 Chemical and physical parameters of the microcosms averaged over the 84-day 10:2 saturated fluorotelomer carboxylic acid (FTCA) study plus pretreatment measurements. Measurements were taken regularly over the 84 day period. At each measurement event the mean of control microcosms was taken. These measures were averaged for all the measurement events taken at that concentration over the 84 day period.

Treatment (µg/L)	Maximum temperature (°C) (n=55)	Minimum temperature (°C) (n=55)	DO[b] (mg/L) (n=54)	pH (n=8)	Alkalinity[c] (mg/L) (n=6)	Hardness[c] (mg/L) (n=6)	Conductivity (µs/cm) (n=6)	PAR[d] $\mu Em^{-2}s^{-1}$ (n=3)
Control[a]	26.4 ± 2.5	21.5 ± 2.0	10.3 ± 3.3	8.3 ± 0.5	227 ± 30	338 ± 14	751 ± 60	403 ± 244
0.1	26.3 ± 2.7	20.6 ± 2.0	10.7 ± 3.4	8.4 ± 0.5	229 ± 30	346 ± 15	746 ± 57	427 ± 253
0.5	26.8 ± 2.5	22.0 ± 2.2	10.7 ± 3.3	8.4 ± 0.5	222 ± 22	327 ± 23	730 ± 58	444 ± 276
1	26.4 ± 2.4	21.7 ± 2.1	9.4 ± 3.1	8.2 ± 0.4	235 ± 42	344 ± 18	775 ± 61	346 ± 219
10	27.7 ± 2.8	21.7 ± 2.0	10.8 ± 4.0	8.4 ± 0.5	220 ± 28	323 ± 20	739 ± 60	413 ± 257
50	26.5 ± 2.6	20.9 ± 2.0	10.3 ± 3.3	8.4 ± 0.5	229 ± 34	341 ± 16	751 ± 64	383 ± 279
100	25.2 ± 2.7	22.2 ± 2.0	10.2 ± 2.7	8.3 ± 0.3	234 ± 38	341 ± 14	750 ± 52	387 ± 263
250	25.1 ± 2.4	21.2 ± 2.1	10.4 ± 3.2	8.4 ± 0.5	219 ± 28	337 ± 14	743 ± 59	392 ± 283
750	26.4 ± 2.6	20.9 ± 2.1	10.7± 3.3	8.4 ± 0.5	228 ± 32	343 ± 15	743 ± 58	348 ± 167
1000	26.5 ± 2.7	20.9 ± 2.0	10.5± 3.5	8.3 ± 0.5	214 ± 32	331 ± 12	736 ± 60	432 ± 227
2000	26.1 ± 2.2	21.3 ± 2.0	10.5± 3.3	8.4 ± 0.5	223 ± 21	333 ± 19	739 ± 60	381 ± 243

[a] The values shown are the mean ± the standard deviation.
[b] DO = dissolved oxygen.
[c] Measured as mg/L of $CaCO_3$.
[d] PAR = photosynthetically active radiation. Meaurements were taken at a depth of 60cm.

Table 3.2 Effective concentrations (μgL^{-1}) required to cause a decrease in the endpoints of interest by 10%, 25%, and 50% (EC_{10}, EC_{25}, and EC_{50}) with associated 95% confidence intervals, as calculated using linear and non-linear modeling techniques using SAS v9.1 (SAS Institute, Cary, NC, USA), for endpoints with significant responses in *Elodea canadensis*, *Lemna gibba* and *Myriophyllum spicatum* exposed to 10:2 saturated fluorotelomer carboxylic acid (FTCA) in aquatic microcosms.

Endpoint	Day	EC_{10} (95% CI)	EC_{25} (95% CI)	EC_{50} (95% CI)	Model[a]	Parameters	Corrected r^2
E. canadensis							
Wet mass stem (g)[b]	14	398 (87.9, 709.0)	996 (219.6, 1772.5)	1992 (439.2, 3544.9)	Linear	b = 0.443; x = 1992.1	0.33
RGR_{wet} ($gg^{-1}day^{-1}$)[b]	14	695 (155.9, 1234.0)	1737 (389.8, 3084.7)	3475 (779.6, 6169.4)	Linear	b = 0.140; x = 3474.5	0.35
Dry mass stem (g)[b]	14	301 (0, 650.9)	754 (0, 1627.3)	1507 (0, 3254.7)	Linear	b = 0.056; x = 1507.2	0.14
RGR_{dry} ($gg^{-1}day^{-1}$)[b]	14	517 (29.3, 1004.0)	1292 (73.1, 2511.0)	2584 (146.3, 5021.9)	Linear	b = 0.132; x = 2584.1	0.24
Wet mass roots (g)[b]	28	0.09 (0, 1.13)	4.714 (0, 38.1)	256.6 (0, 1270.1)	Logistic	b = 0.275; t = 0.2847; x = 256.6	0.44
Wet mass roots (g)[b]	42	1.6×10^{-10} ($0, 1.1 \times 10^{-8}$)	0.000031 ($0, 1.2 \times 10^{-3}$)	5.961 (0, 73.6)	Logistic	b = 0.0904; t = 0.3827; x = 5.9	0.46
Dry mass roots (g)[b]	70	127 (35.9, 217.5)	317 (89.78, 543.7)	633.5 (179.6, 1087.4)	Linear	b = 0.0529; x = 633.5	0.38
L. gibba							
Plant number	7	nc[d]	nc	nc	Linear	b = 3.637; x = 3427.5	0.40
Frond number	7	nc	nc	nc	Linear	b = 25.751; x = 6824.8	0.28
M. spicatum							
Wet mass roots (g)[b]	14	nc	nc	nc	Linear	b = 0.072; x = 1381.2	0.24
Cartotenoids (μgmg^{-1})[b]	28	nc	nc	nc	Linear	b = 0.158; x = 3307.1	0.28
Wet mass roots (g)[b]	70	nc	nc	nc	Linear	b = 3.156; x = 1374.0	0.48
Dry mass stem (g)[b]	70	nc	nc	nc	Linear	b = 0.429; x = 1167.6	0.32
RGR_{dry} ($gg^{-1}day^{-1}$)[a]	70	nc	nc	nc	Linear	b = 0.051; x = 4661.3	0.43
Wet mass roots (g)[b]	84	nc	nc	nc	Linear	b = 3.157; x = 1506.1	0.30
Cartotenoids (μgmg^{-1})[b]	84	nc	nc	nc	Linear	b = 0.148; x = 2899.7	0.29
RGR_{dry} ($gg^{-1}day^{-1}$)[c]	14	215 (31.7, 397.6)	537 (79.3, 993.9)	1073 (158.5, 1987.9)	Linear	b = 0.043; x = 1073.2	0.26

[a] The reparameterized equations used to fit the concentration-responses of 10:2 FTCA-exposed *M. spicatum*, *E. canadensis*, and *L. gibba*: Linear $y = ((-b \times 0.5)/x)x_o + b$; Logistic $y = t/[1 + (x_o/x)^b]$. The variable x is the calculated EC_{50} for the concentration-response relationship modeled, x_o is the actual concentration (µg/L) being evaluated, y is the response or change from control of the endpoint modeled, and b, and t are constants.
[b] Plants grown as individuals. [c] Plants grown in model populations.
[d] These EC_xs are not calculated (nc) as the endpoints of interest demonstrate an increase from controls with increasing concentration.

Table 3.3 Statistically significant ($p<0.05$) differences in biomass and relative growth rates (RGRs) of individually grown *Myriophyllum spicatum* and *Elodea canadensis,* and the plants grown in model populations and communities over 84 days in outdoor microcosms. Biomass and RGR values are the mean (n=11) ± standard deviation.

Endpoint	Time	Individual	Population	Community	*p*- value
M. spicatum					
Wet mass (g)	14	0.490 ± 0.243	0.363 ± 0.155	0.387 ± 0.132	0.239
	42	3.315 ± 0.649	9.735 ± 4.052*	8.015 ± 3.468*	<0.001
	84	6.186 ± 1.945[a]	31.194 ± 11.56[a]*	32.067 ± 16.90[a]*	<0.001
Dry mass (g)	14	0.056 ± 0.027	0.044 ± 0.010	0.045 ± 0.013	0.261
	42	0.459 ± 0.116	1.259 ± 0.560*	0.958 ± 0.451*	0.002
	84	1.079 ± 0.338[a]	5.961 ± 2.594[a] *	6.321 ± 3.784[a]*	<0.001
RGRwet ($gg^{-1}day^{-1}$)	14	0.081 ± 0.031	0.014 ± 0.124	0.065 ± 0.032	0.178
	42	0.074 ± 0.005	0.098 ± 0.012*	0.093 ± 0.012*	<0.001
	84	0.044 ± 0.005[a]	0.063 ± 0.005[a]*	0.062 ± 0.008[a]*	<0.001
RGRdry ($gg^{-1}day^{-1}$)	14	0.052 ± 0.032	0.040 ± 0.017	0.039 ± 0.029	0.457
	42	0.069 ± 0.006	0.091 ± 0.013*	0.084 ± 0.014*	<0.001
	84	0.044 ± 0.005[a]	0.062 ± 0.009*	0.064 ± 0.007[a]*	<0.001
E. canadensis					
Wet mass (g)	14	0.592 ± 0.075	0.414 ± 0.128*	0.336 ± 0.088*	<0.001
	42	1.873 ± 0.677	9.416 ± 3.507*	8.007 ± 5.636*	<0.001
	84	2.990 ± 0.937[b]	14.442 ± 6.927[a]*	14.051 ± 5.130*	<0.001
Dry mass (g)	14	0.074 ± 0.023	0.042 ± 0.011*	0.035 ± 0.009*	<0.001
	42	0.335 ± 0.103	1.128 ± 0.496*	0.889 ± 0.645*	<0.001
	84	0.667 ± 0.229[b]	3.000 ± 1.163[a]*	2.935 ± 1.014*	<0.001
RGRwet ($gg^{-1}day^{-1}$)	14	0.140 ± 0.009	0.113 ± 0.021*	0.098 ± 0.022*	<0.001
	42	0.073 ± 0.010	0.111 ± 0.009*	0.228 ± 0.408*	<0.001
	84	0.042 ± 0.004[b]	0.060 ± 0.005[a]*	0.060 ± 0.005*	<0.001
RGRdry ($gg^{-1}day^{-1}$)	14	0.132 ± 0.021*	0.092 ± 0.020*	0.078 ± 0.021*	<0.001
	42	0.080 ± 0.008	0.108 ± 0.010*	0.099 ± 0.016*	<0.001
	84	0.048 ± 0.004[b]	0.066 ± 0.005 [a]`*	0.066 ± 0.005*	<0.001

[a]Values are the mean (n=10) ± standard deviation.
[b]Values are the mean (n=7) ± standard deviation.
*Statistically significant difference in growth measure from individually grown plants.

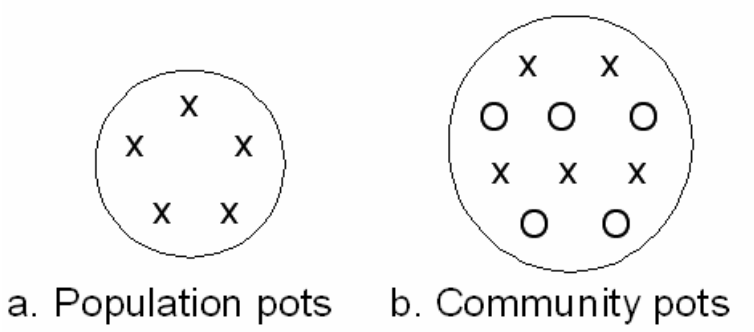

Figure 3.1 Diagram of the planting arrangement used for model populations and communities of *Myriophyllum spicatum and Elodea canadensis*. The density of plants in each population and community pot approximated 1 plant per 40 cm2. (a) *M. spicatum*:*E. canadensis* planting density was 5:0 or 0:5 (monoculture). (b) *M. spicatum* : *E. canadensis* planting density was 5:5 (mixed culture).

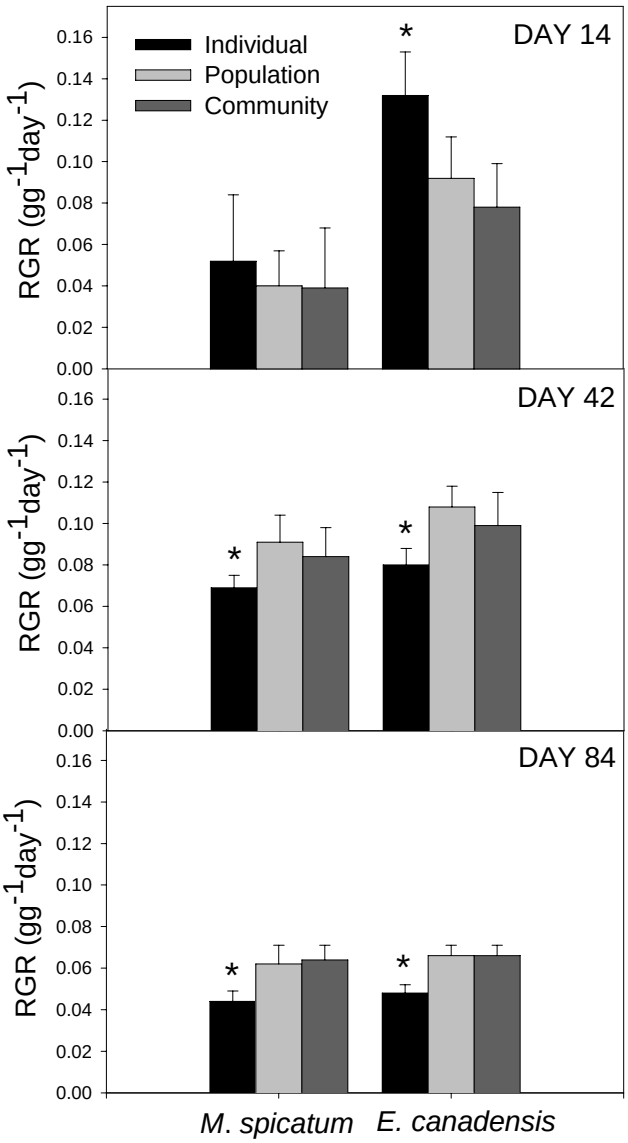

Figure 3.2 Relative growth rates (RGR) (gg-1day-1) calculated from total dry biomass of *Myriophyllum spicatum* and *Elodea canadensis* grown as individuals and in model populations and communities over 84 days. Error bars represent the standard deviation about the mean. An * denotes a statistically significant difference as detected using a Tukey's test ($p<0.05$).

4 EFFECTS OF PLANTING SYSTEM DESIGN ON THE TOXICOLOGICAL SENSITIVITY OF MYRIOPHYLLUM SPICATUM AND ELODEA CANADENSIS TO A CHEMICAL STRESSOR: A CASE STUDY USING ATRAZINE

4.1 ABSTRACT

The triazine herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-trazine) was selected as a chemical stressor in an investigation of how toxicological responses of individually grown macrophytes reflect those of plants grown in more natural model populations and two-species communities. Phytotoxicity of the compound to *Myriophyllum spicatum* L. and *Elodea canadensis* Michx. was assessed under semi-natural field conditions using 12 000 L outdoor microcosms. Exposure concentrations of 25, 50, 100, 250 µg/L plus controls (n=3) were evaluated, selected to fall within a range of concentrations known to produce a toxic response in the tested macrophytes, and effective concentrations required to cause a decrease in biomass endpoints by 10, 25, and 50% were estimated. The sensitivities of aquatic plants to atrazine did not differ substantially between planting systems, and few interactions between the effects of the planting method and atrazine effects on macrophyte biomass were detected using a two-way ANOVA. A lack of significant differences in biomass and relative growth rate measures between plants grown under the various test systems also indicated that interactions between and among species did not influence growth of plants in the model population and communities. Under these test conditions, the use of the "cone-tainer" method provided estimates of toxicity consistent with those from plants grown in assemblages, and potential interactions between plants were not found to modify the response of macrophytes to atrazine.

4.2 INTRODUCTION

In practice, ecotoxicology is largely focused on investigating the effects of contaminants at an organism-level, measuring survival, growth, and reproduction in individuals (Clements and Kiffney, 1994; Forbes and Calow, 1999). While research methods have advanced beyond laboratory-based single-species toxicity testing, regulatory ecotoxicology still commonly relies on these tests to inform the process of ecological risk assessment (Calow and Forbes, 2003). Extrapolation between levels of biological organization is routinely conducted (Power and McCarty, 1997; Preston, 2002) based on the underlying assumption that organism-based responses are predictive of effects at the population, community and ecosystem levels (Cairns, 1983; Preston, 2002), despite concern over the lack of a causal relationship demonstrated in the literature (Clements and Kiffney, 1994; Walthall and Stark, 1997; Forbes and Calow, 1999). Proponents of an ecological viewpoint reason that the interactions between elements of a system are more complex than can be predicted from the examination of the individual ecosystem components (Cairns, 1983).

The toxicity of environmental contaminants to aquatic macrophytes is typically evaluated using the laboratory approach. Effects of the chemical stressor on the larger plant community are estimated from effects measured at the individual-level, such as growth indicators and pigment concentrations, and the results may be used to inform the lower tiers of ecological risk assessment (Davy, 2001). This practice is yet to be validated, however (Hanson and Arts, 2007). In both standardized (ASTM, 1999), and non-standardized (Fairchild et al., 1998; Turgut and Fomin, 2001; Knauer et al., 2006) laboratory-based assays on submersed and emergent aquatics, macrophytes are grown in

isolated test units that do not allow for natural interactions between and among plant species to occur.

Likewise, simulated field-level assessments, ie., microcosm studies, that strive to provide more environmental realism, also have an individual-plant focus. In the past, the monitoring of toxicant effects to aquatic macrophytes was considered a secondary objective of microcosm research, and the inclusion of plants in model ecosystems usually involved the unmanaged growth of plants directly from the bottom sediments. These practices resulted in limited species diversity and high variability within and between treatments (Coors et al., 2006). Research has moved towards more controlled methods for assessing macrophyte toxicity within these model systems. Species composition is explicitly selected, and macrophytes are individually potted (Detenbeck et al., 1996; Hanson et al., 2001; Davies et al., 2003; Brain et al., 2005a; Brain et al., 2005b; Hanson et al., 2005) in attempts to reduce variability within the test systems (Hanson et al., 2003; Coors et al., 2006). Although the macrophtyes are grown in a model ecosystem with multiple trophic levels interacting, the individually potted plants are not exposed to typical intraspecific interactions, such as population density effects (Drake and Unger, 1989; Driever et al., 2005; Wang et al., 2005) and interactions between species, such as shading, allelopathy, and competition for resources (Agami and Waisel, 2002; Wu and Yu, 2004).

Evidence suggests that the responses of organisms to an environmental contaminant may be modified by species interactions within the ecosystem (Simkiss et al., 1993; Sibly et al. 2000; Preston 2002). For example, laboratory testing conducted to determine the short-term toxicity of cadmium, mercury and pentachlorophenol to

freshwater oligochaetes, *Limnodrilus hoffneisteri* and *Tubifex tubifex*, found that responses of species tested in mixtures differed significantly from individually tested species (Chapman et al., 1982). Comparison of the 96-hour lethal concentrations indicated that the worms were less tolerant of contaminants when tested in pure culture rather than in mixed. Also, in an evaluation of the response of aquatic plants, *Egeria densa* and *Ceratophyllum demersum*, to oxytetracycline exposure, researchers observed increased susceptibility of macrophytes to the contaminant when grown as paired plants relative to those grown as individuals (Hanson et al., 2006), thus indicating the possibility that plant interactions may modify toxicant effects. With this in mind, there is a lingering question over whether monitoring effects of contaminants on individually grown plants is associated with potential for over- or under-estimation of effective concentrations for the larger plant community. Initial studies were conducted in aquatic microcosms to compare toxicity of chemicals to plants that are grown and tested as model populations and two–species communities, to those grown as individuals, but a lack of significant toxicity of the tested contaminants meant that direct comparison of sensitivity was not possible (McGregor et al., 2007, McGregor et al., 2008). Significant differences in the relative growth rates and biomass measures of plants grown under each planting system however, indirectly indicated the potential for individual-based test systems to underestimate toxicity in simulated field studies, as those test systems with greater relative growth rates may be more sensitive toxicologically (Huebert and Shay, 1993; Cedergreen et al., 2004).

This current study was initiated in order to more explicitly examine whether the responses of plants grown under widely-used individual-based test systems appropriately

characterize those of more realistically grown plant assemblages. This was done by examining the sensitivity of freshwater macrophytes, *Elodea canadensis* and *Myriophyllum spicatum*, grown under various planting designs, to a recognized chemical stressor of macrophytes, atrazine. As a known phytotoxicant, atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine), was selected to serve as a model toxicant. Like other triazine herbicides, the compound inhibits photosynthesis by preventing electron transfer at the reducing site in photosystem II (Kamrin, 1997). Atrazine is typically applied pre-emergence for crop protection against selected broadleaf and grassy weeds in corn, sorghum, and sugarcane production, and may also be applied preplant and postemergence and in other crop and landscape systems (WSSA, 1989). The compound is mainly absorbed through the roots or foliage of terrestrial plants (Kamrin, 1997).

Since its registration in the United States in 1959, atrazine has become one of the most widely used herbicides in North America (Lytle and Lytle, 2005), with over 35 000 metric tons applied throughout the United States in 1998 (Giddings et al., 2005). The compound is used heavily in the midwestern United States and in southern Ontario, Canada, where corn crops are dominant (Solomon et al., 1996). As approximately 0.1 to 3.0 % of atrazine sprayed on fields is lost into aquatic systems (Jones et al., 1982), the compound has been widely detected as a contaminant in surface waters (Huber, 1993). Atrazine primarily enters waters through surface runoff following precipitation or irrigation, with a propensity for movement in the dissolved state from treated soils (Glotfelty et al., 1984), and has an expected half-life ranging from 3 to 90 plus days in surface waters (Giddings et al., 2005). Environmental concentrations are determined to rarely exceed 20 μg/L in rivers and streams (Solomon et al., 1996), while peak

concentrations have reached as high as 1 mg/L in reservoirs adjacent to treated fields (Kadoum and Mock, 1978).

An extensive body of toxicity and environmental fate data exists for atrazine, along with numerous environmental risk assessments (Eisler, 1989; Huber, 1993; Solomon et al., 1996; Giddings et al., 2005). Laboratory testing and model ecosystem studies have shown that atrazine may cause effects in non-target freshwater plant species, including *E. canadensis* and *M. spicatum* (Forney and Davis, 1981; Johnson, 1986; Fairchild et al., 1998). As discussed in the most recent ecological risk assessment, freshwater macrophytes, freshwater phytoplankton, and saltwater phytoplankton demonstrate comparable sensitivity to atrazine; the geometric means for acute toxicity of each group range between 85 and 123 μg/L (Giddings et al., 2005). While the mean of the data for freshwater macrophytes falls within the range of other plant groups, the data also demonstrate differing sensitivity to atrazine within freshwater macrophytes, associated with varying species and monitored endpoints. In laboratory based investigations, *E. canadensis* was found to be the most sensitive freshwater plant based on the acute estimate of a 14-day EC_{50} (wet weight) of 21 μg/L (Fairchild et al., 1998), while *M. spicatum* was determined to be significantly less sensitive. About 3700 μg/L of atrazine is required to produce a 50% reduction in the number of branches produced by *M. spicatum* after 5 days (Bird, 1993), and about 91 μg/L to inhibit stem dry weight by 50% over 28 days (Kemp et al., 1985). The effects of atrazine on root length, dissolved oxygen concentration, and the number of nodes per centimeter, in an axenic laboratory-culture of *Myriophyllum sibiricum* were also examined (Roshon, 1997). They estimated 14-day IC_{50}s to range from 1130 to 2066 μg/L for the above mentioned endpoints, while

pigment concentration and shoot length endpoints endpoints were stimulated at the highest test concentrations. The unexpected low toxicity of atrazine to *M. sibiricum* is potentially due the presence of a carbon source, sucrose, in the growth media, to a solvent-pesticide interaction, or some type of resistance of the plants, demonstrating that laboratory-based tests can drastically underestimate toxicity under certain conditions.

Data from microcosm and mesocosm studies indicate that exposure to concentrations of 20 μg/L atrazine or less, rarely produce effects in the aquatic plant community, and that recovery from effects typically occurs at exposures of up to 50 μg/L atrazine (Giddings et al., 2005). In general, studies conducted under semi-field conditions found macrophyte communities were significantly affected by concentrations ≥ 100 μg/L atrazine (deNoyelles et al., 1982; Kettle, 1982; Carney, 1983; Johnson, 1986; Kettle et al., 1987; deNoyelles et al., 1989). To ensure that a notable response would occur in the evaluated freshwater macrophytes, the treatment levels were assigned to range between 25 and 250 μg/L atrazine. This facilitated the comparison of sensitivities of plants grown under various planting designs, including individually grown, monocultures and mixed assemblages of *E. canadensis* and *M. spicatum*.

The two main objective of this study were to (1) investigate how macrophyte toxicological responses observed in the individual-based microcosm test reflect responses observed in more natural model populations and two-species communities; (2) examine whether relative growth rate of the plants under these different scenarios can predict the observed sensitivities. We hypothesize that those plants with greater relative growth rates will exhibit a greater sensitivity to atrazine. An ancillary goal of the experiment

was to estimate effective concentrations for atrazine to *E. canadensis* and *M. spicatum* under microcosm conditions, as this is yet to be determined.

4.3 METHODS AND MATERIALS

4.3.1 Microcosms

The 15 outdoor microcosms utilized in this study are located at the University of Guelph Microcosm Facility at the Guelph Turfgrass Institute, Ontario, Canada (Figure 1.1A). Each of the facility's 30 artificial ponds are approximately 1.2 m deep with the 30 artificial ponds are approximately 1.2 m deep with a water depth of 1 m, a surface area of 11.95 m^2, and a diameter of 3.9 m. The microcosms are lined with black, food-grade polyvinyl chloride (Fox Pools Canada, Burlington, ON) and are sunken into the ground with the tops flush with the ground level. They each hold approximately 12 000 L of water which is supplied from an adjacent spring-fed irrigation pond (62 x 62 x 4 m deep).

To establish a model freshwater ecosystem, 35 plastic propagation trays (52 x 25 x 7 cm; Canadian HydroGardens, Ancaster, ON) were added to the bottom of each microcosm, containing an amended sediment mix (Waterdown Garden Supply, Troy, ON). This sediment consisted of a 3-way mixture of sand, loam and organic matter and covered approximately 38% of each microcosm floor.

Water was circulated between the microcosms and the irrigation pond at a rate of 12 000 L/day for 25 days prior to treatment with atrazine. Circulation was undertaken in order to reduce variability within and between microcosms, providing consistent physicochemical properties and biological communities. Six pots of macrophytes (*M. spicatum*) were placed in each microcosm during the water circulation period. These

plants were obtained from several on-site untreated microcosms and were not used in the assessment of effects. Circulation was terminated 2 days prior to treatment (July 16, 2006) for the creation of self-contained systems. The final day of the field study was August 29, 2006 (Day 42).

4.3.2 Atrazine treatment and sampling regime

The treatments applied to the microcosm were 25, 50, 100 and 250 µg/L atrazine, plus controls. Treatment of the microcosms took place on July 18, 2006, with each concentration randomly applied to three separate microcosms (n=3). The atrazine, supplied by Syngenta (96% active ingredient, technical-grade, Syngenta Crop Protection, Inc.,Greensboro, NC), was weighed out and dissolved in 50 mL of acetone (Reagent Grade, Fisher Scientific, Whitby, ON) on the day of treatment. The treatments were mixed in amber bottles, shaken, and added to the microcosms while simultaneously agitating with a stirrer to promote mixing. The same volume of acetone was added to the control ponds.

Water samples for atrazine analysis and routine water chemistry determination were collected using a metal depth-integrated water column sampler (Solomon et al. 1982). Integrated subsamples from a minimum of four randomly selected locations in the microcosms were collected to a volume of approximately 2 L. Water residue samples were taken on days -1, 1, 4, 7, 14, 28, and 42, a 1-L aliquot collected and stored at 4°C until analysis.

4.3.3 Water chemistry and photosynthetically active radiation

Measures of temperature, dissolved oxygen (DO), pH, alkalinity, hardness, and conductivity were taken regularly over the course of the study. Water samples were collected as described above and 500 mL aliquots were stored in amber bottles at 4°C for two days before processing. Maximum and minimum temperatures were measured daily, Monday through Friday, at a water depth of 20 cm. Point temperatures and DO readings were also taken daily at a depth of 50 cm using a HANNA HI-9143 meter (HANNA Instruments, Laval, QB). Due to equipment failure, DO was not measured on the last two days of the study. Water hardness, alkalinity and conductivity were measured on days -1, 21, and 42, and pH was measured weekly. Standard methods and kits by Hach (Hach Company, Loveland, CO) were used to determine hardness and alkalinity. An Accumet Research AR20 pH/Conductivity meter (Fisher Scientific, Whitby, ON) was used to measure pH and conductivity.

Photosynthetically active radiation (PAR) was measured weekly using a Li-Cor Quantum/Radiometer/Photometer model LI-185A (Li-Cor, Lincoln, NE). Readings were taken on clear sunny days between the hours of 12 and 2 pm. Measures were taken at the surface of each microcosm, at the surface in a glass jar, and at a depth of 60 cm in a glass jar in order to normalize the reading.

4.3.4 Analysis of atrazine

Analysis of atrazine residues was performed by enzyme linked immunosorbent assay (ELISA), using the Abraxis Atrazine Assay Kit (Abraxis LLC, Warminster, PA) and associated procedures. Prior to commencement of the assay, the water samples were brought to room temperature and 10 mL aliquots were centrifuged at approximately 805

g for 10 minutes. The residue samples were then diluted in order to fall within the range of the standard curve (0.1-5 μg/L) and the analysis completed. An Ohmicron RPA-1 photometric analyzer (Ohmicron Corp., Newtown, PA) was used to calculate calibration curves and concentrations. Analyses were performed within 48 hours of sampling and normally within 24 hours. Time-weighted averages at each atrazine concentration for the three replicates were calculated.

4.3.5 Macrophyte assessment

4.3.5.1 Individual responses of *M. spicatum* and *E. canadensis*

Myriophyllum spicatum L. (Haloragaceae) and *Elodea canadensis* Michx. (Hydrocharitaceae) used in the field study were harvested from a single untreated on-site microcosm, originally obtained from a local reservoir (Guelph Correctional Facility, Guelph ON, Canada). The plants were grown in 164 mL "cone-tainers" or plastic planting tubes held in a 96-well planting tray (Figure 1.1D) (Steuwe and Sons, Corvallis, OR, USA) for assessment of the response of individual plants. The "cone-tainers" were 21 cm long with a 3.8 cm internal diameter (Figure 1.1G) and had cotton pads placed in the bottom to plug holes. The tubes were filled with the same amended sediment used in the microcosms and soaked for several days in irrigation pond water to allow the soil to settle. Apical shoots of *M. spicatum* and *E. canadensis,* without any side roots or shoots, were cut to 5 cm and planted in the "cone-tainers". Each shoot was planted 2 cm into the soil and surrounded by approximately 0.5 to 1 cm of Turface (Applied Industrial Materials, Buffalo Grove, IL, USA) to secure the plants in the sediment. A total of 6 plants per species were evenly spaced across each planting tray and added to the microcosms one day prior to treatment (July 17, 2006). One tray was placed into the

centre of each pond, to provide maximum sunlight and reduce edge effects. Each species was sampled the day before atrazine treatment and at 14, 28 and 42 days after treatment. On day -1, ten plants of each species were evaluated as 5 cm apical shoots, to obtain their baseline condition. At each other sampling point, two plants of each species were removed randomly from the microcosms, transported back to the laboratory in their respective microcosm water and immediately evaluated. The endpoints evaluated were biomass (wet mass/dry mass) of roots and shoots. Chlorophyll-*a* levels were measured by ethanol extraction using an Ultrospec 3100 *pro* UV/Visible Spectrophotometer (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) by methods that have been described in detail elsewhere (Nusch 1980).

4.3.5.2 Assemblages of *M. spicatum* and *E. canadensis*

Mixed and monocultures of *M. spicatum* and *E. canadensis* were grown in plastic sterilite containers (34.9 x 20.6 x 32.1 cm deep) (Sterilite Corporation, Townsend, MA) to model small populations and two-species communities, in which potential intra- and inter-species interactions could occur. The sterilite containers were filled with sediment and prepared for planting using the same method as the "cone-tainers" (Figure 1.1F).

Monocultures of *M. spicatum* and *E. canadensis* were grown at two densities. The low- density arrangement consisted of either nine *M. spicatum* or nine *E. canadensis* plants per sterilite container, equal to 1 plant per 80 cm^2. This density was selected in order to fall within the range of biomass densities of a nature population of *M. spicatum* studied in a Northern temperate region of the United States (Lillie et al., 1997). The plants were positioned in a 3 x 3 grid configuration (Figure 4.1a). In the high-density arrangement, eighteen shoots of a single species were planted in each sterilite container in

a 6 x 3 configuration (Figure 4.1b). The planting density was equal to 1 plant per 40 cm^2, selected in order to match the density of model populations grown in previous studies (McGregor et al., 2007, McGregor et al., 2008).

Under the two-species mixed growth conditions, the low-density planting rate consisted of nine plants per species for a total of eighteen plants in a 6 x 3 configuration. The species type was alternated each row of plants (Figure 4.1c). The same set-up was used for the high-density community containers, with eighteen plants per species grown (Figure 4.1d).

A total of eighteen sterilite containers, three containers per design, were added to each microcosm and evenly spaced around the "cone-tainer" planting trays in the centre of the ponds. Mixed and monoculture pots were sampled at 14, 28 and 42 days after treatment. The endpoints monitored were root and shoot biomass (wet mass/dry mass). Biomasses of assemblages of plants were standardized to a per plant basis to allow for comparison to individually grown plants.

4.3.6 Statistical analyses

4.3.6.1 Water chemistry and photosynthetically active radiation

For each monitored parameter a one-way analysis of variance (ANOVA) was performed to identify significant differences between treatments using SigmaStat 3.5 (Systat Software 2006, Jandel, San Rafael, CA, USA). ANOVA assumptions of residual normality and homogeneous variance were assessed and confirmed using a Kolmogorov-Smirnov test and a Levene median test, respectively. When significance ($p < 0.05$) was found, the means were compared with the control using Dunnett's test ($\alpha = 0.05$).

4.3.6.2 Relative growth rate comparison and plant toxicity data analysis

Relative growth rates (RGR) were calculated according to Hunt (1990) from wet and dry biomass measures of control plants as follows:

$$RGR = [\ln(W_2) - \ln(W_1)] / t_2 - t_1 \quad (1)$$

where W_1 and W_2 are plant weights at times t_1 and t_2. RGRs of macrophytes grown under each planting design (individual plants, low density populations, high density populations, low density mixes, high density mixes) were compared for day 14, 28, and 42 data, using a one-way ANOVA ($\alpha = 0.05$) in SigmaStat 3.5, and a Tukey's test for pairwise comparisons when significance was found.

Plant toxicity was evaluated using nonlinear regression techniques according to the procedure outlined in Stephenson et al. (2000). To identify distinct concentration-response relationships macrophyte data for all treatments were modelled in SigmaPlot 2000 (SPSS Inc. Chicago, IL, USA) using linear, logistic, and hormetic equations (Hanson et al., 2006). The corrected coefficient of determination (r^2 = 1-[residual sum of squares/corrected sum of squares]) and graphical interpretation of the plots were used to select the best-fitting model. For those biomass endpoints showing a significant concentration response ($p < 0.05$), proc NLIN of SAS v9.1 (SAS Institute, Cary, NC, USA) was used to generate effective concentrations (EC_x) resulting in 10, 25, and 50% differences in measured endpoints from control treatments (EC_{10}, EC_{25}, and EC_{50}), plus confidence intervals. Prior to use in the regression analyses, day 1 mean values were subtracted from later sampling dates for data on wet and dry mass of roots and shoot so that only new growth data were used in the assessment of effects.

4.3.6.3 Analysis of possible plant interactions

The study was conducted using a split-plot design in order to investigate the interaction effects of the main factors, atrazine concentration and planting design, on the biomass (growth) of *M. spicatum* and *E. canadensis*. A two-way ANOVA ($\alpha = 0.05$) was performed using proc mixed of SAS v9.1. Anova assumptions of residual normality and homogeneous variance were assessed and confirmed using the Shapiro-Wilk test (proc univariate normal) and by graphical interpretation of residual plots. Data that did not meet normality requirements were natural ln or square root transformed. When significance of the interaction effect was found, the "slice" command was used in order to identify how atrazine effects changed with planting design. When main factor effects were significant but the interaction effect was not, comparison of means using orthogonal contrasts were conducted to investigate growth differences between test designs (ie. individual versus population 1; individual versus community 1, etc.).

4.4 RESULTS

4.4.1 Atrazine and physico-chemical analysis

The time-weighted averages (TWA) for each atrazine concentration were calculated for the 42 day exposure period. The control microcosms had a TWA of 0.0 ± 0.0 μg/L, the 25 μg/L microcosms had a TWA of 24.5 ± 2.5 μg/L, the 50 μg/L microcosms had a TWA of 49.5 ± 2.8 μg/L, the 100 μg/L microcosms had a TWA of 104.0 ± 5.6 μg/L, and the 250 μg/L microcosms had a TWA of 247.5 ± 12.2 μg/L. These measured concentrations were used as the exposure level for statistical purposes.

Temperature, DO, pH, alkalinity, hardness, conductivity, and photosynthetically active radiation data of the microcosms over the course of the study are provided in Table 4.1 The results presented represent the mean of all measurements taken for each specified parameter at a given atrazine concentration averaged over all sampling times. Dunnett's test for multiple comparisons yielded no significant differences ($\alpha = 0.05$) for maximum temperature, alkalinity, hardness, conductivity, or PAR as compared with control values, and although significant differences were found for minimum temperature (post treatment) a concentration-response trend was not evident. Significant decreases in dissolved oxygen in the 104.5-µg/L and 247.5-µg/L microcosms compared to the control ponds were evident. Trends in DO at each treatment level over 42 days are presented in Figure 4.2.

4.4.2 Macrophyte toxicity

Non-linear regression analysis conducted at all sampling times, for macrophtye species grown as both individuals and in assemblages, found significant concentration-response relationships for 110 of the 120 monitored biomass endpoints, with endpoint inhibition increasing as a function of atrazine concentration. Effective concentrations required to cause a 10%, 25%, or 50% decrease in the endpoint of interest were calculated using the best-fitting model, for the majority of responses modeled in *M. spicatum* and *E. canadensis* (Tables 4.2 and 4.3, respectively). Box plots of the range of EC_{50} estimates for each planting scenario, at each time point are presented in Figure 4.3. Effective concentrations were not estimated for individually *E. canadensis* at Day 42, due to a lack of concentration-response relationships for any of the biomass endpoints. For individually grown *M. spicatum* (days 14, 28, 42) and *E. canadensis* (days 28, 42)

chlorophyll-*a* content was found to increase with increasing atrazine concentration. Effective concentrations required to cause a 110%, 125%, or 150% increase in the endpoint of interest were calculated.

4.4.3 Comparison of RGRs of individual plants to model populations and communities

The RGRs of *M. spicatum* and *E. canadensis* grown in control micocosms as individual plants and in model populations and communities are presented in Table 4.4. Generally, the rate of plant growth between the various planting systems falls within the same range. Few significant differences in RGRs between individually grown plants and those grown in model populations and communities were detected, with the exception of *E. canadensis* grown individually and in model populations at days 28 and 42. Individually grown *E. canadensis* were found to have significant lower ($p < 0.05$) $RGRs_{wet}$ than those of plants grown in low- and high- density model populations. Figure 4.4 presents that RGR_{dry} of plants grown for 42 days under the various planting designs.

4.4.4 Split-plot analysis

There were no significant interactive effects between atrazine concentration and planting design on biomass of *M. spicatum* or *E. canadensis* at days 14 and 28, or for *M. spicatum* plants at day 42, as determined in a two-way ANOVA applied to growth data (Table 4.5). The interactions of these factors significantly affected biomass of *E. canadensis* at day 42. Atrazine was found to significantly affect plant biomass at all time points, as was expected due to the signficant concentration-response relationships reported earlier in this manuscript. Wet and dry biomassess of roots and shoots however,

were not universially affected by the planting design factor. Generally, biomass of *M. spicatum* and *E. canadensis* plants at day 42, and biomass of *M. spicatum* roots and *E. canadensis* shoots at days 14 and 28, were significantly impacted by planting design, while planting design was not significant for *E. canadensis* root, or *M. spicatum* shoot biomass, at days 14 and 28.

4.5 DISCUSSION

Change in the biomass of macrophytes is widely used as both an endpoint in plant toxicity testing assays (Lewis, 1995; ASTM, 1999), and as an indicator of relations between and among plant species in competition and density-dependence investigations (Agami and Waisel, 2002; Demirezen et al., 2007). Thus, analysis of the plant biomass data using a mixed factorial model and an associated two-way ANOVA procedure allowed for simultaneous examination of changes in biomass related to atrazine effects, and planting design effects used as an indirect indicator of plant relations, and the interaction effects between these two main factors. At days 14 and 28, neither species demonstrated a significant interaction between factors, nor did *M. spicatum* at day 42, establishing that the effect of atrazine on plant biomass over these growth periods was unaltered by the method under which plants were grown and toxicity assessed. The effect of atrazine on biomass was consistent across all types of planting design, and vice-versa, for those biomass endpoints that did not demonstrate an interaction between factors but found the main effects of factors significant (Table 4.5). Orthogonal contrasts comparing mean biomass measures between planting systems, revealed few significant differences between design types (ie. individual vs. population 1, population 1 vs

population 2, etc.), and no consistent trends across biomass endpoints for planting systems that did demonstrate statistically different means.

The effect of atrazine on wet mass at day 28 and biomasses on day 42, was not uniform across *E. canadensis* planting methods, indicated by the significant interaction between main factors. The "slice" command used to investigate how atrazine effects vary across planting methods, determined that individually grown *E. canadensis* did not demonstrate significant differences between mean biomass measures at each concentration level of atrazine, and thus did not respond to atrazine exposure. Plants grown in model populations and communities however, did demonstrate differences in biomass means between concentrations. It was hypothesized that potential interactions between plants grown in mixed and monoculture assemblages would impact biomass and in turn modify the response of plants to atrazine, but the lack of interactions between atrazine and planting method effects, except in the case when individually grown *E. canadensis* did not respond to atrazine, indicate that relations between plants did not impact the response of plants to atrazine.

Significant concentration-response relationships ($p < 0.05$), modeled using linear and logistic equations, were found for most of the effect measures monitored. These analyses were conducted independently of the two-way ANOVA. The pronounced effect of atrazine on the growth of *E. canadensis* and *M. spicatum*, demonstrated by these non-linear regressions, was expected at the monitored range of exposures. Effective concentrations however, were not estimated when the models were not a good fit to macrophyte response data, as was the case for various endpoints in individually grown *E. canadensis* at days 28 and 42, model populations of *E. canadensis* at day 28, and model

communities of *M. spicatum* at day 42. In these cases, the regression models did not appropriately characterize the large variability between replicates at one or more monitored concentrations. This is not likely an indication that atrazine was not phytotoxic, but rather a failure of the test system when there are anomalies in growth data. The individually grown *E. canadensis* were "chewed-up" in several of the control ponds resulting in low biomass measures, while plants in the 25 μg/L microcosms simply did not grow at all, therefore the decreasing trend across atrazine concentrations could not be made out from uncharacteristically low growth at the lowest concentrations. Aproximately 40**%** of all significant *M. spicatum* concentration-responses relationships had an associated coefficient of determination over 0.8, compared with over 70**%** of the responses monitored in *E. canadensis*. This indicates that generally, stronger concentration-responses were observed for *E. canadensis*, as a greater proportion of variability in the data is accounted for. Despite that fact that low level exposures of atrazine have been demonstrated to cause a stimulatory response in laboratory based tests on *M. sibiricum* (Roshon, 1997) and in general, a hormetic response to low levels of herbicides is commonly observed in plants (Ries, 1976), the hormetic model was not good fit with macrophyte data, with only five *M. spicatum* responses adequately described by the model. The increase in chlorophyll-*a* content of individually grown macrophtyes does however, correspond with the stimulatory response of pigments observed in *M. sibiricum* cultures (Roshon, 1997), while pigment content was not monitored when plants were grown in assemblages.

Changes in water quality parameters (Table 4.1), specifically significant decreases in DO, serve as an indicator of atrazine-induced reduction in primary productivity at the

104.5-µg/L and 247.5-µg/L exposure levels (Solomon et al., 1996). These trends are consistent with observations in the literature, demonstrating reduced oxygen production in model freshwater ecosystems exposed to atrazine applications of ≥ 100 µg/L (Brockway et al., 1984; Hamilton et al., 1989; Detenbeck et al., 1996). The large drop in DO concentration observed on days 15-16, across all treatment levels (Figure 4.2), likely stems from particularly overcast conditions noted on the day previous to monitoring, and an associated drop in photosynthetic activity.

Relative comparisons of EC_{50}s established that toxicological sensitivity of aquatic plants did not differ substantially between macrophytes grown under the various planting designs. Averages of EC_{50} values across four endpoints (wet root, dry root, wet shoot and dry shoot masses) for each sampling day and species were calculated, and boxplots of the data presented in Figure 4.3. The considerable overlap of 95% confidence intervals surrounding EC_{50} estimates indicates that sensitivities fall within the same range. Consistently wide intervals around EC_x values however, suggest that uncertainty is associated with these estimates and that precision is generally low. Differences in sensitivity of macrophytes between planting methods was found to vary greatest between *E. canadensis* at 14 days, with about a 6-fold difference between the lowest average EC_{50},14.9 µg/L for low density model populations, and the highest average EC_{50}, 86.5 µg/L for high density model community. The smallest range of toxicological sensitivities exist between *M. spicatum* at day 42, varying 2-fold between individually grown plants with an average EC_{50} of 63.1 µg/L, and low density model populations with an average EC_{50} of 112.4 µg/L; average EC_{50}s of the other three planting methods falling between these values. No particular planting method proved to be consistently more or less

toxicologically sensitive across time and species. These ranges in EC_{50} values are relatively small in comparison to between species differences in sensitivities to environmental contaminants. In an assessment of the response of aquatic plants to a mixture of tetracyclines established 13- and 57-fold differences in EC_{25} and EC_{10} values between *L. gibba* and *M. spicatum* (Brain et al., 2005b), and the EC_{50}s of eleven test species exposed to metsulfuron-methyl were found to vary by a factor of 56 (Cedergreen et al., 2004). Considering that the responses of surrogate species are deemed representative of other aquatic plants (Lewis et al., 1995; Brain et al., 2004), despite potentially large differences in sensitivities (Fairchild et al., 1998), the variation in responses between macrophytes grown under various planting methods observed here, is negligible in terms of the risk assessment process.

Differences between RGRs of *E. canadensis* in control microcosms were found at days 28 and 42 (Figure 4.4). The RGR_{wet}s of plants grown in low- and high-density monocultures were significantly higher then plants grown in individual "cone-tainers", while differences between *E. canadensis* in model communities and individually grown plants were not evident. Significant differences were not detected between RGRs of individually grown *E. canadensis* and *M. spicatum*, and those plants grown in model populations and communities, at all other time points (Table 4.4). Because growth of macrophytes between planting systems was generally within the same range, the plants were expected to demonstrate similar responses to toxicant exposure with respect to growth endpoints, as was in fact demonstrated through comparison of sensitivities of macrophytes between planting systems. Those individually grown *E. canadensis* that demonstrated significant lower RGRs, did not respond to atrazine exposure and could not

be described by a concentration-response model, demonstrating a potential link between RGR trends and toxicity observations.

The growth rate observations in this study are not totally consistent with results from previous investigations (McGregor et al., 2007, McGregor et al., 2008), in which significantly lower RGRs were observed for *E. canadensis* and *M. spicatum* plants grown as individuals compared to those grown in model populations and communities over 35, 42, and 84 days. Several factors may have contributed to the differences in RGRs trends between studies. The two experiments conducted over the summer of 2005 (McGregor et al., 2007, McGregor et al., 2008) utilized "cone-tainers" that were 116 mL in volume, while the current investigation used larger "cone-tainers" (164 mL), for individual macrophyte tests. Although planting densities between model populations and two-species communities were kept constant between investigations, larger individual test containers, may mean that lower RGRs of individually grown plants in previous studies was potentially avoided by eliminating resource limitations due to "cone-tainer" size constraint.

Differences in statistical sensitivity between studies may have also contributed to inconsistency in RGR trends. The previous assessments investigated larger sample sizes of n=15 and n=11 (McGregor et al., 2007, McGregor et al., 2008) compared to that of control data from the current study (n=3), and thereby demonstrated smaller standard deviations about mean RGR measures. In the current study, we also found that sensitivity of the multiple-comparison tests were mainly below the desired statistical power of 0.8. Potentially, small differences in RGRs between test systems, while perhaps

biologically relevant, would not be have been detected by hypothesis testing conducted using one-way ANOVAs and Tukey's comparisons.

In general, macrophyte growth over the course of the study was not as successful as has been observed in previous field seasons. RGRs of both *E. canadensis* and *M. spicatum* grown individually and in model populations and communities, are significantly lower than RGR values from previous investigations conducted at the same experimental site over similar experiment lengths (Hanson et al., 2002; McGregor et al., 2007, McGregor et al., 2008). A number of factors may have contributed to this observation, such as differences in nutrient content of the waters and underlying sediments between studies, differences in environmental conditions between field seasons, and a later date of commencement during the summer season (Ward et al., 1987; Madsen and Sand-Jensen, 1994). While these differences are not surprising considering the wide variability in RGRs of these macrophytes in the natural environment (Pokorny et al., 1984; Lillie et al., 1997), the reduced rate of plant growth led to diminished biomass accumulation in plant stands, and may have consequently limited interactions among macrophytes. Mixed and monocultures of aquatic plants were grown for comparison to individually grown plants, but also to elucidate plant interactions as manifested through changes in biomass and RGRs. Because comparisons of total biomass and RGRs between planting methods found few significant differences, it appears that for the range of densities investigated the macrophyte species are indifferent to the presence of other plants. These results do not correspond with observations from Abernethy et al. (1996), in which the investigation of interspecific interactions between the two plant species demonstrated significant loss in biomass of *M. spicatum* when grown in mixed cultures compared with monoculture

control plants, indicating that *E. canadensis* is the more competitive species. This may be partly related to slower growth of macrophytes over the field study, as plants stands may not have reached a biomass at which interactions between macrophytes were evident. Also, the use of higher initial planting densities and a longer duration of monitoring as compared to our investigation, with monocultures of one plant per 31.7 cm^2 and mixed assemblages of one plant per 15.8 cm^2 monitored over 84 days (Abernethy et al., 1996), may help to explain differences in results. Other population density and community investigations typically monitor macrophyte growth over longer durations, or until biomass filled planting containers and plants have reached carrying capacity (Agami and Waisel, 1985; Agami and Waisel, 2002; Barrat-Segretain and Elger, 2004) to allow for establishment of plant interactions within stands.

Further investigation of the effects of interactions between species to modify the response of macrophytes to a chemical contaminant is recommended, despite the fact that toxicological sensitivities of macrophytes to atrazine were generally within the same range between planting designs. Because interactions between plants, manifested as changes in growth rate and biomass, were not observed under these particular experimental conditions, future studies should focus on monitoring biomass and RGR of macrophytes over longer growth periods, at higher planting densities, and in newly planted assesblages versus established plant stands, in order to better address the question of whether interactions among macrophytes may modify sensitivity to a chemical toxicant.

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Table 4.1 Chemical and physical parameters of the microcosms averaged over the 42-day atrazine study plus pretreatment measurements[a].

Treatment (µg/L)	Minimum temperature (°C)		Maximum temperature (°C)		DO (mg/L)		pH[b]	Alkalinity (mg/L)[c]	Hardness (mg/L)[c]	Conduc-tivity (µS/cm)	PAR ($mE^{-2}sec^{-1}$)
	pre - (n = 4)	post - (n = 28)	pre - (n = 4)	post - (n = 28)	pre - (n = 4)	post - (n = 25)	(n = 7)	(n = 3)	(n = 3)	(n = 3)	(n = 4)
Control	22.8 ± 2.9	22.2 ± 2.2	25.8 ± 1.8	25.0 ± 2.4	10.3 ± 0.4	11.4 ± 1.5	8.7 ± 0.4	177 ± 30	305 ± 18	657 ± 26	503 ± 107
25	22.4 ± 2.8	24.4 ± 2.3[d]	25.5 ± 2.3	25.2 ± 2.3	10.4 ± 0.4	11.6 ± 1.6	8.8 ± 0.4	167 ± 34	295 ± 30	630 ± 17	510 ± 113
50	22.6 ± 2.7	24.1 ± 2.0[d]	26.2 ± 1.7	25.3 ± 2.0	10.4 ± 0.3	11.0 ± 1.6	8.7 ± 0.5	161 ± 44	295 ± 33	647 ± 15	577 ± 134
100	22.7 ± 2.4	22.1 ± 2.3	25.7 ± 2.6	25.2 ± 2.1	10.4 ± 0.4	9.0 ± 1.4[d]	8.5 ± 0.6	184 ± 13	311 ± 17	703 ± 29	513 ± 148
250	22.5 ± 2.3	22.0 ± 2.1	26.3 ± 1.8	25.6 ± 2.0	10.3 ± 0.3	7.0 ± 1.7[d]	8.3 ± 0.5	199 ± 5	333 ± 22	727 ± 41	510 ± 142

DO, dissolved oxygen; PAR, photosynthetically active radiation

[a] The values presented are the means ± standard deviations of measurements taken for three replicates at each exposure concentration for each measurement event. These means were then averaged for all the measurement events taken at that concentration over the 42 day period.
[b] The means ± standard deviations of measurements calculated by conversion to corresponding hydrogren ion concentrations.
[c] Measured as mg/L of $CaCO_3$.
[d] Significantly different mean ($p < 0.05$) when compared with control using Dunnett's test ($\alpha = 0.05$).

Table 4.2 Effective concentrations (μg/L) required to cause a decrease in the endpoints of interest by 10%, 25%, and 50% (EC_{10}, EC_{25}, and EC_{50}) with associated 95% confidence intervals, as calculated using linear and non-linear modeling techniques using SAS v9.1 (SAS Institute, Cary, NC, USA), for endpoints with statistically significant responses in *Elodea canadensis* exposed to atrazine in aquatic microcosms[a].

Day	Endpoint	Planting Design	EC_{10} (95% CI)	EC_{25} (95% CI)	EC_{50} (95% CI)	Model[d]	Parameters	Corrected r^2
14	Chloro-*a*	Ind	Nc	nc	nc		nc	nc
	Wet root	Ind	41.0 (0, 173.3)	58.3 (0, 182.9)	82.8 (0, 196.0)	Logistic	b = 3.1298; t = 0.0219; x = 82.8	0.86
		Pop1	0.4 (0, 4.8)	1.6 (0, 13.9)	6.2 (0, 35.2)	Logistic	b = 0.7988; t = 0.0205; x = 6.2	0.98
		Pop2	5.5 (0, 17.8)	12.7 (0, 30.9)	29.3 (5.9, 52.6)	Logistic	b = 1.3159; t = 0.0137; x = 29.3	0.96
		Comm1	8.5 (0, 61.9)	23.0 (0, 115.8)	62.7 (0, 217.2)	Logistic	b = 1.0972; t = 0.0202; x = 62.7	0.59
		Comm2	22.8 (8.6, 37.1)	57.1 (21.6, 92.6)	114.2 (43.2, 185.3)	Linear	b = 0.0104; x = 114.2	0.35
	Dry root	Ind	29.3 (0, 59.9)	73.2 (0, 149.7)	146.5 (0, 299.4)	Linear	b = 0.0027; x = 146.5	0.45
		Pop1	0.4 (0, 6.9)	1.3 (0, 18.3)	4.6 (0, 43.3)	Logistic	b = 0.8571; t = 0.0024; x = 4.6	0.98
		Pop2	3.9 (0, 17.3)	10.8 (0, 34.7)	30.2 (0, 66.1)	Logistic	b = 1.0728; t = 0.0012; x = 30.2	0.85
		Comm1	nc	nc	0.001 (0, 0.4)	Logistic	b = 0.0739; t =0.0027; x = 0.001	0.42
		Comm2	22.5 (9.8, 35.2)	56.3 (24.5, 88.0)	112.5 (49.0, 176.0)	Linear	b = 0.0011; x = 112.5	0.48
	Wet shoot	Ind	0.006 (0, 0.4)	0.4 (0, 15.4)	23.2 (0, 296.9)	Logistic	b = 0.2673; t = 0.2283; x = 23.2	0.81
		Pop1	1.2 (0, 6.0)	5.1 (0, 18.3)	21.8 (0, 49.6)	Logistic	b = 0.7592; t = 0.2623; x = 21.8	0.88
		Pop2	25.9 (0, 71.4)	45.2 (0, 97.7)	78.9 (18.9, 138.8)	Logistic	b = 1.9757; t = 0.1777; x = 78.9	0.91
		Comm1	7.1 (0, 53.2)	20.9 (0, 107.2)	61.9 (0, 214.2)	Logistic	b = 1.0126; t = 0.1002; x = 61.9	0.72
		Comm2	47. 9 (0, 146.7)	61.7 (0, 151.0)	79.5 (2.3, 156.7)	Logistic	b = 4.3312; t = 0.0481; x = 79.5	0.97
	Dry shoot	Ind	8.6 (0, 37.9)	19.5 (0, 62.5)	44.2 (0, 103.8)	Logistic	b = 1.3462; t = 0.0165; x = 44.2	0.86
		Pop1	17.1 (0, 37.2)	21.5 (7.5, 35.6)	27.2 (13.7, 40.7)	Logistic	b = 4.7348; t = 0.0109; x = 27.2	0.99
		Pop2	28.0 (0, 99.5)	35.0 (0, 92.2)	43.7 (8.3, 79.2)	Logistic	b = 4.9281; t = 0.0098; x = 43.7	0.99
		Comm1	9.1 (0, 52.0)	18.2 (0, 74.2)	36.2 (0, 105.1)	Logistic	b = 0.0034; t = 0.0042; x = 36.2	0.39
		Comm2	27.2 (0, 126.1)	32.8 (0, 117.3)	39.6 (0, 103.0)	Logistic	b = 5.8223; t = 0.0040; x = 39.6	0.99
28	Chloro-*a*[b]	Ind	nc	82.9 (5.0, 160.8)	165.8 (10.1, 321.5)	Linear	b = 1.3424; x = 165.8	0.74
	Wet root	Ind	nc	nc	nc		nc	nc
		Pop1	9.0 (0, 36.2)	13.4 (0, 39.6)	20.0 (0.7, 39.3)	Logistic	b = 2.7465; t =0.1791; x = 20.0	0.99

		Pop2	11.8 (0, 27.1)	16. 4 (3.4, 29.4)	22.7 (14.4, 31.1)	Logistic	b = 3.3517; t = 0.1533; x = 22.7	0.99
		Comm1	30.9 (0, 88.7)	48.7 (0, 109.1)	76.6 (14.0, 139.2)	Logistic	b = 2.4223; t = 0.0504; x = 76.6	0.88
		Comm2	20.2 (9.9, 30.5)	50.5 (24.7, 76.3)	100.9 (49.3, 152.5)	Linear	b = 0.0612; x = 100.9	0.38
	Dry root	Ind	nc	nc	nc		nc	nc
		Pop1	5.8 (0, 32.6)	9.6 (0, 39.7)	15.8 (0, 43.2)	Logistic	b = 2.1900; t = 0.0164; x = 15.8	0.99
		Pop2	10.5 (0, 26.8)	15.1 (0.4, 29.7)	21.6 (11.6, 31.6)	Logistic	b = 3.0466; t = 0.0135; x = 21.6	0.99
		Comm1	33.0 (0, 70.0)	44.8 (11.6, 78.0)	61.0 (27.1, 94.8)	Logistic	b = 3.5708; t = 0.0050; x = 61.0	0.96
		Comm2	5.1 (0, 22.3)	9.7 (0, 31.4)	18.4 (0, 40.5)	Logistic	b = 1.7023; t = 0.0134; x = 18.4	0.99
	Wet shoot	Ind	4.8E-10 (0, 1.5E-7)	0.00011 (0, 0.02)	24.7 (0, 546.4)	Logistic	b = 0.0891; t = 0.3655; x = 24.7	0.83
		Pop1	4.4 (0, 16.023)	9.5 (0, 25.9)	20.3 (1.6, 39.1)	Logistic	b = 1.4347; t = 1.3464; x = 20.3	0.99
		Pop2	9.0 (0, 18.0)	16.4 (5.8, 27.1)	30.0 (18.6, 41.4)	Logistic	b = 1.8250; t = 1.1458; x = 30.0	0.99
		Comm1	nc	nc	nc		nc	nc
		Comm2	10.1 (0, 36.5)	20.8 (0, 56.1)	42.7 (0, 88.0)	Logistic	b = 1.5291; t = 0.2607; x = 42.7	0.97
	Dry shoot	Ind	30.0 (1.3, 58.7)	75.0 (3.4, 146.7)	150.1 (6.7, 293.5)	Linear	b = 0.0418; x = 150.1	0.97
		Pop1	4.5 (0, 21.4)	9.1 (0, 31.6)	18.3 (0, 42.4)	Logistic	b = 1.5743; t =0.1172 ; x = 18.3	0.99
		Pop2	7.3 (0, 18.3)	12.8 (0.3, 25.3)	22.6 (10.9, 34.3)	Logistic	b = 1.9342; t = 0.1209; x = 22.6	0.99
		Comm1	nc	nc	nc		nc	nc
		Comm2	10.5 (0, 26.0)	19.4 (0.68, 38.0)	35.9 (14.4, 57.3)	Logistic	b = 1.7821; t = 0.0464 ; x = 35.9	0.97
42	Chloro-a[b]	Ind	nc	80.0 (8.5, 151.4)	159.9 (17.0, 302.9)	Linear	b = 1.1121; x = 159.9	0.54
	Wet root	Ind	nc	nc	nc		nc	nc
		Pop1	6.2 (0, 31.8)	10.4 (0, 39.2)	17.6 (0, 43.4)	Logistic	b = 2.0955; t =0.8724; x = 17.6	0.99
		Pop2	19.4 (1.7, 37.0)	27.4 (10.5, 44.2)	38.6 (22.7, 54.5)	Logistic	b = 3.1936; t = 0.4066; x = 38.6	0.99
		Comm1	20.8 (0, 54.1)	29.1 (0, 60.2)	40.6 (12.5, 68.7)	Logistic	b = 3.2826; t = 0.1600; x = 40.6	0.99
		Comm2	4.2 (0, 19.0)	9.8 (0, 32.4)	23.1 (0, 51.3)	Logistic	b = 1.2907; t = 0.1462; x = 23.1	0.98
	Dry root	Ind	nc	nc	nc		nc	nc
		Pop1	5.3 (0, 30.7)	9.3 (0, 39.7)	16.6 (0, 45.6)	Logistic	b = 1.9213; t = 0.0864; x = 16.6	0.99
		Pop2	5.9 (0, 19.4)	11.4 (0, 28.6)	22.3 (4.4, 40.1)	Logistic	b = 1.6491; t = 0.058; x = 22.3	0.98
		Comm1	21.0 (8.9, 33.0)	52.4 (22.3, 82.6)	104.8 (44.6, 165.1)	Linear	b = 0.0222; x = 104.8	0.46
		Comm2	4.1 (0, 16.5)	9.5 (0, 28.4)	22.3 (0, 45.8)	Logistic	b = 1.2950; t = 0.0215; x = 22.3	0.99
	Wet shoot	Ind	nc	nc	nc		nc	nc
		Pop1	6.8 (0, 23.6)	14.5 (0, 37.7)	31.1 (2.7, 59.6)	Logistic	b = 1.4420; t = 2.7193; x = 31.1	0.97
		Pop2	nc	nc	62.6 (42.3, 82.9)	Logistic	b = 3.5941; t = 1.6819; x =	0.96

	Comm1	39.1 (0, 140.1)	59.9 (0, 160.3)	91.9 (0, 189.1)	Logistic	b = 2.5733; t =0.4981; x = 91.9	0.99
	Comm2	23.3 (10.8, 35.8)	58.2 (27.0, 89.4)	116.4 (54.1, 178.8)	Linear	b = 0.3625; x = 116.4	0.67
Dry shoot	Ind	nc	nc	nc		nc	nc
	Pop1	2.4 (0, 14.1)	7.0 (0, 29.6)	20.8 (0, 55.7)	Logistic	b = 1.0140; t = 0.334; x = 20.8	0.87
	Pop2	17.8 (4.5, 31.2)	30.0 (15.1, 44.9)	50.4 (33.7, 67.1)	Logistic	b = 2.1156; t = 0.2582; x = 50.4	0.95
	Comm1	28.6 (0, 105.1)	47.9 (0, 132.4)	80.1 (0, 172.5)	Logistic	b = 2.1365; t = 0.1012; x = 80.1	0.97
	Comm2	2.4 (0, 13.2)	8.0 (0, 31.2)	26.9 (0, 67.3)	Logistic	b = 0.9047; t = 0.1039; x = 26.9	0.83

CI, Confidence interval; Ind, Individually grown plants; Pop1, Population low density; Pop2, Population high density; Comm1, Community low density; Comm2, Community high density; Chloro-*a*, Chlorophyll-*a*

[a] The reparameterized equations used to fit the concentration-responses of monensin exposed *M. spicatum*, and *E. canadensis*: Linear $y = ((-b \times 0.5)/x)x_o + b$; Logistic $y = t/[1 + (x_o/x)^b]$; Hormetic $y = [t(1+hx_o)]/ (1+[(x+hx_o)/(1-x)](x_o/x)^b]$. The variable x is the calculated EC_{50} for the concentration-response relationship modeled, x_o is the actual concentration (µg/L) being evaluated, y is the response or change from control of the endpoint modeled, and b, t, and h are constants.

[b] Effective concentrations (µg/L) required to cause an increase in the endpoints of interest by 110%, 125%, and 150% (EC_{110}, $EC1_{25}$, and EC_{150}) with associated 95% confidence intervals, fit using the linear equation: Linear $y = ((b \times 0.5)/x)x_o + b$

Table 4.3 Effective concentrations (µg/L) required to cause a decrease in the endpoints of interest by 10%, 25%, and 50% (EC_{10}, EC_{25}, and EC_{50}) with associated 95% confidence intervals, as calculated using linear and non-linear modeling techniques using SAS v9.1 (SAS Institute, Cary, NC, USA), for endpoints with statistically significant responses in *Myriophyllum spicatum* exposed to atrazine in aquatic microcosms[a].

Day	Endpoint	Planting Design	EC_{10} (95% CI)	EC_{25} (95% CI)	EC_{50} (95% CI)	Model[d]	Parameters	Corrected r^2
14	Chloro-*a*[b]	Ind	29.9 (20.9, 39.0)	74.8 (52.2, 97.5)	149.7 (104.3, 195.0)	Linear	b = 0.6449; x = 149.7	0.99
	Wet root	Ind	21.8 (0, 52.8)	36.0 (1.8, 70.1)	59.4 (21.7, 97.1)	Logistic	b = 2.1893; t = 0.7622; x = 59.4	0.92
		Pop1	18.4 (0, 47.2)	32.3 (0, 65.9)	56.7 (17.5, 95.8)	Logistic	b = 1.9567; t = 0.5426; x = 56.7	0.82
		Pop2	23.9 (0, 50.6)	39.1 (10.1, 68.1)	63.9 (32.2, 95.6)	Logistic	b = 2.2337; t = 0.489; x = 63.9	0.90
		Comm1	15.1 (0, 30.2)	26.5 (8.9, 44.1)	46.5 (26.1, 66.9)	Logistic	b = 1.9487; t = 0.444; x = 46.5	0.97
		Comm2	24.7 (0, 55.1)	35.7 (7.3, 64.1)	51.5 (25.0, 77.9)	Logistic	b = 2.9887; t = 0.4183; x = 51.5	0.74
	Dry root	Ind	27.7 (0, 75.2)	43.7 (0, 93.7)	69.0 (16.1, 121.8)	Logistic	b = 2.4067; t = 0.0417; x = 69.0	0.88
		Pop1	26.5 (12.9, 40.2)	66.3 (32.1, 100.5)	132.7 (64.3, 201.0)	Linear	b = 0.0275; x = 132.7	0.50
		Pop2	25.6 (0, 53.2)	41.7 (11.8, 71.5)	67.8 (35.4, 100.2)	Logistic	b = 2.2532; t = 0.0298; x = 67.8	0.90
		Comm1	13.4 (0, 29.5)	23.4 (5.0, 41.9)	40.9 (20.0, 61.8)	Logistic	b = 1.9724; t = 0.0264; x = 40.9	0.99
		Comm2	24.0 (12.0, 36.0)	59.9 (29.9, 90.0)	120.0 (59.8, 180.0)	Linear	b = 0.0204; x = 120.0	0.47
	Wet shoot	Ind	75.4 (0, 196.8)	188.6 (0, 492.0)	377.2 (0, 984.0)	Linear	b = 0.5696; x = 377.2	0.30
		Pop1	27.7 (13.2, 42.2)	69.3 (33.1, 105.4)	138.5 (66.2, 210.8)	Linear	b = 1.2575; x = 138.5	0.29
		Pop2	26.1 (0, 65.4)	50.2 (0.7, 99.6)	96.4 (34.8, 157.9)	Logistic	b = 1.6825; t = 1.0424; x = 96.4	0.79
		Comm1	12.4 (0, 37.5)	36.8 (0, 83.0)	109.4 (23.9, 194.9)	Logistic	b = 1.0079; t = 1.2698; x = 109.4	0.97
		Comm2	26.1 (15.1, 37.1)	65.3 (37.7, 92.8)	130.5 (75.4, 185.7)	Linear	b = 0.8469; x = 130.5	0.69
	Dry shoot	Ind	35.7 (7.2, 64.3)	89.3 (17.9, 160.7)	178.6 (35.8, 321.4)	Linear	b = 0.0673; x = 178.6	0.43
		Pop1	24.0 (15.2, 32.7)	59.9 (38.0, 81.8)	119.8 (76.1, 163.5)	Linear	b = 0.1006; x = 119.8	0.51
		Pop2	27.6 (0, 62.617)	42.7 (6.5, 79.0)	66.2 (28.3, 104.2)	Logistic	b = 2.5083; t = 0.1008; x = 66.2	0.82
		Comm1	3.6 (0, 13.4)	14.1 (0, 38.0)	55.8 (5.0, 106.6)	Logistic	b = 0.7975; t = 0.1139; x = 55.8	0.92
		Comm2	18.7 (1.1, 36.3)	31.7 (11.9, 51.6)	53.9 (31.5, 76.3)	Logistic	b = 2.0750; t = 0.0853; x = 53.9	0.88
28	Chloro-*a*[b]	Ind	21.0 (15.1, 26.9)	52.4 (37.6, 67.2)	104.9 (75.3, 134.5)	Linear	b = 1.0226; x = 104.8	0.94
	Wet root	Ind	6.4 (0, 14.3)	14.6 (2.9, 26.3)	33.5 (18.1, 48.9)	Logistic	b = 1.3271; t = 2.2309; x = 33.5	0.94
		Pop1	22.2 (7.0, 37.5)	55.5 (17.4, 93.6)	111.0 (34.8, 187.3)	Linear	b = 0.8680; x = 111.0	0.25

		Pop2	17.1 (0, 50.9)	25.7 (0, 59.6)	38.7 (4.7, 72.7)	Logistic	b = 2.6884; t = 1.3293; x = 38.7	0.99
		Comm1	21.9 (1.6, 42.1)	26.4 (7.4, 45.4)	31.8 (8.9, 54.7)	Logistic	b = 5.8401; t = 1.3301; x = 31.8	0.99
		Comm2	23.3 (12.2, 34.4)	58.2 (30.4, 86.0)	116.5 (60.9, 172.1)	Linear	b = 0.4201; x = 116.5	0.46
	Dry root	Ind	1.7 (0, 10.2)	6.5 (0, 27.3)	25.2 (0, 65.4)	Logistic	b = 0.8142; t = 0.2445; x = 25.2	0.87
		Pop1	21.1 (4.5, 37.7)	52.8 (11.4, 94.1)	105.5 (22.7, 188.3)	Linear	b = 0.0841; x = 105.5	0.37
		Pop2	24.4 (0, 75.8)	31.2 (0, 76.4)	39.9 (3.8, 75.9)	Logistic	b = 4.4753; t = 0.1333; x = 39.9	0.99
		Comm1	18.2 (0, 46.2)	22.4 (4.0, 40.8)	27.6 (6.9, 48.3)	Logistic	b = 5.2685; t = 0.168; x = 27.6	0.99
		Comm2	3.4 (0, 39.3)	7.3 (0, 58.9)	15.5 (0, 75.7)	Logistic	b = 1.4515; t = 0.1619; x = 15.5	0.99
	Wet shoot	Ind	30.3 (17.6, 42.9)	75.6 (44.0, 107.3)	151.3 (88.0, 214.5)	Linear	b = 1.1559; x = 151.3	0.61
		Pop1	24.0 (6.2, 41.8)	60.0 (15.5, 104.6)	120.1 (31.0, 209.2)	Linear	b = 1.9232; x = 120.1	0.31
		Pop2	37.7 (0, 139.3)	57.2 (0, 158.1)	86.9 (0, 184.4)	Logistic	b = 2.6307; t = 1.2715; x = 86.9	0.96
		Comm1	26.8 (6.1, 47.5)	67.0 (15.2, 118.8)	133.9 (30.3, 237.5)	Linear	b = 1.7578; x = 133.9	0.18
		Comm2	25.9 (10.9, 40.9)	64.7 (27.2, 102.3)	129.5 (54.4, 204.6)	Linear	b = 1.0324; x = 129.5	0.33
	Dry shoot	Ind	4.8 (0, 17.6)	14.2 (0, 38.6)	42.5 (1.6, 83.5)	Logistic	b = 1.0025; t = 0.1960; x = 42.5	0.78
		Pop1	22.6 (6.6, 38.5)	56.4 (16.6, 96.3)	112.8 (33.1, 192.5)	Linear	b = 0.2842; x = 112.8	0.43
		Pop2	43.3 (0, 143.6)	57.8 (0, 150.9)	77.2 (0, 163.1)	Logistic	b = 3.7953; t = 0.1551; x = 77.2	0.96
		Comm1	21.0 (9.8, 32.3)	52.6 (24.5, 80.6)	105.1 (49.0, 161.3)	Linear	b = 0.2354; x = 105.1	0.44
		Comm2	35.8 (0, 107.5)	51.3 (0, 120.8)	73.5 (5.1, 141.8)	Logistic	b = 3.0615; t = 0.1749; x = 73.5	0.71
42	Chloro-a^b	Ind	24.6 (9.4, 39.8)	61.5 (23.5, 99.4)	122.9 (47.1, 198.8)	Linear	b = 0.8665; x = 122.0	0.79
	Wet root	Ind	25.0 (8.9 41.2)	62.6 (22.2 103.0)	125.2 (44.4 206.0)	Linear	b = 1.3906; x = 125.2	0.57
		Pop1	nc	nc	28.2 (0, 236.3)	Hormetic	b = -0.0513; t = 0.5160; h = -0.0447; x = 28.2	0.56
		Pop2	nc	nc	35.6 (0 , 251.6)	Hormetic	b = 1.0022; t = 0.8844; h =-0.0447; x = 35.6	0.53
		Comm1	20.8 (0, 54.1)	29.1 (0, 60.2)	40.6 (12.5, 68.7)	Logistic	b = 3.2826; t = 0.16; x = 40.6	0.99
		Comm2	22.9(3.7, 42.1	57.2 (9.3, 105.2)	114.4 (18.5, 210.4)	Linear	b = 1.0049; x = 114.4	0.63
	Dry root	Ind	22.9 (4.6, 41.2)	57.2 (11.4, 103.1)	114.5 (22.8, 206.1)	Linear	b = 0.1934; x = 114.5	0.43
		Pop1	nc	nc	24.6 (0, 435.0)	Hormetic	b = 1.0073; t = 0.0924; h = -0.0409; x = 24.6	0.32
		Pop2	nc	nc	nc		nc	nc
		Comm1	21.0 (8.9, 33.0)	52.4 (22.3, 82.6)	104.8 (44.6, 165.1)	Linear	b = 0.0222; x = 104.8	0.46
		Comm2	42.3 (0, 121.0)	52.1 (0, 122.9)	64.0 (0, 154.7)	Logistic	b = 5.3061; t = 0.1674; x = 64.0	0.95

Wet shoot	Ind	34.8 (9.4, 60.1)	86.9 (23.5, 150.3)	173.8 (47.0, 300.5)	Linear	b = 1.0201; x = 173.8	0.41
	Pop1	nc	nc	53.1 (0. 157.4)	Hormetic	b = 0.8781; t = 1.4204; h = -0.0731; x = 53.1	0.12
	Pop2	nc	nc	nc		nc	nc
	Comm1	36.0 (0, 136.1)	52.1 (0, 149.8)	75.5 (0, 170.8)	Logistic	b = 2.9648; t = 0.3721; x = 75.5	0.99
	Comm2	25.8 (1.9, 49.7)	64.5 (4.7, 124.2)	129.0 (9.5, 248.4)	Linear	b = 1.2199; x = 129.0	0.60
Dry shoot	Ind	13.6 (0, 32.5)	22.7 (2.0, 43.4)	37.8 (15.4, 60.3)	Logistic	b = 2.148; t = 0.2596; x = 37.8	0.88
	Pop1	21.3 (5.2, 37.4)	53.3 (13.0, 93.5)	106.6 (26.0, 187.1)	Linear	b = 0.2862; x = 106.6	0.16
	Pop2	nc	nc	54.8 (0, 136.8)	Hormetic	b = 0.9117; t = 0.1119; h = -0.0759; x = 54.8	0.71
	Comm1	26.8 (0, 101.5)	45.1 (0, 128.5)	75.9 (0, 168.2)	Logistic	b = 2.1099; t =0.1317; x = 75.9	0.97
	Comm2	23.4 (6.3 40.5)	58.4 (15.7, 101.2)	116.9 (31.3, 202.5)	Linear	b = 0.1899; x = 116.9	0.65

CI, Confidence interval; Ind, Individually grown plants; Pop1, Population low density; Pop2, Population high density; Comm1, Community low density; Comm2, Community high density; Chloro-*a*, Chlorophyll-*a*

[a] The reparameterized equations used to fit the concentration-responses of monensin exposed *M. spicatum*, and *E. canadensis*: Linear $y = ((-b \times 0.5)/x)x_o + b$; Logistic $y = t/[1 + (x_o/x)^b]$; Hormetic $y = [t(1+hx_o)]/ (1+[(x+hx_o)/(1-x)](x_o/x)^b]$. The variable x is the calculated EC_{50} for the concentration-response relationship modeled, x_o is the actual concentration (µg/L) being evaluated, y is the response or change from control of the endpoint modeled, and b, t, and h are constants.

[b] Effective concentrations (µg/L) required to cause an increase in the endpoints of interest by 110%, 125%, and 150% (EC_{110}, $EC1_{25}$, and EC_{150}) with associated 95% confidence intervals, fit using the linear equation: Linear $y = ((b \times 0.5)/x)x_o + b$.

Table 4.4 Relative growth rates of *Myriophyllum spicatum* and *Elodea canadensis* grown in control (0 µg/L atrazine) microcosms as individual plants and in model populations and communities.

Endpoint		Individual	Low density population	High density population	Low density community	High density community	*p*-value
E. canadensis							
Day 14	RGR_{wet} ($gg^{-1}day^{-1}$)[a]	0.060 ± 0.057 [a]	0.082 ± 0.008	0.061 ± 0.021	0.040 ± 0.036	0.025 ± 0.013	0.301
	RGR_{dry} ($gg^{-1}day^{-1}$)[a]	0.041 ± 0.023	0.033 ± 0.017	0.027 ± 0.022	0.017 ± 0.016	0.014 ± 0.011	0.414
Day 28	RGR_{wet} ($gg^{-1}day^{-1}$)[a]	0.049 ± 0.016	0.088 ± 0.017 [b]	0.085 ± 0.005 [b]	0.019 ± 0.015	0.047 ± 0.011	<0.001
	RGR_{dry} ($gg^{-1}day^{-1}$)[a]	0.033 ± 0.036	0.066 ± 0.023	0.069 ± 0.012	0.014 ± 0.013	0.047 ± 0.006	0.029
Day 42	RGR_{wet} ($gg^{-1}day^{-1}$)[a]	0.029 ± 0.025	0.077 ± 0.015 [b]	0.066 ± 0.002 [b]	0.040 ± 0.014	0.040 ± 0.008	0.014
	RGR_{dry} ($gg^{-1}day^{-1}$)[a]	0.034 ± 0.031	0.069 ± 0.015	0.066 ± 0.003	0.041 ± 0.018	0.045 ± 0.008	0.140
M. spicatum							
Day 14	RGR_{wet} ($gg^{-1}day^{-1}$)[a]	0.126 ± 0.009	0.132 ± 0.018	0.132 ± 0.003	0.141 ± 0.023	0.120 ± 0.022	0.619
	RGR_{dry} ($gg^{-1}day^{-1}$)[a]	0.112 ± 0.013	0.118 ± 0.020	0.120 ± 0.003	0.129 ± 0.022	0.111 ± 0.018	0.692
Day 28	RGR_{wet} ($gg^{-1}day^{-1}$)[a]	0.096 ± 0.005	0.063 ± 0.038	0.079 ± 0.027	0.084 ± 0.028	0.061 ± 0.016	0.452
	RGR_{dry} ($gg^{-1}day^{-1}$)[a]	0.102 ± 0.006	0.067 ± 0.044	0.078 ± 0.033	0.094 ± 0.030	0.079 ± 0.033	0.683
Day 42	RGR_{wet} ($gg^{-1}day^{-1}$)[a]	0.047 ± 0.021	0.048 ± 0.024	0.045 ± 0.016	0.023 ± 0.014	0.041 ± 0.026	0.596
	RGR_{dry} ($gg^{-1}day^{-1}$) [ac]	0.067 ± 0.006	0.053 ± 0.023	0.050 ± 0.020	0.037 ± 0.018	0.052 ± 0.026	0.518 [c]

E. canadensis, *Elodea canadensis*; *M. spicatum*, *Myriophyllum spicatum*; RGR, relative growth rate

[a] Values are the mean (n=3) ± standard deviation.
[b] Statistically significant difference ($p < 0.05$) from individually grown plants as determined by analysis of variance using Tukey's test of SigmaStat 3.5.
[c] Natural ln transformed data as did not meet assumptions of equal variance.

Table 4.5 Individual and interactive effects of atrazine concentration and planting design on wet and dry biomass of roots, shoots and total biomass of *Myriophyllum spicatum* and *Elodea canadensis* grown under microcosm conditions, determined using a mixed model two-way analysis of variance ($\alpha = 0.05$) in SAS v9.1.

Dataset		**Main effects** Atrazine *p*-value	Pdesign *p*-value	**Interaction** Atrazine*pdesign *p*-value
E. canadensis				
Day 14	Wet root	0.0028*	0.3139	0.8018
	Wet shoot	0.0008*	0.0099*	0.9771
	Total wet	0.0005*	0.0167*	0.9846
	Dry root	0.0132*	0.0401*	0.3133
	Dry shoot	<.0001*	0.0074*	0.8526
	Total dry	0.0299*	0.0299	0.9173
Day 28	Wet root[a]	0.0005*	0.9502	0.3693
	Wet shoot	<.0001*	<.0001*	0.0014*
	Total wet	<.0001*	<.0001*	0.0042*
	Dry root[a]	0.0003*	0.8053	0.4900
	Dry shoot	<.0001*	0.5531	0.2189
	Total dry	<.0001*	0.6569	0.2669
Day 42	Wet root[a]	<.0001*	<.0001*	0.0009*
	Wet shoot	0.0004*	<.0001*	0.0001*
	Total wet	0.0003*	<.0001*	0.0002*
	Dry root[a]	0.0003*	0.0020*	0.0135*
	Dry shoot	0.0002*	<.0001*	0.0174*
	Total dry	0.0002*	<.0001*	0.0144*
M. spicatum				
Day 14	Wet root	<.0001*	0.0010*	0.9694
	Wet shoot	<.0001*	0.0001*	0.0113*
	Total wet	<.0001*	0.0482*	0.5595
	Dry root	<.0001*	0.0011*	0.9326
	Dry shoot	<.0001*	0.0907	0.0830
	Total dry	<.0001*	0.0654	0.3964
Day 28	Wet root	0.0003*	<.0001*	0.0817
	Wet shoot	0.0029*	0.2190	0.3389
	Total wet	0.0006*	0.0582	0.3159
	Dry root[a]	0.0110*	0.0019*	0.5266
	Dry shoot	0.0007*	0.6833	0.4465
	Total dry	0.0016*	0.2752	0.4536
Day 42	Wet root[a]	0.0001*	0.0187*	0.7913
	Wet shoot	0.0138*	0.0048*	0.1810
	Total wet	0.0032*	0.0003*	0.2286
	Dry root[a]	0.0001*	0.0187*	0.7913

Dry shoot	0.0001*	0.1855	0.1697
Total dry	0.0006*	0.0073*	0.6759

M. spicatum, *Myriophyllum spicatum*; *E. canadensis*, *Elodea canadensis*; Pdesign, planting design.

[a] Dataset does not meet ANOVA assumptions of residual normality and homogeneous variance.
* Statistically significant effect.

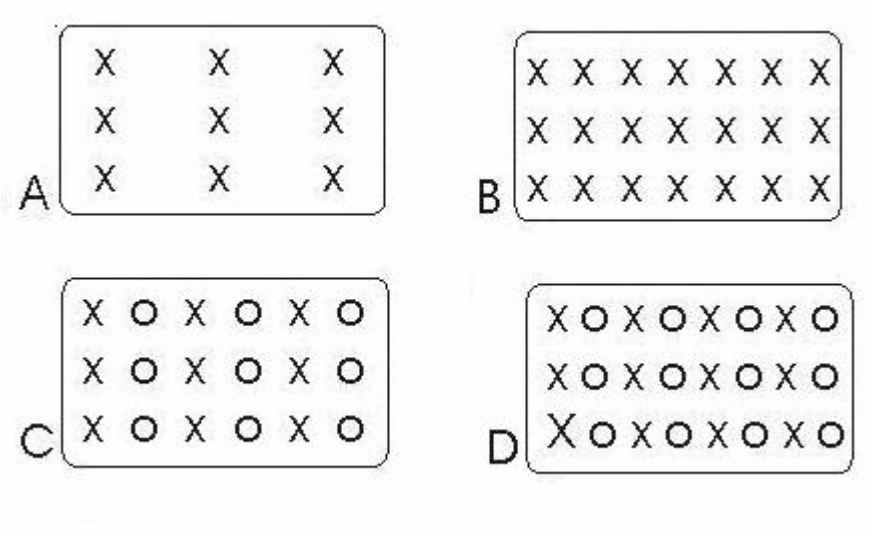

Figure 4.1 Planting arrangement used for model populations and communities of *Myriophyllum spicatum* and *Elodea canadensis*. (A) Low density model populations are approximately 1 plant per 80 cm^2 of either *M. spicatum* or *E. canadensis* (monoculture). (B) High density model populations are approximately 1 plant per 40 cm^2 of either *M. spicatum* or *E. canadensis* (monoculture). (C) Low density two-species assemblages are approximately 1 plant per 40 cm^2, alternating *M. spicatum* or *E. canadensis* (mixed culture). (D) High density two-species assemblages are approximately 1 plant per 20 cm^2, alternating *M. spicatum* or *E. canadensis* (mixed culture).

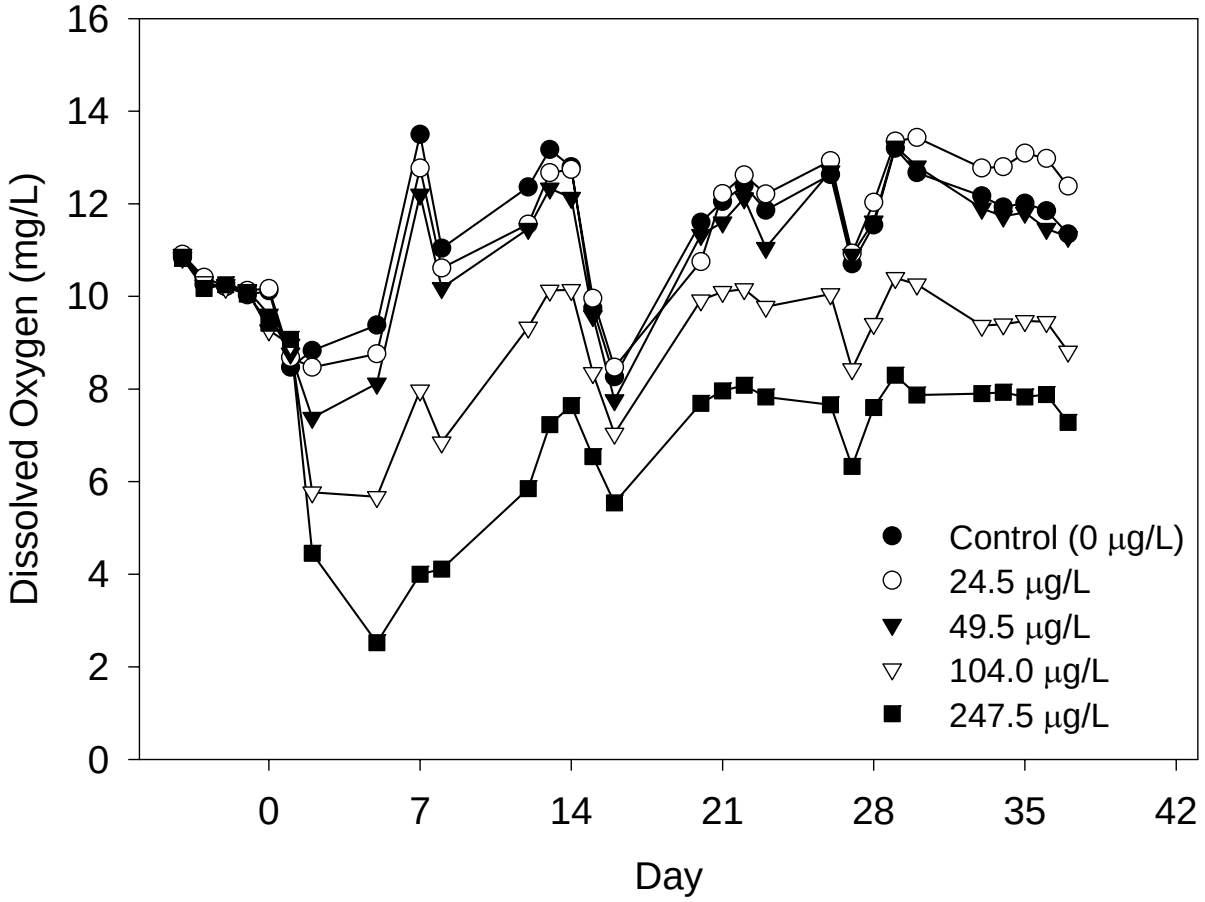

Figure 4.2 Dissolved oxygen (mg/L) levels at a depth of 50 cm in aquatic microcosms treated with various levels of atrazine over a 42-day exposure duration.

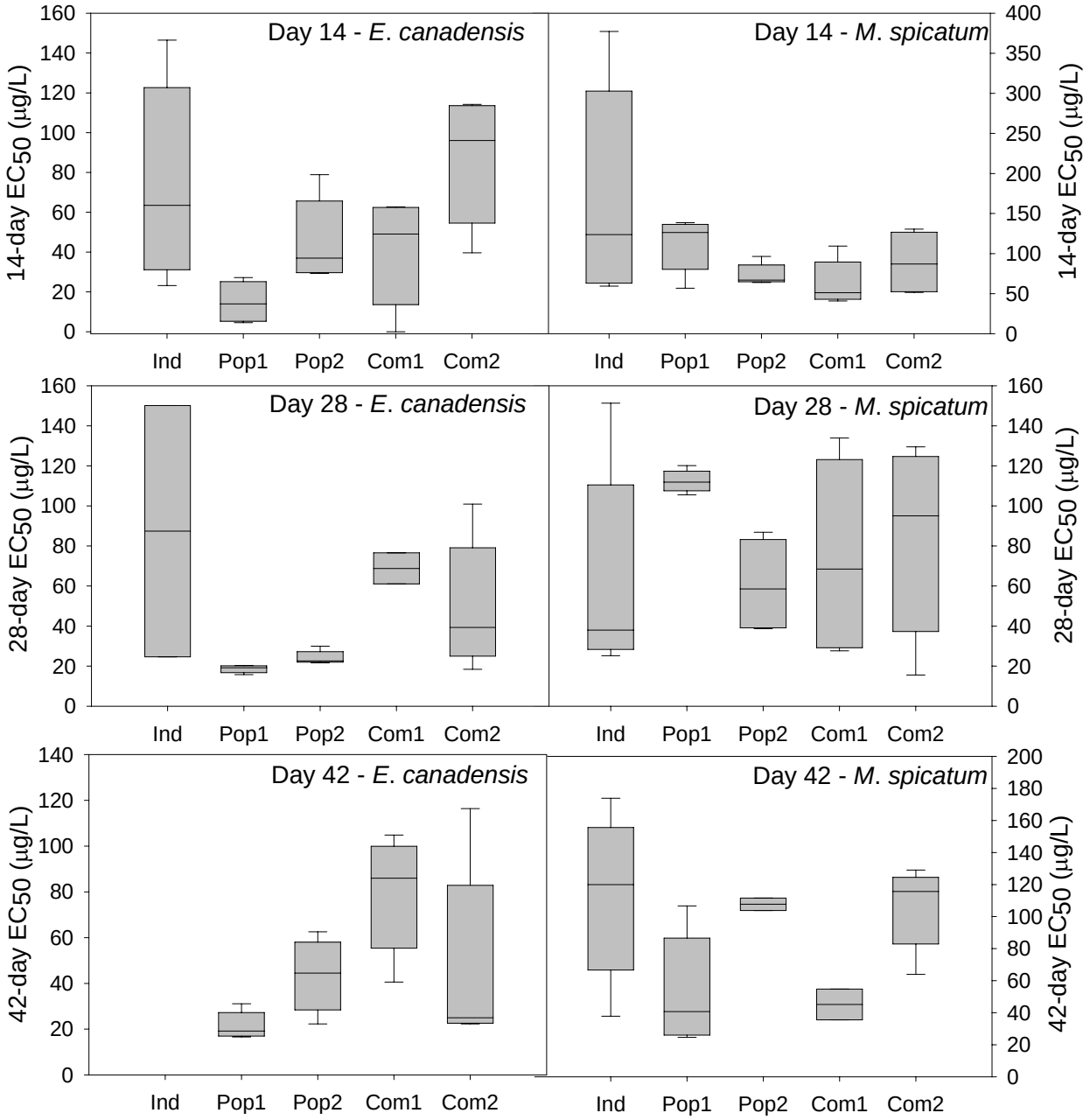

Figure 4.3 Box plots of effective concentrations resulting in 50% differences in macrophyte growth from control treatments (EC_{50}). The 25th and 75th centiles are shown as the box ends, the whisker bars are the 10th and 90th centiles, and the solid line within the box represents the median.

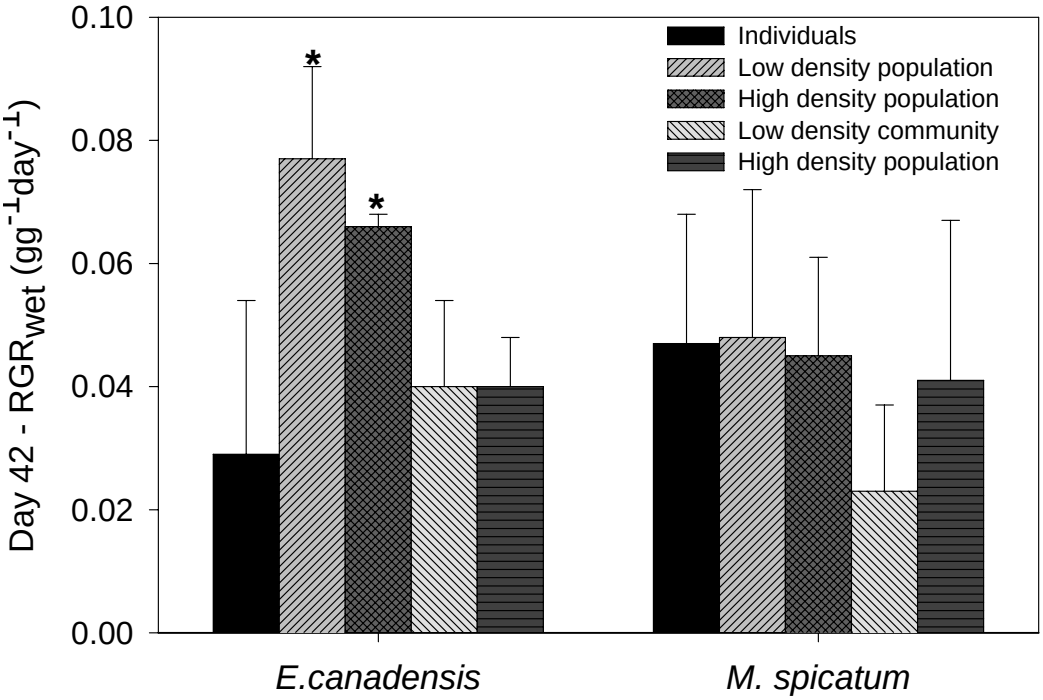

Figure 4.4 Relative growth rates (RGR) ($gg^{-1}day^{-1}$) of *Myriophyllum spicatum* and *Elodea canadensis* grown as individuals and at two densities in model populations and two-species communities over 42 days in 12,000-L outdoor microcosms. Error bars represent the standard deviation about the mean. An asterisk (*) denotes a statistically significant difference as compared to the individuals, detected using a Tukey's test ($p < 0.05$).

5 SUMMARY AND GENERAL CONCLUSIONS

5.1 TOXICITY ASSESSMENTS

A main objective of our research was to evaluate the toxicity of monensin and the 10:2 FTCA to common freshwater plants, *Lemna gibba, Myriophyllum spicatum*, *Elodea canadensis*, and *Egeria densa*, under microcosm conditions. We also wanted to assess how well the results of standard laboratory-based plant assays reflect field level responses of aquatic macrophytes to environmental contaminants, by comparing the sensitivities of plants tested in microcosms to those evaluated in the laboratory. Due to emerging concern over atrazine effects, toxicity of the compound to rooted freshwater macrophytes grown under various planting designs was also evaluated. Despite the fact that atrazine toxicity to aquatic plants has been extensively examined, this assessment provides microcosm-derived effective concentrations for *Myriophyllum spicatum* and *Elodea canadensis* not previously estimated.

Both pharmaceuticals and fluorinated organic compounds have been in use in for many years, but it is only recently, with the development of analytical methods capable of detecting and quantifying their occurrence in the environment, that they have been identified as significant contaminants of aquatic ecosystems (Daughton and Ternes, 1999; Martin et al., 2004). Each class of compounds presents a unique set of challenges in terms of their quantification in environmental matrices, and the characterization of toxicity and environmental fate. Pharmaceuticals are developed to be biologically active and to elicit a response in target organisms at low doses, but their effects on non-target organisms are often unexamined or poorly understood (Halling-Sorenson, 1998). The widespread occurence of fluorinated organics and an

inadequate understanding of their behaviour and effects in the environment has highlighted the need for more research in this area (Martin et al., 2004).

The beneficial effects of ionophore antibiotics, such as monensin toxicity in target organisms are widely known (Duffield et al., 1997; McGuffey et al., 2001), while assessment of the impact of the unintentional release of parent compound into surface waters and the potential risk to non-target organisms are virtually unexamined (Hillis et al., 2007). Likewise, despite the detection of fluorotelomer carboxylic acids in the environment, a single study exists considering the toxicity of telomer acids to three aquatic organisms (Phillips et al., 2007). The results of their study provide evidence that the acids, including the 10:2 FTCA, are more toxic than their ultimate breakdown products (the perfluorinated carboxylic acids) which have been the primarly focus of toxicity research to date. Despite concern over the potential effects of these compounds, little ecotoxicological data exists for either chemical, let alone effects at the field-level. Thus, results from our microcosm-based investigations represent a contribution to the body of data required before a comprehensive assessment of their risk to the aquatic environment may be carried out.

The experiments conducted to determine toxicity of monensin and the 10:2 FTCA to freshwater macrophytes, found that there were no consistent effects on any of the endpoints monitored over the duration of the studies. The use of these data in a basic hazard quotient risk assessment indicates that concentrations of monensin currently detected in the aquatic environment, do not pose a risk to these freshwater macrophytes. Although results of the 10:2 FTCA investigation demonstrate that the compound did not elicit biologically significant responses at tested concentrations,

the potential risk posed to aquatic plants cannot be directly assessed because the actual concentrations of the compound in surface waters is yet to be reported in the literature. Concentrations of perfluorodecanoic acid (PFDA), the expected breakdown product of the 10:2 fluorotelomer acids (Dinglasan et al., 2004), have been detected in surface waters at the low ng/L range (So et al., 2007). As Scott et al. (2006) found that concentrations 10:2 FTCA in North American precipitation samples were signifantly correlated with PFDA, using concentrations of PFDA in surface water samples as a surrogate for 10:2 FTCA we conclude that current concentrations of the acids are not likely to pose a threat to freshwater plants using a basic hazard quotient (HQ) method. A HQ of 0.002 is obtained using 2 μg/L as the NOEC under seminatural field conditions, and the highest detected concentration of PFDA in surface waters of 3.8 ng/L (So et al., 2007).

The actual concentrations of the chemicals in microcosm waters were not quantified successfully in either study, and nominal concentrations were used in the analyses toxicity. Although the presence of monensin in the test systems was established through assessment of water samples using verified methods (Hao et al., 2006), unexpected delays in analysis times lead to invalidation of detection results. Instead, environmental fate data from a previous investigation conducted at the same testing facility under similar experimental conditions was deemed reflective of compound partitioning and degradation behaviour under our study conditions (Lissemore, 2005). This indicates that macrophytes were likely exposed to monensin over the course of the study, as the compound was found to be stable under previous test conditions.

Although formulation of the 10:2 FTCA test compound and treatment of the microcosms was conducted according to established methods (Achilefu et al., 1995), and researchers remain confident that 10:2 FTCA was added to the microcosms (C. Butt, 2007), unanticipated detection issues were encountered in the laboratory and have yet to resolved. Research is presently underway to investigate why detection of the compound in water samples was unsuccessful, with efforts focused around partitioning properties and fate of the fluorinated surfactant in waters and sediments (Myers and Mabury, 2007).

The laboratory based investigation of the toxicity of saturated and unsaturated carboxylic acids mention analytical issues surrounding precipitation of compound out of solution and/or adherence to sample containers in relation to their encounted water sample storage issues (MacDonald, 2005), suggesting that similar behaviour may have occurred in our investigation despite measures taken to avoid these issues. Additionally, a recent study determined that fluorotelomer alcohols, the precursors to telomer acids, have a greater propensity for sorbtion to organic phases and less of a tendency to remain in the water phase than originally presumed (Goss et al., 2006), and accordingly there is potential that the closely related FTCAs may exhibit similar partitioning behaviour. The absence of supportive water residue analysis data yields uncertainty over the level 10:2 FTCA exposure to macrophytes. Although the measurement and confirmation of concentrations by analytical means is preferential, owing to the paucity of data on this class compounds, these toxicity results should not be overlooked. Worth noting, findings of other toxicity investigations in the literature have also relied on the use of nominal concentrations (Brain et al., 2004).

In both experiments, it was hypothesized that monitored concentrations of monensin and 10:2 FTCA would prove phytotoxic to aquatic macrophytes when evaluated in a chronic exposure microcosm-based testing scheme, in turn indicating that the acute laboratory tests are not necessarily predictive of simulated field-level responses. The compounds did not prove to be toxic to freshwater macrophytes under the testing regime however, and results were in general correspondence with laboratory-based single species testing (Brain et al., 2005; Phillips et al., 2007). In these cases, the baseline screening assays proved to be protective of the larger macrophyte community, indicating that the lower tiers of an ecological risk assessment which rely on the laboratory derived toxicity data, would be accurate in concluding that the compounds pose no risk to freshwater aquatic macrophytes at current environmental concentrations (Solomon, et al., 2000; Davy et al., 2001).

Results of the atrazine toxicity assessment were consistent with observations in the literature. Both *E. canadensis* and *M. spicatum* demonstrated inhibited growth in response to increasing atrazine concentration. Laboratory based tests conducted using *E. canadensis* have reported EC_{50}s ranging from 21 to 109 μg/L atrazine for exposures lengths of between 14 and 28 days (Forney and Davis, 1981; Fairchild et al., 1998). We found EC_{50} values ranging from 4.6 to 147 μg/L (day 14), 15.8 to 150 μg/L (day 28), and 16.6 to 116 μg/L (day 42), for *E. canadensis*. Studies have shown that *M. spicatum* is less sensitve to atrazine than *Elodea* under laboratory conditions, with EC_{50} values ranging from 91 to 3700 μg/L over test lengths of 24 hours to 28 days (Forney and Davis, 1981; Jones and Winchell, 1984; Kemp et al., 1985; Bird, 1993). Under our simulated field conditions EC_{50} values for *M. spicatum* were

estimated to range between 40.9 and 377 μg/L (day 14), 15.5 and 151 μg/L (day 28), and 24.6 and 173.8 μg/L (day 42), proving to be less sensitve than *E. canadensis* at earlier time points, and comparable over longer exposure durations. The atrazine toxicity data obtained from this investigation will be used further in an ecotoxicological risk assessment focusing on review of potential risk of atrazine posed to the macrophyte component of freshwater ecosystems.

5.2 RESPONSES OF INDIVIDUALLY GROWN PLANTS VERSUS ASSEMBLAGES

The common approach of using individually grown macrophytes in the evaluation of contaminant toxicity leads to the question of whether this practice is appropriate, and if observed responses are reflective of those expected in higher levels of biological organization. Thus, an additional objective of our research was to investigate how well plant responses observed in the individual-based microcosm test reflect responses observed in model populations and two-species communities, using biomass and relative growth rate measures as mechanisms for comparison, and monensin, 10:2 FTCA, and atrazine as chemical stressors.

From our studies, neither plants grown individually nor those from mixed and monoculture assemblages demonstrated a phytotoxic response to monensin or the 10:2 FTCA. As both toxicity assessment systems arrived at the same conclusions, responses of individually grown plants corresponding with model populations and communities, the use of the "cone-tainer" method was protective of aquatic plants under these conditions. Significant differences found in biomass and RGR measures of macrophytes propagated as individuals and those in model populations and

communities however, suggest that had inhibition of growth by contaminant effects been observed, the response of growth related endpoints may have varied between systems as well. The correlation between decreasing RGRs and toxicological sensitivity of macrophytes to a chemical stressor has been observerd in the literature (Cedergreen et al., 2004). Because individually grown plants in these two studies demonstrated lower RGRs then assemblages of plants, accordingly, they may prove to be less sensitive towards a chemical toxicant.

In order to further test this hypothesis an evaluation of toxicity was conducted using a known plant stressor, atrazine, under similar conditions. Generally, results from the individual-based plant assays corresponded with results from tests conducted on plants grown in model populations and communities. Sensitivities to atrazine (measured as EC_xs) were within a comparable range between test systems, suggesting that the "cone-tainer" method provides a reasonable estimation of contaminant effects under these circumstances. Unlike the previous studies, this investigation detected few statistically significant differences between biomass or RGRs of plants grown under various planting scenarios. As growth between systems was generally within the same range, a difference in macrophyte sensitivity related to varying growth between systems was not expected.

The final objective of our research was to investigate how intra- and inter-species plants relations may modify their response to a toxiciant. Results from the first two microcosm-based studies could not directly speak to this question, as monensin and the 10:2 FTCA did not prove to be phytotoxic to tested macrophytes and differences between responses of plants grown under the various planting

systems were not observed. In the final investigation, evidence did not suggest that interactions between plants occurred, as expressed through changes in biomass and RGRs. The lack of impact of plant relations on growth meant that interaction effects of planting method and the main effects of atrazine were not observed, and that individual plants may respond to a toxicant in much the same fashion as a plant in the model populations or commuities.

5.3 FUTURE RESEARCH DIRECTIONS

Further investigation of the potential for interactions between aquatic macrophytes to influence their response to an environmental stressor is recommended. While our research indicated that responses of aquatic plants tested using the individual "cone-tainer" method correspond with observations of plants from model populations and communities, due to a lack of relations between extra and conspecific neighbours we did not directly examine whether such interactions could modify response to a toxicant. Subsequent testing should be performed under conditions in which relations between freshwater plants are apparent, using higher planting densities and over longer durations of growth (Barrat-Segretain and Elger, 2004) in order to allow for establishment of these interactions. The establishment of model populations and communities of plants in planting containers prior to evaluation, instead of conducting testing on newly planted assemblages may also be worth investigation, as the developmental stage of the plants has been shown to influence the outcome of competitive investigations, as has the spatial pattern in which the macrophytes were planted (Barrat-Seretain, 2005). While not observed in our study, negative interactions between *E. canadensis* and *M. spicatum* have been

documented (Abernethy et al., 1996). The causes for reductions in biomass were not clearly examined however, and delineation between mechanisms such as competition between species over a common resource, shading, allelopathy, etc. not provided. Also, experiments have investigated growth interactions of *E. canadensis* and *M. spicatum* with other macrophyte species (Agami and Waisel, 2002; Barrat-Segretain and Elger, 2004), but these relationships do not speak directly to how the species will interact with one another (Barrat-Segretain, 2005). Thus, experiments aimed at determining the mechanisms by which *E. canadensis* and *M. spicatum* influence the growth of the other species, and how this may modify observed toxicity is also warranted. Finally, there are uncertainties associated with the occurrence and environmental fate of the 10:2 FTCA. Further investigation of the chemical characteristics of the compound and associated partitioning behaviour is required, before appropriate characterization of potential risk posed to the aquatic environment may be performed.

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6 APPENDIX

Example SAS coding used in nonlinear regression analysis for field derived aquatic macrophytes data is shown below:

```
data first;
input dose endpoint;
cards;
input data here
;
proc print;
Run;

[LINEAR MODEL]:

proc nlin data=first;
parameters b=0.37 x= 50;
model endpoint=((-b*0.1)/x)*dose+b;
output out=second p=pred r=resid;
run;
proc plot data=second(obs=15);
     plot endpoint*dose pred*dose='p' /overlay;
     plot resid*dose / vref=0;
run;

proc
nlin data=first;
parameters b=0.37 x= 100;
model endpoint=((-b*0.25)/x)*dose+b;
output out=second p=pred r=resid;
run;
proc plot data=second(obs=15);
     plot endpoint*dose pred*dose='p' /overlay;
     plot resid*dose / vref=0;
run;

proc
nlin data=first;
parameters b=0.37 x= 200;
model endpoint=((-b*0.5)/x)*dose+b;
output out=second p=pred r=resid;
run
;
proc plot data=second(obs=15);
     plot endpoint*dose pred*dose='p' /overlay;
     plot resid*dose / vref=0;
run;


[LOGISTIC MODEL]:

proc nlin data=first;
parameters t=0.02 x= 50 b=5;
model endpoint=t/(1 +(.1/.9)*(dose/x)**b);
```

```
output out=third p=pred r=resid;
run;
proc plot data=third(obs=15);
     plot endpoint*dose pred*dose='p' /overlay;
     plot resid*dose / vref=0;
run;
proc nlin data=first;
parameters t=0.02 x= 50 b=5;
model endpoint=t/(1 +(.25/.75)*(dose/x)**b);
output out=third p=pred r=resid;
run;
proc plot data=third(obs=15);
     plot endpoint*dose pred*dose='p' /overlay;
     plot resid*dose / vref=0;
run;
proc nlin data=first;
parameters t=0.02 x= 100 b=5;
model endpoint=t/(1 +(dose/x)**b);
output out=third p=pred r=resid;
run;
proc plot data=third(obs=15);
     plot endpoint*dose pred*dose='p' /overlay;
     plot resid*dose / vref=0;
run;


[HORMETIC MODEL]:

proc nlin data=first;
parameters t=0.02 x= 100 b=5 h=1;
model endpoint=(t*(1+h*dose))/(1+((0.1+h*dose)/0.9)*(dose/x)**b);
output out=forth p=pred r=resid;
run;
proc plot data=forth(obs=15);
     plot endpoint*dose pred*dose='p' /overlay;
     plot resid*dose / vref=0;
run;
proc nlin data=first;
parameters t=0.02 x= 100 b=5 h=1;
model endpoint=(t*(1+h*dose))/(1+((0.25+h*dose)/0.75)*(dose/x)**b);
output out=forth p=pred r=resid;
run;
proc plot data=forth(obs=15);
     plot endpoint*dose pred*dose='p' /overlay;
     plot resid*dose / vref=0;
run;
proc nlin data=first;
parameters t=0.02 x= 100 b=5 h=1;
model endpoint=(t*(1+h*x))/(1+((0.5+h*dose)/0.5)*(dose/x)**b);
output out=forth p=pred r=resid;
run;
proc plot data=forth(obs=15);
     plot endpoint*dose pred*dose='p' /overlay;
     plot resid*dose / vref=0;
run;
```