EVALUATING THE FIELD AND LABORATORY EFFICACY OF A TOXICITY TEST FOR THE AQUATIC MACROPHYTE *ELODEA CANADENSIS*

by

Neil C. Rentz

A Thesis submitted to the Faculty of Graduate Studies of

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ABSTRACT

EVALUATING THE FIELD AND LABORATORY EFFICACY OF A TOXICITY TEST FOR THE AQUATIC MACROPHYTE *ELODEA CANADENSIS*

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Growth parameters and toxicity of the herbicide diuron were assessed for the macrophyte *E. canadensis* under both field and laboratory conditions. Under controlled laboratory conditions, non-axenic *E. canadensis* shoots were grown in AAP, Hutner's, Hoagland's, Smart and Barko, and Andrew's media as well as in DI water with endpoints of biomass (wet/dry), plant length, root (mass, length, number), chlorophyll-a content, percent of plants survived, and relative growth rate (RGR) (based on wet/dry mass and plant length) measured at the end of 14-d to determine which media type promoted strongest growth. AAP media performed most consistently and exhibited significantly higher growth compared to many of the other media. This was likely due to a N:P ratio that did not provide an excess of N and P to encourage the growth of algal contamination, which was a critical issue in this laboratory study. A toxicity test with *E. canadensis* using AAP as growth media showed a definite toxicological response to diuron and higher toxicological sensitivity compared to *L. gibba* and *L. minor*. Although this showed promise for the use of *E. canadensis* in further laboratory testing, caution needs to be taken in data interpretation since the endpoints measured exhibited higher variation compared to *Lemna* species. No response in *E. canadensis* was observed using the herbicide atrazine, likely due to interference caused by algal contamination. A microcosm study exposing *E. canadensis* to diuron was also conducted to determine the field efficacy of the laboratory toxicity test. *E. canadensis* grown at low population

densities and in young, non-established stands showed higher relative growth rates (RGR) compared to high density, established populations. Individual shoots in cone-tainers showed the lowest RGRs. This was likely due to intraspecific competition and established stands reaching carrying capacity, thus experiencing a reduction in growth. A clear toxicological response was observed in the field, with higher populations densities and non-established stands showing slightly higher sensitivity compared to lower densities and established populations. Individually grown shoots in cone-tainers showed lower sensitivity compared to populations. These results support the hypothesis that higher RGRs can lead to greater toxicological sensitivity, a phenomenon previously described in literature. A comparison between laboratory and field toxicity data for diuron exposure showed no consistent trends in sensitivity between the two test systems, thus laboratory data were not predictive of field data. This indicates that if *E. canadensis* is to be used as a surrogate for other macrophytes for further laboratory testing, work needs to be done to reduce variation in these laboratory assays.

PREFACE

Chapters 2 and 3 of this thesis are organized as manuscripts to be submitted for publication in scientific journals. For this reason, some repetition of introductory and methodological material occurs throughout. All chapters and the associated manuscripts were written by Neil Rentz, as indicated by the primary authorship below.

Chapter 2:

Rentz N.C., Solomon K.R., Hanson M.L. 2009. A toxicity bioassay for the freshwater macrophyte *Elodea canadensis* Michx. Pest Manag Sci. (to be submitted).

Chapter 3:

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Table 1.1 Acronyms used in text, figures and tables

Acronym	Definition
LOEC	Lowest observable effect concentration
NOEC	No observable effect concentration
EC_x	Effective concentration causing an impact of X% from control on an organism exposed to a toxicant
LC_x	Lethal concentration causing death to X% of an organism population exposed to a toxicant
IC_x	Inhibitory concentration causing inhibition of X% of an organism population exposed to a toxicant
CI	Confidence Interval
RGR	Relative growth rate
CPMU	N-(3-chlorophenyl)-N-methylurea
DCPMU	(N-(3,4-dichlorophenyl)-N'-methlyurea
3,4 DCA	3,4-dichloroaniline
DCPU	N-3,4-dichlorophenylurea
PQ	Plastoquinone
K_{ow}	Octanol-water partition coefficient
K_{oc}	Organic carbon sorption coefficient
DI water	Deionized water
Hoag	Hoagland's media
Hut	Hutner's media
And	Andrew's media
GP	Smart and Barko General purpose media
CV	Coefficient of variation
MSD	Minimum significant difference
MDD	Minimum detectable difference
Chl-a	Chlorophyll-a
ANOVA	Analysis of variance

1 INTRODUCTION

1.1 THE IMPORTANCE OF AQUATIC MACROPHYTE TESTING

Aquatic macrophytes are important species in terms of structure, function, and overall health of aquatic ecosystems. These plants produce oxygen, assist in nutrient cycling, improve water quality by assimilating heavy metals (Gouder de Beauregard and Mahy, 2002), stabilize sediments with their root structure (Wang and Freemark, 1995), and serve as a primary energy source in food webs (Lewis, 1995b). Macrophytes also support the primary functioning of wetland ecosystems including habitat provision, floodwater retention and groundwater recharge (Environment Canada, 2005). With the use of agricultural herbicides the effects on non-target aquatic macrophytes in wetlands is a relevant concern (Wang and Freemark, 1995). Applications of these chemicals can result in spray drift and runoff into adjacent wetlands (Battaglin et al., 2002; Lockhart et al, 1989). Once in adjacent water bodies, herbicides can have detrimental effects on aquatic macrophytes including reduction in biomass which leads to reduced habitat for aquatic invertebrates, food for waterfowl, and sediment stability, amongst other secondary effects (Davy et al., 2001). These potential impacts give rise to the need for phytotoxicity tests, which can determine the potential impacts of environmental contaminants on aquatic macrophyte species (Fairchild et al., 1997).

In spite of their obvious importance to aquatic systems, aquatic macrophytes have not always held a prominent place in ecotoxicology and risk assessment. Evaluation of toxicity of various compounds in the environment have used invertebrates and fish as surrogates for lower organisms such as aquatic plants (Lewis, 1995a, b). The practice of

considering these organisms as the most sensitive species for toxicity testing reveals two main deficiencies. One of these deficiencies is that due to the great variation in sensitivities to contaminants, there is no single or group of species that is universally the most sensitive to most contaminants (Rand et al., 1995, Wang and Freemark, 1995). The second deficiency is that lower organisms that provide crucial ecosystem functions are not accurately represented (Lewis, 1995b). With these deficiencies in mind, several studies have described greater sensitivity to aquatic macrophytes compared to other organisms. Taraldsen and Norberg-King (1990) found the duckweed *Lemna minor* to be more sensitive to a herbicide factory effluent compared to *Ceriodaphnia dubia.* In a study on the toxicity of various herbicides, Roshon et al. (1999) found rooted, submersed aquatic macrophytes to be more sensitive as compared to fish and invertebrate species, clearly due to the specific modes of action of herbicides on plant species and in many cases, much more sensitive than algae or duckweed assays. Considering their sensitivity to various contaminants and role in maintaining ecosystem function, they have been vastly underrepresented in toxicology testing. Hence, it is important to more adequately protect aquatic macrophytes by expanding available test methodologies. As stated by Catallo (1993), the role of ecotoxicology in aquatic systems is to study effects of toxic substances in these systems with a special interest in how significant these effects are to the structure and function of the ecosystem as a whole. In order to capture effects to the ecosystem as a whole, aquatic macrophytes must be carefully considered in toxicity assessments.

1.2 MACROPHYTES IN PESTICIDE RISK ASSESSMENT

The testing of non-target aquatic plants for use in the registration of new chemicals was proposed by the United States Environmental Protection Agency in 1978, with the first regulations published in 1982 (Davy et al., 2001). This regulation consisted of three distinct tiers of assessment. The first used the maximum-labeled dosage of a chemical to determine if the plant's appearance and growth were affected. If $\geq 50\%$ reduction of aquatic plant growth was observed, second tier assessments, involving concentration-response analysis to determine EC_{25}, EC_{50} and NOEC concentrations were conducted. In the third tier of assessment, field studies would be conducted on sensitive species to further examine adverse effects. In developing this regulation, only one aquatic macrophyte was required for testing, *Lemna gibba,* along with four algal species. Although regulations have undergone many revisions over the years, the fact that *Lemna* species are the only required aquatic macrophyte required for testing remains the same (Davy et al., 2001). In 1993, Canada adopted its own guidelines for non-target plant testing and evaluation. These guidelines, much like those of the USEPA, rely heavily on *Lemna* as the representative aquatic macrophyte. Currently, the Canadian agency (Pest Management and Regulatory Agency – PMRA) and the USEPA have harmonized methodologies for non-target plant testing to use the three tier model with sole emphasis on *Lemna* as the only tested aquatic macrophyte (Davy et al., 2001). These methodologies have come under criticism since *Lemna* cannot be representative of the diverse array of macrophytes in the environment. Explanations for this criticism are many including that since *Lemna* is a monocotyledon, dicotyledons are not represented (Hanson et al., 2009; Knauer et al., 2008; Davy et al., 2001). Also, sediment-bourne

exposure routes are not captured as *Lemna* floats and is therefore not interacting significantly with sediment (Davy et al., 2001). In recognizing these deficiencies, new requirements have been proposed for non-target aquatic macrophyte testing. These recommendations include an increase in the vascular plant species required for testing from one to three species (one submersed and two emergent) as well as using a four tier assessment framework (Davy et al., 2001). The first level is a deterministic assessment where risk quotients are used to determine if a chemical is phytotoxic. This involves dividing the estimated environmental concentrations by an acute EC_x value. A risk quotient of > 1 indicates a significant level of risk warranting level two assessments. These second level assessments, or refined assessments, evaluate concentration-response data from most sensitive species to determine where most uncertainty occurs. Third level assessments, or expanded assessments, use keystone species in acute and chronic tests that are specific to a region of concern. The fourth and final level, or comprehensive assessment, involves mesocosm/microcosm field based studies with multiple species to get a more accurate description of toxicity under natural conditions. With these proposed revisions to current methods of non-target aquatic macrophyte testing, it is hoped that these important species will be better represented in a risk assessment context.

1.3 WHAT IS A BIOASSAY?

A foundation of risk assessment is the toxicity test or bioassay, as it is commonly called in aquatic toxicology, that is conducted to assess toxicity to a specific macrophyte. A bioassay, as defined by Finney (1978), is "an experiment for estimating the nature, constitution, or potency of a material (or of a process), by means of the reaction that follows its application to living matter". In short, a specific concentration(s) of a

chemical is applied to an organism to determine a specific response or effect, usually an adverse one. Adverse effects that occur at relatively low concentrations are of the greatest concern, since this indicates high sensitivity of a specific species of test organism to that chemical (Rand et al., 1995). Effects on the organism are measured via endpoints, which are based on important biological processes like growth and reproduction (Rand et al., 1995). With macrophytes, these can be measurable parameters such as biomass, relative growth rate, and chlorophyll-a content. It is crucial that endpoints chosen are of ecological relevance and indeed show a response to the contaminant of concern over the set exposure period. These tests can vary in their duration of exposure. Short-term exposures to a chemical, usually at lethal concentrations, are termed acute toxicity tests, while exposure over longer periods usually at sub-lethal concentrations are chronic toxicity tests. Typically, response data are interpreted in two common ways. One is by calculating EC_x values. This is the effective concentration, or the concentrations required to cause an effect in X% of the test organisms. LC_x values are similar to EC_x except it is the concentration that caused lethality to X% of the population. IC_x values again, are similar to LC_x and EC_x, except represent inhibition of a specific endpoint occurring in X% of the population. A common value for X used in these calculation is 50% of the test organism population, but can be a variety of values depending on what level is deemed acceptable for the compound being tested. Another common way data are interpreted is by comparing responses in treatments to controls to derive NOEC/LOEC or no observable effect concentrations and lowest observable effect concentrations. These describe the concentration threshold where no effects (NOEC) and where effects start to occur (LOEC) (Rand et al., 1995). The use of NOEC and LOEC has undergone criticism

with regards to their biological meaning. Since these values rely heavily upon significance levels tested and can only be one of the specific concentrations tested, their significance is purely statistical and has little significance in the biological responses of test organisms (Hanson et al., 2002). It has been suggested that an EC_x value, particularly one with a low X value (EC_{10}), be used as a surrogate for the NOEC/LOEC (Hanson et al., 2002). This will capture the low concentrations exhibited by NOEC/LOEC but be based upon concentration-response data, which are more biologically significant.

1.4 CURRENT LABORATORY STANDARD METHODS

One criterion of a well-developed and efficient bioassay is that it be widely accepted by the scientific community (Rand et al., 1995). One way to achieve this is through developing standard methods. The use of bioassays, much like any test method, can be subject to great variation. In order to establish consistency and reduce variation within macrophyte tests, standard methods need to be established (Hanson and Arts, 2007). Creating standard methods are advantageous as they create uniform methodologies that can be used by a variety of laboratories and personnel, they can be replicated easily, and they allow for comparisons of data amongst different agencies that use the tests (Rand et al., 1995). Standard methods have been published for aquatic macrophyte testing, but those available represent very few species. Formalized and accepted standard methods have only been developed for *Lemna* spp., a floating macrophyte and *Myriophyllum sibiricum* (which is also applicable to *M. spicatum*), a submersed rooted macrophyte. For *Lemna* spp., numerous standardized protocols have been developed by Environment Canada (2007), the American Society for Testing and

Materials (ASTM) (2005a), the Organization for Economic Co-operation and Development (OECD) (2002), and the United States Environment Protection Agency (USEPA) (1996), along with other, less formal methods (Greenberg et al., 1992). All of these methods remain consistent in their core methodology; they prescribe a 7-day exposure period within Erlenmeyer flasks or plastic Petri dishes with a set number of plants (3-5 plants with 3-4 fronds each) and fronds in each exposure, incubated at 25°C, illuminated at a range of 57 – 135 μmol m^{-2} s^{-1}. Various growth media are recommended for use in these *Lemna* spp. tests including M-Hoagland's (USEPA, 1996; ASTM, 2005a), AAP 20X (USEPA, 1996; ASTM, 2005a), Hogland's E+ (ASTM, 2005a; Environment Canada, 2007), Swedish Standard Media (SIS) (OECD, 2002), and Hutner's (Greenberg et al., 1992) with replacement of the media occurring at day 3 or 5 if a static renewal method is implemented. In regards to other available test species methodologies, only one method for the rooted submersed macrophyte *Myriophyllum sibiricum* has been formalized (ASTM, 2005b). This test places a small apical segment of the macrophyte in a borosilicate glass tube with a small amount of root substrate (Turface or sand) with modified Andrew's growth media over a 14-d period. Illumination ranges between 100 to 150 μmol m^{-2} s^{-1} with a 16:8h light/dark cycle with temperature maintained at 25/20°C light/dark. All current methodologies recommend using a range of test concentrations, allowing for the calculations of EC_x, IC_x, or LC_x values from concentration-response curves. Also, in using a range of concentrations, the concentrations which cause effects and/or that are found in the environment can be bracketed by the outside limits of this range.

These methodologies have proven to be useful in obtaining statistical precision and toxicological sensitivity in toxicity testing. Exposure of perflourinated compounds (Boudreau et al., 2003), haloacetic acids (Hanson and Solomon, 2004), and various herbicides (Roshon et al., 1999) to *Lemna* spp. and *Myriophyllum* spp. were conducted using these standard methods and had coefficients of variation (CV) for most endpoints ranging from 10 – 30% and 95% confidence intervals being within 10% of the calculated EC_x value. Although these standard methods show very acceptable statistical resolution, there are many methods for testing macrophytes that alter such parameters like test chamber size, lighting intensity, or the presence of natural sediments (Cedergreen et al., 2004a; Fairchild et al., 1998; Feiler et al., 2004; Belgers et al., 2007). Although it is important to continue to develop new ways of testing macrophytes, it is important that some consistency is reached in the methods used. If this does not occur, it will be increasingly difficult to make comparisons between various studies for the same species (Rand et al., 1995).

In spite of the success of current standard methods for macrophyte testing, a major deficiency exists in the number of methods currently available that are published and accepted by the scientific community. Current standard methods only exist for *Lemna* and *Myriophyllum* species. Just as relying on one or a very small number of species is a problem in risk assessment due to the misrepresentation of various macrophytes with diverse morphological characteristics (Hanson et al., 2009; Knauer et al., 2008; Davy et al., 2001), so is it a problem in the number of standard test methods available. Various other macrophytes have been used in toxicity testing such as *Elodea canadensis*, *Ceratophyllum demersum*, and *Potamogeton crispus* (Fairchild et al., 1998;

Cedergreen et al., 2004a) and there are studies evaluating the efficacy of laboratory culturing (Knauer et al., 2006). All of these macrophytes were shown to be sensitive to various contaminants and were successful in culturing procedures. The great diversity of macrophyte species with regards to their morphological characteristics, chemical sensitivity, and overall use in laboratory tests indicate the potential for the development of new standardized methods.

1.5 TEST ORGANISM – *ELODEA CANADENSIS*

The primary test organism for this study was Canadian Pondweed, *Elodea canadensis* Michx. *Elodea canadensis* Michx. is a rooted, dioecious, monocotyledonous, submersed aquatic macrophyte with characteristic dichotomously branched shoots with whorls of three leaves 6 – 17 mm long and 1 – 5 mm wide. *E. canadensis* is native to Canada, distributed widely with highest densities in southern British Columbia, southern Manitoba, southeastern Ontario, and southern Quebec; as well it is invasive in Europe, United Kingdom, Asia, South Africa, Australia and New Zealand (Spicer and Catling, 1988; Kozhova and Izhboldina, 1993; Simpson, 1984). It is found in relatively cool habitats (10 – 24°C) in alkaline waters with a pH range of 6.5 – 10, and it grows at depths of 1 – 8 m with roots preferentially anchored in fine sediments compared to sandy substrates (Spicer and Catling, 1988; Bowmer et al., 1995). It also has the capacity for overwintering (1 – 4°C), where it forms vegetative propagules that rest on the sediment and grow into adult plants the following spring (Bowmer et al., 1995). A study conducted by Pokorny et al. (1984) found a natural population of *E. canadensis* growing in a constructed fish pond in Czechoslovakia (7.02 ha, 1.5 m mean depth) to have a mean RGR of 0.028 $gg^{-1}d^{-1}$. *Elodea* tends to grow well in co-dominant communities with

Ceratophyllum demersum, *Myriophyllum spicatum*, *Myriophyllum exalbescens*, *Ultricularia vulgaris*, and *Potamogeton* spp. Dominant, dense stands of *E. canadensis* have been shown to grow and out-compete other primary producers. Allelopathic activity has been described as a reason for this observation. Erhard et al. (2006) demonstrated inhibition of algal species in the presence of crude extracts of allelopathic compounds from *E. canadensis*. As *E. canadensis* dies back, it is the most rapidly decaying of most submersed vascular plants, decaying in 47 – 57-d. *E. canadensis* is largely considered a nuisance species due to dense submersed beds that may limit the use of water bodies for recreational purposes and slow water flow which can impact irrigation for agriculture (Spicer and Catling, 1988). *E. canadensis* is considered an excellent food source for other organisms with a crude protein content of 27% (Spicer and Catling, 1988; D'Agaro et al., 2004). The plant is a nutrient sink, obtaining much of its nutrients from the sediments and absorbing elemental metals via stems and leaves (Spicer et al., 1988). Iron has been described as the single most important micronutrient that is limiting to the growth of *E. canadensis*.

E. canadensis has been used in studies characterizing the effects of metals on macrophytes (Stoyanova, 1993; Mal et al., 2002; Brown and Rattigan, 1979), the use of macrophytes for phytoremediation of environmental contaminants (Rice et al., 1997), as well as the toxicity of herbicides in the laboratory (Fairchild et al., 1998; Cedergreen, 2004a,b; Hofmann et al., 1990), the toxicity of herbicides in the field (McGregor et al., 2007), and the toxicity of phenolic compounds (Stom et al., 1981).

Previous studies have also shown that *E. canadensis* can be successfully cultured in the laboratory, even from non-axenic material. A laboratory study conducted by

Knauer et al. (2006) successfully cultured *E. canadensis* from non-axenic material in the laboratory with no visible signs of bacterial or algal contamination present with increases in plant length of 70 – 75% over the 21 day test duration, indicating reasonable growth in this test system.

In spite of this body of ecotoxicological research conducted with this macrophyte, its widespread distribution, and its ability to be cultured in the laboratory, no standard methods have been published for it. Establishing these methods for this species is important since *E. canadensis* is a rooted, submersed species that can describe effects from sediment based and water column exposures, both of which cannot be described by *Lemna*.

1.6 MODEL CONTAMINANTS

1.6.1 Diuron

1.6.1.1 Physical properties and general use

Diuron (N'(3,4-dichlorophenyl)-N'N-dimethylurea) (Appendix 1) is classified as a phenylurea herbicide and is mainly applied as a pre-emergence herbicide to kill a variety of common broadleaf weeds and grasses in crops such as alfalfa, small grains, and a variety of fruit crops (USEPA, 2003b; Ware and Whitacre, 2004). It is also used to defoliate right-of-ways and to clear ornamental and economical fish ponds of aquatic vegetation (USEPA, 2003b). This herbicide is a white, crystalline, and odorless in its solid form (Vencill, 2002). It has the capacity to become sorbed to soil with a K_{oc} of 485 ml/g and an aerobic soil half-life ranging from 90 - 365 days (Vencill, 2002; PAN-UK, 2005). Also, with a K_{ow} of 589, diuron has the potential to partition into lipids within organisms. Diuron is applied at a variety of different rates depending on the formulation.

Agricultural application rates range from 0.2-6.4 lbs active ingredient/acre, while non-agricultural (roadside/railroad defoliating) is about 0.8-12 lbs active ingredient/acre (USEPA, 2003b). Typical formulations of this herbicide are mixtures including bromacil, sodium chlorate, sodium borate, imazapyr, paraquat salt, and isopropylamine salt (Vencill, 2002).

1.6.1.2 Mode of action

The mode of action of diuron is via the inhibition of photosynthesis. It effectively blocks the transport of electrons from the photosystem II acceptor Q to plastoquinone (PQ) (Ware and Whitacre, 2004). This leads to the cessation of ATP and NADPH production, both of which are required for carbon dioxide fixation (Ware and Whitacre, 2004). Since the Q_A acceptor is no longer oxidized, singlet oxygen and triplet chlorophylls are formed. These two species then can interact with lipids creating lipid radicals that can oxidize proteins and lipid molecules. This will lead to cell death through membrane leakage and chlorophyll loss (Vencill, 2002).

1.6.1.3 Sales and usage

Diuron is still commonly used today in North America, though sales and usage data for Canada presented by Brimble et al. (2005) showed relatively low usage. Data for British Columbia, Alberta, Ontario, and Nova Scotia were presented. Alberta used the most diuron with a value of 9.9×10^3 kg used in 1998. BC, Ontario, and Nova Scotia used significantly less with values of 770, 299, and 10 kg in 2003. Total usage of diuron in Canada represented by these data was 1.1×10^4 kg/year. In comparison to the highest used herbicide, glyphosate, with a Canada wide usage of 4.6×10^7 kg, diuron use is quite

low. In a United States Environmental Protection Agency report on american pesticide usage by Kiely et al. (2004) of the Office of Prevention, Pesticides, and Toxic Substances, Diuron ranked as the 7th most popular pesticide in 2001 and the 8th most popular pesticide in 1999. The usage was 4.4 – 8.8 million kg for 2001 and 1999. These values greatly exceed the usage in Canada, likely due to the higher production of fruit crops and longer growing season in the United States, leaking to higher diuron usage in the United States than in Canada (USEPA, 2003b; Brimble et al., 2005).

1.6.1.4 Environmental concentrations and guidelines

Environmental concentrations of diuron have been quantifiedin both experimental and natural settings as well as environmental monitoring in agricultural settings. Powell et al. (1996) conducted simulated rainfall after diuron application to roadside plots to determine concentrations in runoff water. It was found that after 3.6 kg ai/ha of diuron was applied and simulated rainfall occurred, concentrations in runoff water ranged from 144 – 1.8 x 10^3 µg/L six weeks after application. During that period, a maximum of 5.4% of the original application was removed via runoff. Also, a maximum 419 µg/L diuron was detected in runoff of a second simulated rainfall event six weeks after application. This study also investigated actual runoff events from rainfall following the same application of diuron as the simulated rainfall events. Over the 13-week monitoring period, concentration of diuron in runoff ranged from 46 – 2.8 x 10^3 µg/L with a maximum of 8.4% of the original application lost to runoff. Thirteen weeks after application, 46 µg/L diuron was detected in runoff water. A monitoring study conducted by Rupp et al. (2006) investigated the concentrations of diuron present in runoff and tile drain water of two grass seed fields. Diuron application for the two fields ranged from

1.1 – 2.5 kg ai/ha. For one of the fields, a concentration of 2.2 x 10^3 µg/L diuron was found in runoff after rainfall occurring 45-d after application. Concentrations remained at levels >940 µg/L 68-d after application with exponential decreases in concentrations to 6 µg/L 130-d after application. For the other test field, the first rainfalls occurred at 27 and 29-d after application leading to concentrations in runoff of 120 µg/L. Much like the first field, over 130-d concentrations exponentially decreased to levels < 10 µg/L. The first field showed a more rapid decline in diuron runoff concentrations likely due to its greater slope and tile drainage system. Concentrations of diuron collected in the tile drainage water in the first test field were much lower than found in surface runoff, with averages of 1.7 µg/L. Diuron is also used as an antifouling biocide on ships to prevent growth of living organisms on the hull (Lambert et al., 2006). Water sampled from ports in the East Angia region of the UK found maximum diuron concentrations of 0.25 µg/L. Similar monitoring in ports of Japan conducted by Okamura et al. (2003) found diuron in 86% of samples collected with a concentration of 3.05 µg/L. Environmental monitoring of diuron has also been reported in a variety of agricultural areas. The South Florida Water Management District monitors 32 surface water sampling sites in areas used extensively for sugar cane and citrus fruit production. During the December 1998 sampling period, only two sites contained diuron above the detection limit of 0.4 – 0.8 µg/L. These sites showed concentrations of 0.64 and 1.2 µg/L (Pfeuffer, 1998). A review of data reported by the United States Geological Survey (USGS) found that out of 942 samples from agricultural applications (with 7.96% positive detections) and 315 samples from urban application (with 13.02% positive detections), mean concentrations of diuron were below levels of detection (0.05 µg/L) for both urban and agriculture use patterns (APVMA,

2005). A monitoring study in Australia over a 5 year period (1990 – 1995) covered an area of >7.0 x 10^5 ha mainly used for citrus crops. It was found that surface runoff samples, although showing ~40% detections of diuron, reached maximums of only 20 µg/L with creeks draining large irrigation areas reaching maximums of 7.5 µg/L (APVMA, 2005). Collections of runoff from one particular citrus farm with 4.5 kg ai/ha applied showed diuron concentrations ranging from 1.2 – 20 µg/L with a mean of 10.9 µg/L after the first rainfall event. It is clear from these collected data that diuron from agricultural applications like citrus farming leads to much lower concentrations in surface water compared to more intensive applications like roadside ditches or grass seed farms.

Very few regulatory levels of diuron have been established. The Pesticide Management Regulatory Agency of Canada has not set a maximum residue limit for diuron, but states that under the Food and Drug Regulations subsection B.15.002(1) residues cannot exceed 0.1 mg/L on food products (PMRA, 2006). Since diuron is consistently found in the surface waters of the United Kingdom, a non-statutory Environmental Quality Standard for diuron of 0.1 µg/L was established (PAN-UK, 2005). In Australia the ADI (acceptable daily intake) is 6.0 x 10^{-3} mg/kg bw/day (APVMA, 2005). Other than these few guidelines, no others are published for diuron.

1.6.1.5 Environmental fate

Diuron is degraded primarily through biodegradation via fungal species (Vencill, 2002; Tixier et al., 2000). The biodegradation process usually starts with the removal of N-methyl groups (Dellamatrice and Monteiro, 2004; Tixier et al., 2000). When one methyl group is removed, diuron loses 50% of its herbicidal activity, with no activity remaining after the second group is removed. These two metabolites, (N-(3,4-

dichlorophenyl)-N'-methlyurea (DCPMU) and N-3,4-dichlorophenylurea (DCPU), can then be transformed to 3,4-dichloroaniline (3,4-DCA), ammonia, and carbon dioxide under aerobic conditions (Dellamatrice and Monteiro, 2004; Giacomazzi and Cochet, 2004) (Structures shown in Appendix 3). Under anaerobic conditions, diuron can be biodegraded to N-(3-chlorophenyl)-N-methylurea (CPMU) (Giacomazzi and Cochet, 2004). Some species that have been identified as diuron degraders include *Arthrobacter* sp., *Sphingomonas* sp., and *Acinetobacter* sp (Dellamatrice and Monteiro, 2004). DCPMU, DCPU, and 3,4 DCA present an ecological concern with regards to their toxicity. A study conducted by Tixier et al. (2000), used a variety of fungal species to degrade diuron to produce DCPMU and DCPU. Upon verification that the degradation did produce these metabolites, they were synthesized in the laboratory and were subject to various microbiological toxicity tests. In all tests conducted, the two metabolites showed higher toxicity than the parent diuron with regards to cell division processes and enzyme activity (Tixier et al., 2000).

Although diuron has been reported as being only weakly photodegraded, studies have been conducted to describe its occurrence in the environment. Jirkovsky et al. (1997) exposed diuron to light of various wavelengths ranging from 254-365 nm on different surfaces such as clay and silica. Dry, aerobic conditions were represented through irradiation on silica as well as aquatic system conditions in aqueous solution. A vast array of degradation products can be formed as a result of photolysis depending greatly upon the type of surface used as well as the wavelength of irradiation (Jirkovsky et al., 1997). This indicates that potential production of toxic degradation products can vary in the environment depending on whether the chemical is bound to soil or in

aqueous solution. Overall, in natural sunlight, photodegradation of diuron is a minor degradation process (Giacomazzi and Cochet, 2004). Diuron can also undergo hydrolysis. In neutral solution at 25°C, diuron exhibits very slow hydrolysis to yield 3,4-dichloroaniline (3,4-DCA) as a byproduct (Giacomazzi and Cochet, 2004).

Diuron has been shown to have potential to become sorbed to soil. Studies show diuron having the strongest sorption to soil in comparison to other pesticides like atrazine, bromacil, chlortoluron, and carbofuran (Liyanage et al, 2006; Inoue et al., 2006; Madhun et al., 2006). The presence of metals have shown to modify the adsorption of diuron to soils. Gonzalez-Pradas et al. (1994) demonstrated that the presence of cadmium at low diruon concentrations can increase diuron sorption. Also, at higher diuron concentrations, the presence of zinc can increase diuron sorption. This is likely due to the modification of hydrophobic interactions with the soil by these metals (Gonzalez-Pradas et al., 1994).

Due to diuron's capacity to be adsorbed to soil, it presents a relatively low risk of leaching into groundwater. This was verified by a study conducted by Delphin and Chapot (2006). Leaching experiments were conducted in silt loam soils for atrazine, metolachlor and diuron. The study injected the pesticides at 30 and 80 cm depths. Diuron was found at the 80 cm depth in quantities much lower than other herbicides tested. This is likely due to higher degradation rates in surface soils and a higher organic carbon sorption coefficient (K_{oc}) of diuron compared to atrazine and metolachlor. Caracciolo et al. (2005) calculated the groundwater ubiquity score (GUS) for diuron from microbiologically active soil to be in the range of 1.8-2.8, identifying it as having relatively low leaching potential.

1.6.1.6 Freshwater aquatic macrophyte toxicity

The toxicity of diuron to aquatic macrophytes has been characterized for a limited number of species. *Lemna gibba* and *L. minor* were the most highly represented in literature. Dewez et al. (2002) verified that the mechanism for toxicity of diuron to its target organisms indeed occurs in a similar fashion for the non-target macrophyte *Lemna gibba*. The study reported the increases of chlorophyll-a florescence as a result of inhibition of photosystem II from exposure to diuron and its transformation products (DCPMU and DCPU), indicating a significant inhibition of photosynthetic activity. Such inhibition was not found in the transformation product DCA since the methylurea group is not present, indicating that this group determines the extent of photosystem II inhibition. Teisseire et al. (1999) exposed *L. minor* to diuron and found that concentrations inhibiting 50% and 90% of growth, measured as relative growth from frond numbers, were 25±3 and 60±2 µg/L respectively for a 7 day exposure. Exposure concentrations as low as 5 µg/L showed growth inhibition. Another effect observed in this study characteristic of phenylurea herbicides such as diuron, is the increase in chlorophyll with increasing diuron exposure. This is due to the formation of shade-type chloroplasts that have broad grana and greater stacking of thylakoids (Teisseire et al., 1999). These chloroplasts have been found to be much less efficient at photosynthesis than normal chloroplasts (Teisseire et al., 1999). The aquatic macrophytes *Myriophyllum spicatum* and *Apium nodiflorum* were exposed to diuron for 14-days in a study conducted by Lambert et al. (2006). *A. nodiflorum* showed high sensitivity with regards to root growth with an EC_{50} of 2.6×10^{-4} µg/L. Relative growth rate of *M. spicatum* was also quite sensitive to diuron with a NOEC of 5.0×10^{-4} µg/L and and EC_{50} of 5 µg/L. An

interesting phenomenon related to macrophyte toxicity is something known as the "diuron effect" that has been described by Teisseire and Vernet (2000). This effect involves diuron increasing antioxdative enzyme activity that can protect the organism from oxidative stress from copper contamination (Teisseire and Vernet, 2000). *Lemna minor* exposed to diuron showed slight stimulation of pyrogalol peroxidase, glutathione reductase, glutathione S-reductase, and guaiacol peroxidase enzyme activity. Although this enzyme stimulation cannot be directly attributed to the antioxidative defense against copper, it still presents an interesting interaction worthwhile of further study. Other than these few studies on macrophtye toxicity, no other toxicity assessments of diuron has been conducted on aquatic macrophytes.

1.6.2 Atrazine

Atrazine (6-chloro-N^2-ethyl-N^4-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine) is a triazine herbicide applied pre-emergence for the treatment of broadleaf weeds with some grassy weeds (PMRA, 2007). A list of the physical and chemical properties of atrazine is found in the Appendix 2. Atrazine is mainly used in North America for the protection of a variety of corn crops (PMRA, 2007). Triazine herbicides are photosystem II inhibitors (Fuerst and Norman, 1991). Within photosystem II, atrazine binds to the Q_B binding niche on the D1 protein via hydrogen bonds, Van Der Waals forces, and hydrophobic interactions (Fuerst and Norman, 1991). By acting as an analog of plastoquinone, atrazine blocks electron flow to the D1 reaction center. This blockage of electron flow leads to a cascade of reactions leading to the formation of singlet oxygen that cause lipid peroxidation, the main cause of phytotoxicity (Fuerst and Norman, 1991). Atrazine, along with related triazine herbicides, are among the top ten most used

herbicides in Canada representing 5.8 x 10^5 kg active ingredient/year, as determined from provincial pesticide usage data from 1998 – 2003 (Brimble et al., 2005). In the United States, annual usage is estimated at 3.5 x 10^7 kg active ingredient/year (From data collected from 1990 – 1997) (USEPA, 2003a).

Environmental concentrations of atrazine in surface water have been reported in various sources. In rivers and streams, atrazine has most commonly been found in the range of 5 – 20 µg/L with concentrations of 0.4 – 5 µg/L found in lakes and reservoirs, from data collected in the Midwestern United States (Solomon et al., 1996). Data collected in Canada in the provinces of Ontario and Quebec, two provinces that use the most atrazine, showed average surface water concentrations of 0.02 – 5.75 µg/L, with highest occurrences of < 0.1 – 2.0 µg/L (PMRA, 2007).

Atrazine is present in the aquatic environment with solubility of 33 mg/L but has a low potential of bioaccumulation with a log K_{ow} of 2.7 (Solomon et al., 1996, PMRA, 2007). Atrazine is moderately/highly mobile in soil with a K_{oc} ranging from 25 – 155, indicating its potential to runoff into adjacent water bodies. Phototransformation or hydrolysis are not important modes of transformation in aquatic environments since atrazine is stable at pH 5, 7 and 9 and has a photolysis half life of 335 days under natural light (Solomon et al., 1996, PMRA, 2007). Microbial degradation is the primary form of degradation in both soil and aquatic environments (PMRA, 2007). In laboratory studies, biodegradation half-lives of atrazine range from 77 – 159 days in anaerobic soil and 40 – 115 days in aerobic soil conditions, indicating moderate persistence in soil (PMRA, 2007). Laboratory biodegradation studies in aquatic systems showed atrazine half-lives of 80 - > 400 days in aerobic aquatic conditions and 330 – 608 days in anaerobic aquatic

conditions (PMRA, 2007). Field studies have also characterized the fate of atrazine in the aquatic environment. Artificial lake experiments have shown atrazine to be moderately persistent with a half-life of 150 days (PMRA, 2007).

Various water quality guidelines exist for atrazine in North America. In Canada, a guideline for the protection of aquatic life is 1.8 µg/L, while a guideline for protection of agricultural waters is 10 µg/L for irrigation water and 5 µg/L for livestock water (Kegley et al., 2008). A maximum contaminant level (MCL) of 3.0 µg/L has been set in the United States for drinking water (Kegley et al., 2008).

The toxicity of atrazine to aquatic macrophytes has been described in both laboratory and field conditions. In a laboratory study conducted by Fairchild et al. (1998), atrazine was more toxic to aquatic macrophytes than alachlor and metolachlor over a 14-d exposure period. Macrophyte species showed high sensitivity to atrazine with EC_{50}s of 22 µg/L (*Ceratophyllum demersum*), 24 µg/L (*Najas* spp.), 92 µg/L (*Lemna minor*), 21 µg/L (*Elodea canadensis*), and 132 µg/L (*Myriophyllum heterophyllum*). Kemp et al. (1985) found showed similar atrazine toxicity to macrophytes over a 28-d exposure period. It was found that IC_{50} values for *Potamogeton perfoliatus* and *Myriophyllum spicatum* were 55 and 117 µg/L respectively, for total photosynthesis (based on O_2 measurements) with IC_{50}s of 30 and 91 µg/L respectively for final biomass. These results indicate a higher sensitivity of atrazine to *Potamogeton* spp. and *Elodea* spp. with lower sensitivity to *Myriophyllum* spp. Toxicity of atrazine under field conditions to aquatic macrophytes has also been described. In a 42-d outdoor microcosm exposure of atrazine to *Elodea canadensis* and *Myriophyllum spicatum* populations, EC_{50}s ranged from approximately 5 – 60 µg/L and 25 – 130 µg/L

respectively. Individual plants were also tested in this study, but showed lower sensitivity compared to plants grown in populations. Field data suggests, much like laboratory data, that *Myriophyllum* spp. show lower sensitivity to atrazine than other species.

1.7 CHARACTERISTICS OF A GOOD BIOASSAY

Rand et al. (1995) identified some common characteristics that make bioassays efficient and able to generate useful data for risk assessment. Four of the most paramount characteristics are that the test should be repeatable and statistically sound, include ecologically relevant endpoints measured under realistic exposure durations, be sensitive enough to detect effects, and be predictive of results observed in the field.

1.7.1 Repeatability and statistically sound methods

Repeatability is the ability of a test to be conducted by a variety of laboratories or personnel achieving similar results with similar statistical precision (Rand et al., 1995). Standard methods are an effective way of achieving repeatability. As previously described, some standardized methods exist for aquatic macrophyte testing, published by a variety of institutions, both governmental and private. Unfortunately, although some studies have followed these methodologies for rooted submersed macrophytes (Hanson and Solomon, 2004; Roshon et al., 1999), many other studies do not follow any standard guidelines, relying instead on ad hoc approaches (Cedergreen et al., 2004a; Fairchild et al., 1998; Feiler et al., 2004; Belgers et al., 2007), leading to inconsistency in literature. If a consistent and replicable body of comparable toxicity data for aquatic macrophytes is to be developed for use in risk assessment applications, consistency in methods is vital.

Quantifying variability in bioassays is also very important in order to make the tests statistically sound. This allows for the assay to be statistically sensitive as well as toxicologically sensitive. One useful statistical parameter for comparing the statistical resolution of endpoints both within a study and between different studies is the coefficient of variation (CV) (Hanson et al., 2003). The CV is a standard measure of variation in bioassays allowing for easy comparison between endpoints regardless of their magnitude of response (Sokal and Rohlf, 1995). CV is calculated by dividing the sample standard deviation by the mean of the specific endpoint and multiplied by 100 to express it as a percent (Sokal and Rohlf, 1995). Another commonly used measure of variation in macrophyte tests is minimum detectable difference (MDD). This parameter is the minimum amount of change in control organisms of a particular endpoint that must occur for effects to be considered significant (Sokal and Rohlf, 1995). The minimum significant difference (MSD) is similar to the MDD, except it calculates a percent change from control for a specific standard error derived from an ANOVA analysis of a particular endpoint (Sokal and Rohlf, 1995). With CV, MSD and MDD, a low value indicates a small change from controls in order to detect ecologically significant effects for that endpoint. Typically, a macrophyte bioassay should detect changes of $\geq$ 20% change in plant biometrics from controls to be considered ecologically significant and this value is based on observed impacts on plant species (Christman et al., 1994).

These measures of variation have been documented in a variety of macrophyte studies and have shown the high statistical resolution that can be achieved with these tests. In the laboratory, Roshon et al. (1999) found CVs ranging from 8% – 78% for *M. sibiricum* exposed to various herbicides for endpoints of shoot growth, root dry mass, and

root number. Of the three endpoints, root dry mass showed the consistently lowest CVs ranging from 8% – 55%. A laboratory study conducted by Knauer et al. (2006) evaluated the potential for non-axenic shoots of *E. canadensis* and *M. spicatum* to be used in a toxicity test. Both species were cultured in small test chambers in M4 media with various endpoints being measured to determine statistical sensitivity. Endpoints included root number, total plant length, root length, length of side shoots, and fresh weight. Total plant length was the endpoint that showed the lowest variation for both species with CVs ranging from 3 – 20%. Laboratory assessments of haloacetic acid toxicity to macrophtyes conducted by Hanson and Solomon (2004) found lowest CVs, MSDs, and MDDs for plant length and node number for *M. sibiricum* and *M. spicatum*, ranging from 6 – 8% for CV, 9 – 11% for MSDs, and 11 – 15% for MDDs. Toxicity assessments for *L. gibba* were also conducted which found frond growth rate and chlorophyll-a having the lowest CVs, MSDs, and MDDs, ranging from 5 – 7% for CVs, 11 – 13% for MSDs, and 10 – 13% for MDDs (Hanson and Solomon, 2004). Laboratory exposures of linuron to *E. canadensis* conducted by Snel et al. (1998) used biomass and photosynthetic efficiency endpoints, with photosynthetic efficiency CVs ranging from 1 – 2% and a biomass CV of 13%. MDDs for photosynthetic efficiency and biomass were <1 – 7% and 40%, respectively.

Variation in macrophyte endpoints in the field has also been described. Hanson et al. (2003) analyzed data from four separate outdoor microcosm studies that exposed *M. sibiricum* and *M. spicatum* to a variety of haloacetic acids. Lowest CVs were observed for node number and plant length for both species, with CVs ranging from 1 – 16% for node number and plant length ranging from 3 – 27%. These same endpoints also showed

lowest MDDs for both species with node number ranging from 7 – 25% and plant length ranging from 13 – 49%. These results were comparable with those described by Brain et al. (2005) for *M. spicatum* exposed to the pharmaceutical tylosin in outdoor microcosms. Node number and plant length showed CVs ranging from 4 – 9 % and 5 – 15% respectively with root number and pigment endpoints (chlorophyll-a, chlorophyll-b, and carotenoids) also showing low variation (2 – 12% for both endpoints). Lowest MSD and MDDs were observed for node number and caroteniod pigments ranging from 17 – 43% (MSD) and 9 – 21% (MDD) for node number and 12 – 36% (MSD) and 6 – 25% (MDD). *Lemna gibba* was also used in this study and showed very low variation in the growth rate endpoint with a CV of 4%, an MDD of 7% and an MSD of 2%.

All of these results describe the utility of macrophyte tests with regards to their statistical sensitivity. Both in laboratory and field settings macrophytes have the ability of producing precise toxicity data for a variety of compounds. Consistent results in multiple studies (ie. Hanson et al. 2003 and Brain et al., 2004) show that these tests can be repeatable and still yield high statistical resolution for different compounds.

1.7.2 High sensitivity to detect effects

The ability of a bioassay to have high toxicological sensitivity is a very important factor of any toxicity test and has been under great debate. Currently, *Lemna* is the only aquatic macrophyte required for the registration of new chemicals by many nations including the USA, Canada, and the EU (Davy et al., 2001). This has been greatly criticized since *Lemna*, or any one species, cannot be representative of the great diversity of macrophyte species that occur naturally in the environment (Davy et al., 2001; Wang and Freemark, 1995). Greater sensitivity of other macrophytes compared to *Lemna* spp.

has been described in literature. For example, in an interspecies comparison of toxicity of sulfonylurea and imazolidinone herbicides, Roshon et al., (1999) reported that rooted macrophytes often showed higher sensitivity than floating macrophytes such as *Lemna* spp. Also, Fairchild et al., (1998) showed three other species of aquatic macrophytes (*Ceratophyllum sp.*, *Najas sp.*, and *Elodea sp.*) to be more sensitive to four herbicides than *L. minor*. Cedergreen et al. (2004a, 2004b) also found *Batrachium trichophyllum* to be more sensitive to metsulfuron methyl and *Ceratophyllum submersum* being more sensitive to terbutylazine than *L. minor* and *L. trisulca.* An evaluation of macrophtye toxicity data by Vervliet-Scheebaum et al. (2006) found the rooted submersed macrophytes *M. spicatum* and *E. canadensis* to be more sensitive then *L. minor* for compounds whose mode of action was to target amino acid synthesis and auxin simulators.

In spite of the concern with using only *Lemna* in current testing methods, certain evidence proves its utility as a generally sensitive species for a variety of compounds. An investigation of macrophyte toxicity data conducted by Rentz and Hanson (2009) found when *Lemna* laboratory toxicity data were converted to a 14-d duration from their typical 7-d duration and compared to laboratory toxicity data of other rooted submersed macrophytes (14-d exposure) that *Lemna* data were within one order of magnitude of other macrophyte toxicity data. Therefore, if either a 10-fold uncertainty factor is applied or if the duration of *Lemna* tests is increased to 14-d, *Lemna* are capable of capturing sensitivities of these other macrophytes. *Lemna* laboratory data were also compared to field data of other macrophytes and a similar trend was observed. Although other macrophyte data in the field showed high sensitivity, applying a 10-fold uncertainty

factor to *Lemna* laboratory data would capture sensitivity of other macrophytes in the field. This investigation provides compelling evidence that *Lemna* could potentially continue being used in risk assessment without the need for the use of other species.

Past methods of toxicity assessment have also used algae as surrogates for higher vascular plants. This needs to be conducted with caution, since differences in sensitivity between algae and vascular plants are highly variable (Fletcher, 1990). Fletcher (1990) investigated toxicity data of algae and vascular plants from exposures to over 1500 different chemicals. It was found that for many compounds (~500), responses were unique to vascular plants and may not even elicit effects in algal species. From compounds that showed responses in both algae and vascular plants, there was no clear trend in toxicity. In some compounds, vascular plants were more sensitive while in others algae were more sensitive. This evidence supports the need for more aquatic macrophyte testing in risk assessment.

Various exposure routes also must be considered with regards to the sensitivity of the tests. Macrophytes that are rooted in sediment such as *Myriophyllum* and *Elodea* species can absorb compounds via the sediment, whereas a floating macrophyte like *Lemna* cannot. This issue was addressed in a study conducted by Feiler et al. (2004). *M. aquaticum* was grown in natural contaminated sediment and *L. minor* in the pore water of that same sediment. It was found that levels of growth inhibition in test matrices were higher for *M. aquaticum* compared to *L. minor*. This indicates possible differential sensitivity between the two species due to different exposure routes.

Along with the factor of species selection, the inherent growth rate of the species selected for a test that can influence toxicological sensitivity. For a test to be as rapid as

possible, strong and rapid development of the macrophyte is ideal. One of the reasons *Lemna* is so prevalent in toxicity testing is its very rapid growth rate compared to other macrophytes (Taraldsen and Norberg-King, 1990). Aside from leading to a rapid test, higher relative growth rates (RGR) have been shown to lead to greater chemical sensitivity. Cedergreen et al. (2005b) exposed both rooted submersed and floating macrophytes to metsulfuron-methyl and described a negative correlation between EC_{50} and RGR. This indicated that plants that grew faster were more sensitive to metsulfuron-methyl. This was described by Huebert and Shay (1993) with *Lemna* species as well. This study found that duckweed with a faster doubling time required a lower concentration of the toxicant to reach EC_{50} compared to duckweed with slower doubling times for a given time period. Since RGR is known to influence toxicity, keeping RGRs high in bioassays is very important in order to capture highest sensitivity. In laboratory tests, a major driver of this is the choice of growth media. Various growth media are used in macrophyte testing including Modified Andrew's (ASTM, 2005b), AAP (OECD, 2002), Hoagland's Solution (ASTM, 2005c), Hutner's (Greenberg et al., 1992), a general purpose media by Smart and Barko (1985) and M4 media (Knauer et al., 2006), M-Hoagland's (USEPA, 1996; ASTM, 2005a), AAP 20X (USEPA, 1996; ASTM, 2005a), Hogland's E+ (ASTM, 2005a; Environment Canada, 2007), and Swedish Standard Media (SIS) (OECD, 2002). With so many different media to use for macrophyte testing, it is important to determine which one will provide the strongest macrophyte development for a particular species, and therefore provide the greatest sensitivity. Unfortunately, very few studies have been conducted that investigate various media for one species to determine differential growth in each media. Roshon et al. (1996) conducted a

comparison of growth media for the macrophyte *Myriophyllum sibiricum*. This study used a general macrophyte media formulated by Murashige and Skoog (1962), Hogland's, Gaudet's, Modified Andrew's and Hard Water media and measured area under the growth curve, plant length, root number, root length, wet weight, chlorophyll a and b, carotenoids, membrane permeability and plant area as endpoints. At the end of the 14-d growth period, they deemed Hoagland's and Modified Andrew's media to be the most suitable for growth of this macrophyte, with Modified Andrew's showing the lowest variability (Roshon et al., 1996). Others have observed successful growth of *Elodea canadenesis* in the laboratory (Knauer et al. 2006). In this case, non-axenic segments were grown in M4 media over a 21-day period showing no visual bacterial or algal contamination. The plant length endpoint for both species showed greatest increases throughout the test (70 – 75% increases) with low CVs (3 – 14%). Wet mass showed greater variation than length endpoints with CVs of 19 – 39%. The test also measured the influence of the presence of sediment on growth. It was found that plant length was 2 -3 times longer in sediment test chambers compared to test chambers without sediment, with CVs for plant length of 8 – 19% (Knauer et al., 2006). Turgut and Fomin (2001) also conducted a comparison of media types on the growth of *Myriophyllum aquaticum*. Hoagland's and Andrew's media were compared in this study and Hoagland's media was found to promote strongest growth for most endpoints measured including shoot and root length, chlorophyll-a, chlorophyll-b, and carotenoids (Turgut and Fomin, 2001). Other than these few studies, no other investigations of media type on growth have been conducted. With the compelling evidence that higher RGR can lead to higher sensitivity,

it is important that media type be evaluated prior to a toxicity test in order to achieve the highest sensitivity possible.

If a complete and accurate description of sensitivity to a chemical is to be obtained, it is important to consider all of these factors prior to a test. Relying on a single species may not show the greatest sensitivity for the chemical of concern, which is why new methods encourage the use of more than one species (Davy et al., 2001). Also, it is important to consider growth rate, and how it can be influenced by growth media type. Since higher RGRs have been shown to lead to greater sensitivity, a media and test species that will lead to the most rapid RGR is ideal.

1.7.3 Ecologically relevant endpoints and realistic exposure durations

Aside from endpoints being sufficiently sensitive to detect effects, they also must be ecologically relevant. Endpoints used in macrophyte testing methods are numerous and related to a variety of developmental and physiological parameters (Vervliet-Scheebaum, 2006). One of the most common endpoints currently used is biomass for both wet and dry mass of root and shoot. This is a simple endpoint to measure and has been considered ecologically relevant since it is an estimation of plant productivity (Davy et al., 2001; Wetzel, 2001). Other common endpoints include plant length, root number, root length, and pigment endpoints including chlorophyll-a content, chlorophyll-b content, and carotenoid content (Hanson et al., 2003; Brain et al., 2005; McGregor et al., 2007; Cedergreen et al., 2004b). Other endpoints that measure biochemical processes within a plant have been recommended, since they are the first to respond, even before growth-based endpoints (Davy et al., 2001). These include oxygen evolution, carbohydrate levels, enzyme activity, and chlorophyll fluorescence (Davy et al., 2001).

These have been used in toxicity tests, but to a much lesser extent than other endpoints (Teisseire and Vernet, 2000; Teisseire et al., 1999; Snel et al., 1998; Marwood et al., 2001). With such a wide selection of endpoints to choose for a particular test, it can be difficult to choose endpoints that are both ecologically relevant, economical and simple to measure. Although biomass and length endpoints are very easy to measure, it has been suggested that a suite of endpoints should be used in a test, due to the variation in both statistical and toxicological sensitivity of these endpoints (Hanson and Solomon, 2002; Arts et al., 2008). In doing this, a variety of plant parameters are considered and various levels of sensitivity can be captured.

Test durations are another aspect of testing that is highly variable amongst tests and depend on the initial goals of the test. For acute laboratory tests, which are very common for macrophytes, current methods usually range from 7 – 14-d for laboratory tests (ASTM, 2005a,b; Greenberg et al., 1992; Hanson and Solomon, 2004), which is enough time to elicit effects in most cases (Rand et al., 1995). In other studies that assess the toxicity or fate of a compound or its breakdown products, a test may have to take place over a longer duration, to last through the compounds life cycle in the test environment (Lewis, 1995a), thus requiring a chronic exposure. This occurs commonly in field based microcosm test which can range from 7 - > 42-d in duration (i.e., McGregor et al., 2007; Hanson et al., 2005; Brain et al., 2005). The life cycle of the test organism plays a role in the test duration as well. With chronic toxicity tests, an entire growth cycle of a macrophyte can potentially be exposed to a chemical (Rand et al., 1995). Test duration in both acute and chronic tests must be carefully considered since with increasing duration, test organism sensitivity to the chemical can decrease (Lewis,

1995b). This is likely due to the degradation of the compound with time or the partitioning into other compartments in the environment, which is a process that has been described elsewhere (McGregor et al., 2007; Rice et al., 2004). Therefore, for the test duration to be relevant many factors must be considered and the decision on test duration will depend on the data requirements for the specific test.

1.7.4 Predictive of results found in the field

A major component of the utility of laboratory toxicity tests is their ability to predict effects in the field. With the difference of complexity between the laboratory and the field, it is necessary that a predictive link be found between these two unique test systems so that simple laboratory tests may be used to extrapolate effects at the field level (Hanson and Arts, 2007). This could lead to much more efficient risk assessment methods and a way to validate laboratory tests. With this vast difference in tests systems, it is difficult to make extrapolations between them, simply due to much unpredictability and variation (Rand et al., 1995). In spite of this, it is crucial that these linkages are found since field studies more accurately represent environmental conditions. If this can be done, laboratory tests, which are simple, controllable, and inexpensive may accurately predict the sensitivity found in field conditions. Currently guidelines on the extrapolation of single-species laboratory tests to population and community studies in the field have yet to be developed (Hanson and Arts, 2007). In spite of this, some work has been done to compare laboratory and field results to determine if any relationships can be seen. Hanson et al. (2003) compared the statistical sensitivity of various endpoints measured for *M. sibiricum* in the laboratory and the field and found that CVs for the field and laboratory were not significantly different. This indicates promise in the two systems

maintaining consistent levels of variability for macrophyte tests. Knauer et al., (2008) conducted growth studies of *Myriophyllum* species in the laboratory and the field and found that RGRs were comparable between the two test systems with low CVs for both test systems. Rentz and Hanson (2009) conducted an analysis of *Lemna* toxicity data from the laboratory and under field conditions. They found that, for a variety of compounds, *Lemna* laboratory data were within one order of magnitude of *Lemna* field data as well as other macrophyte field data. In some cases field data were more sensitive compared to laboratory data, but if a 10-fold uncertainty factor is applied, *Lemna* laboratory data can capture much of the sensitivity found in field conditions. These studies give convincing evidence that laboratory studies can predict field effects and that the two test systems can have comparable levels of variation. In order to establish a clearer connection between the laboratory and field, parallel toxicity tests in both laboratory and field should be conducted with a variety of species to determine if these linkages can be clearly established.

1.8 THE IMPORTANCE OF MICROCOSM STUDIES

Current standard methods focus on individual organism laboratory based tests and risk assessment procedures, but the use of field studies can reveal more than simple laboratory studies and have been used more frequently. Many outdoor microcosm studies have been conducted using aquatic macrophytes (Hanson et al., 2003; Hanson et al., 2005; Brain et al., 2005; McGregor et al., 2007) with emphasis on the study of herbicide, pharmaceutical, and haloacetic acid effects on these plants. According to Rand et al. (1995), an outdoor microcosm is a fabricated tank that is generally 2000 – 15000L in volume that are more representative of natural conditions compared to laboratory

conditions. These large test chambers are self-contained environments that remain under a great amount of control by the experimenter, but are still representative of actual field conditions. This level of control allows for replication of the test systems as well as monitoring the system before and after the additions of contaminants (Rand et al., 1995). With their large size relative to laboratory tests, they can contain population and community levels of organization with a variety of organisms simultaneously and can monitor relationships and interactions at these levels of biological organization (Rand et al., 1995). With higher levels of biological organization existing in these test systems, indirect effects of contaminants can be monitored, which is something that cannot be done with laboratory tests. These indirect effects are those that occur as a result of direct effect on organisms in the test system (Rand et al., 1995; Fleeger et al., 2003). For example, if a contaminant leads to the direct effect of mortality of invertebrate populations, the indirect effect of a declining macrophyte population may occur since competing algae species proliferate in the absence of invertebrates. These complex levels of interaction are important to consider in toxicity assessments since they are the types of assemblages that occur in the environment. Unfortunately, very few studies with macrophytes have been conducted at the population or community level. McGregor et al. (2007, 2008) used *M. spicatum* and *E. canadensis* in model population and community assemblages as well as individuals to assess toxicity of the pharmaceutical monensin and the herbicide atrazine. The study found no significant differences in sensitivity between planting systems, but in one study, found that RGR was significantly higher for model populations compared to individuals. Other than these studies, little has been done in microcosm studies to specifically investigate higher levels of biological organization with

regards to toxicity in macrophytes. It is important to consider these assemblages in toxicity tests since they represent natural assemblages in nature. Although these systems simulate natural ecosystems, they also have some drawbacks to their use. One drawback is they cannot be used for fish studies with larger fish studies since they are restrictive in their size. Also, their initial construction and maintenance can be expensive. Finally, although they are considered fairly representative of certain freshwater ecosystems in their macrophyte communities (Williams et al., 2002), they still are not natural systems, and this needs to be considered in data interpretation.

1.9 RESEARCH OBJECTIVES

In order to address some of the concerns with the deficiencies in current methodologies, this study ventured to assess the toxicity of a commonly tested macrophyte, *E. canadensis*, a species that has no established test methods for it. Toxicity was assessed in the laboratory and the field, considering various planting types in the field studies. The specific objectives of this study were:

1) Determine which commonly used plant growth media promotes the strongest development in *Elodea canadensis* under controlled laboratory conditions and determine any influence of supplemental nitrogen and phosphorus and the presence and absence of root substrate in the test systems on growth.
2) Assess the effects of two herbicides (atrazine and diuron) on the aquatic macrophyte *Elodea canadensis* grown in the growth media that promoted strongest growth and assess the statistical sensitivity, or variation, of the measured endpoints.

3) Compare results to those of a parallel assay of *Lemna gibba* and *Lemna minor* to determine relative sensitivities of these plants. Obtaining comparative sensitivities to *Lemna* is crucial since they represent the only species that are required for toxicity testing in pesticide registration.

4) Determine the relationship between relative growth rate (RGR) and toxicological sensitivity, specifically within newly developing populations of different densities, individual plants, and between established and non-established stands and determine if these planting designs themselves influence toxicity.

5) Assess the toxicity of diuron to *E. canadensis*, *L. gibba*, and *M. spicatum* under field conditions.

6) Compare toxicity results from the laboratory to microcosm studies using atrazine and diuron to determine if laboratory results can predict toxicity in field conditions.

1.10 HYPOTHESES

1) Modified Andrew's media will provide strongest growth. This was observed by Roshon et al. (1996) with *Myriophyllum sibiricum*. *E. canadensis* is considered co-dominant with and ecologically similar to *Myriophyllum* species in aquatic systems (Spicer and Catling, 1988; Abernethy et al., 1996) and could therefore respond in a similar fashion.

2) Atrazine and diuron will both show a toxicological response in *E. canadensis*, since they are both photosynthetic inhibitors (Ware and Whitacre, 2004; Solomon et al., 1996). Diuron will show greater sensitivity than atrazine. An EC_{50} (wet mass) for atrazine exposure to *Myriophyllum* was 132 µg/L compared to 5 µg/L

(RGR) for diuron found by Lambert et al. (2006). Since *Myriophyllum* is ecologically similar to *E. canadensis* (Spicer and Catling, 1988; Abernethy et al., 1996) it may have similar sensitivity. No toxicity data for *E. canadensis* exposed to diuron were available. Laboratory assays under controlled conditions will show low variation. Hanson and Solomon (2004) conducted laboratory assays with *Myriophyllum* species, a submersed macrophyte common to *E. canadensis* (Spicer and Catling, 1988; Abernethy et al., 1996) and found very low CVs (4 - 20% of control) for most endpoints measured. Therefore, *E. canadensis* may exhibit similar variation.

3) *Lemna* species will show greater sensitivity than *E. canadensis* due to their rapid growth rate and high capacity for assimilation and bioconcentration of chemicals (Korner and Vermaat, 1998; Greenberg et al., 1992). Higher growth rates can also allow test systems to detect toxicity at lower concentrations (Cedergreen et al., 2004b; Huebert and Shay, 1993).
4) Plants exhibiting higher RGRs will be more sensitive. Therefore, non-established populations, which are still in the exponential growth phase (Forbes et al., 2001) and low density populations that have less intraspecific competition (Agami and Reddy, 1990) will both have higher RGRs and therefore be less sensitive. Again, higher RGRs allow for detection of toxicity at low concentrations, thus exhibiting higher sensitivity (Cedergreen et al., 2004b; Huebert and Shay, 1993).
5) *M. spicatum* will be the most sensitive to diuron followed by *E. canadensis* then *L. gibba.* Teisseire et al. (1999) found an EC_{50} for RGR from frond number of 25 µg/L for *L. minor* while Lambert et al. (2006) found and EC_{50} for relative growth

rate of 5 µg/L for *M. spicatum*. Therefore, it is predicted that *M. spicatum* will be more sensitive than *Lemna* species. Also, since *Myriophyllum* species are similar to *E. canadensis* in their morphology (Spicer and Catling, 1988; Abernethy et al., 1996), *E. canadensis* will exhibit similar sensitivity to *M. spicatum*. No diuron toxicity data were available for *E. canadensis*, therefore, previous toxicity data for this macrophyte could inform this hypothesis.

6) Laboratory toxicity will show greater toxicological sensitivity compared toxicity observed in the field for *E. canadensis*. Fairchild et al. (1998) reported a wet mass EC_{50} for *E. canadensis* of 21 µg/L after 14-d of exposure to atrazine from a controlled laboratory study. McGregor et al. (2008) reported a wet mass EC_{50} for *E. canadensis* exposed to atrazine of 23.2 µg/L in the field. Although these two toxicities are quite similar, incorporating the large variation in field studies, laboratory conditions will provide the highest sensitivity.

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2 A TOXICITY BIOASSAY FOR THE FRESHWATER AQUATIC MACROPHYTE *ELODEA CANADENSIS* MICHX

2.1 ABSTRACT

Duckweed, (*Lemna* spp.) is currently the only macrophyte test required for the registration of plant protection products and other pesticides within the current regulatory risk assessment framework, despite concerns that it is not fully representative of other macrophyte species that have sediment exposure routes and are dicotyledons. Hence, there have been repeated calls for the formal evaluation of different plant species to complement the *Lemna* spp. test as required. To this end, we conducted a study to evaluate the potential of the submersed rooted macrophyte *Elodea canadensis* to be used in a non-axenic laboratory toxicity assay. The aims of the study were to determine the growth media that promotes the strongest development of *E. canadensis* in the laboratory; use that media to conduct toxicity tests with two commonly used herbicides, diuron and atrazine; and compare those results with *Lemna gibba* and *L. minor* for the same compounds. AAP media was found to promote strongest development across many standard endpoints as well as to restrict the amount of algal contamination present in test chambers. Sensitivities to diuron were *E. canadensis* > *L. minor* > *L. gibba* and for atrazine were *L. gibba* > *L. minor* > *E. canadensis*. Overall, tests with *E. canadensis* exhibited high variability and low statistical sensitivity indicating further refinement of the approach is required. Regardless, *E. canadensis* exhibited greater toxicological

sensitivity to diuron and this indicates its possible utility as an additional test species when concerns about the data from *Lemna* spp. alone arise.

2.2 INTRODUCTION

Aquatic plants represent a valued ecological component due to their ability to produce oxygen, assist in nutrient cycling, provide food for organisms such as invertebrates (D'agaro et al., 2004) and waterfowl (Schmieder et al., 2006), improve water quality by assimilating heavy metals (Gouder de Beauregard and Mahy, 2002), stabilize sediments with their root structure (Wang and Freemark, 1995), and quite simply, through their role as primary producers (Lewis, 1995). In order to sustain these functions and the structure of the plant communities that perform them, a variety of test methods for assessing the toxicity of various environmental contaminants on aquatic macrophytes have been reported. Still, formalized and accepted standard methods have only been developed for *Lemna* spp., a floating macrophyte and *Myriophyllum sibiricum* (which is also applicable to *M. spicatum*), a submersed rooted macrophyte. For *Lemna* spp., numerous standardized protocols have been developed by Environment Canada (2007), the American Society for Testing and Materials (ASTM) (2005a), the Organization for Economic Co-operation and Development (OECD) (2002), and the United States Environment Protection Agency (USEPA) (1996), the European and Mediterranean Plant Protection Organization (EMPPO) (2003), along with other, less formal methods (Greenberg et al., 1993). All of these methods remain consistent in their core methodology; they prescribe a 7-day exposure period within Erlenmeyer flasks or plastic Petri dishes with a set number of plants (3-5 plants with 3-4 fronds each) and fronds in each exposure, incubated at 25°C, illuminated at a range of 57 – 135 μmol m^{-2}

s^{-1}. Various growth media are recommended for use in these *Lemna* spp. tests including M-Hoagland's (USEPA, 1996; ASTM, 2005a), AAP 20X (USEPA, 1996; ASTM, 2005a), Hogland's E+ (ASTM, 2005a; Environment Canada, 2007, Acreman, 2001), and Swedish Standard Media (SIS) (OECD, 2002) with replacement of the media occurring at day 3 or 5 if a static renewal method is implemented. In regards to other available test species methodologies, only a method for the rooted submersed macrophyte *Myriophyllum sibiricum* has been formalized (ASTM, 2005b). This test places a small apical segment of the macrophyte in a borosilicate glass tube with a small amount of root substrate (Turface or sand) with modified Andrew's growth media over a 14-d period. Illumination ranges between 100 to 150 μmol $m^{-2} s^{-1}$ with a 16:8h light/dark cycle with temperature maintained at 25/20°C light/dark. For both rooted and floating species, these tests typically require a minimum of five tests concentrations and a control in order to plot concentration-response relationships from which IC_x and EC_x values can be obtained.

Although the aforementioned test methods can be efficient, replicable, and statistically and toxicologically sensitive, they do present a variety of possible shortfalls. Perhaps the main concern that is voiced is that one species will never be consistently the most responsive or sensitive to any particular contaminant, whether under laboratory or field conditions. This is of particular concern since *Lemna* spp. is not rooted in sediment, thus eliminating this route of exposure (Breitholtz et al., 2006; Davy et al., 2001). A study conducted by Feiler et al. (2004) grew *M. aquaticum* in natural contaminated sediment and *L. minor* in the pore water of that same sediment. It was found that levels of growth inhibition from contamination in the test matrix in one natural sediment were

higher for *M. aquaticum* compared to *L. minor*. This indicates possible differential sensitivity between the two species due to different exposure routes. Also, since it is a monocotyledon, it may not be representative of dicotyledons as many herbicides are designed to be specific to monocots or dicots depending on the crop and target pest (Hanson et al., 2009; Knauer et al., 2008; Davy et al., 2001). Greater sensitivity of other macrophytes compared to *Lemna* spp. has been described in literature. For example, in an interspecies comparison of toxicity of sulfonylurea and imazolidinone herbicides, Roshon et al., (1999) reported that rooted macrophytes often showed higher sensitivity than floating macrophytes such as *Lemna* spp. Also, Fairchild et al., (1998) showed three other species of aquatic macrophytes (*Ceratophyllum sp.*, *Najas sp.*, and *Elodea sp.*) to be more sensitive to four herbicides than *L. minor*. Cedergreen et al. (2004a, 2004b) also found *Batrachium trichophyllum* to be more sensitive to metsulfuron methyl and *Ceratophyllum submersum* being more sensitive to terbutylazine than *L. minor* and *L. trisulca*. This said, there is some evidence that laboratory *L. gibba* is reasonably representative of both field responses of itself and other plant species with the application of standard uncertainty factors (Rentz and Hanson, 2009).

To begin addressing these aforementioned concerns, we investigated the potential of a commonly distributed submersed, rooted, monocotyledonous macrophyte, *Elodea canadensis* Michx., to be cultured in the laboratory and used in a toxicity bioassay. *E. canadensis* is native to Canada, distributed widely with highest densities in southern British Columbia, southern Manitoba, southeastern Ontario, and southern Quebec; as well it is invasive in Europe, Asia, South Africa, Australia and New Zealand (Spicer and Catling, 1988). It is found most abundantly in the Great Lakes of Canada and the United

States (Spicer and Catling, 1988). It has also been used previously in ecotoxicology studies on the effects of metals on macrophytes (Stoyanova, 1993; Mal et al., 2002), the use of macrophytes for phytoremediation of environmental contaminants (Rice et al., 1997), as well as the effects of herbicides on macrophytes (Fairchild et al., 1998; Cedergreen, 2004a,b; Arts et al., 2008). A study that used endpoint distributions to compare overall sensitivity across many endpoints found *E. canadensis* to be more sensitive than four other submersed macrophytes to pentachlorophenol (Arts et al., 2008). In spite of its wide distribution and use as an organism to measure toxicity, no work to our knowledge has been reported on attempts to standardize test methods for this species.

When developing a new bioassay, many criteria have to be taken into consideration in order to make the test useful for risk assessment. Amongst them, the test must be economical, statistically sound, sufficiently sensitive to detect significant effects, have effects occur in realistic exposure durations and over a range of concentrations, and have the ability to predict effects in a field setting (Rand et al., 1995). Current *Lemna* spp. methods fulfill these criteria well and any new bioassay should perform in a similar fashion or better in order to advocate for its wider adoption. Therefore, the development of a bioassay for *E. canadensis* will be assessed on its ability to meet these general requirements. Ideally, it will require no more specialized equipment than a *Lemna* spp. assay; it should show sufficient growth within a reasonable time frame to detect effects, in this case, 14-d, much like the current ASTM *Myriophyllum* spp., assay; exhibit toxicity that is equally or more toxicologically sensitive than *Lemna* spp, for two model toxicants, the herbicides diuron and atrazine; be statistically sensitive as determined by coefficients of variation, minimum detectable differences (MDD) and minimum significant

differences (MSD) for respective endpoints (Hanson et al., 2003; Hanson and Solomon, 2004); and be able to predict effects with reasonable accuracy those observed in the field for the model herbicides, as evaluated using previously performed microcosm studies (McGregor et al., 2008).

To begin, an appropriate media for strong growth of the plants in the laboratory is vital. Poor growth due to lack of appropriate nutrients or an excess of those nutrients may confound toxicity data (Huebert and Shay, 1993). Also, it has been shown that macrophytes with higher relative growth rates (RGRs) can be more sensitive to contaminants of concern (Cedergreen et al., 2004b, Huebert et al., 1993). Therefore, in order to precisely and accurately capture chemical sensitivity, it is necessary to use media that will provide rapid growth rates and replicable growth of macrophytes (Roshon et al., 1996). A variety of growth media have been used in macrophyte toxicity tests including Modified Andrew's (ASTM, 2005b), AAP (OECD, 2002), Hoagland's Solution (ASTM, 2005c), Hutner's (Greenberg et al., 1992), a general purpose media by Smart and Barko (1985) and M4 media (Knauer et al., 2006). To date, very little has been done to systematically determine which media promotes strongest development for a specific macrophyte. Roshon et al. (1996) conducted a comparison of growth media for the macrophyte *Myriophyllum sibiricum*. This study used a general macrophyte media formulated by Murashige and Skoog (1962), Hogland's, Gaudet's, Modified Andrew's and Hard Water media and measured area under the growth curve, plant length, root number, root length, wet weight, chlorophyll a and b, carotenoids, membrane permeability and plant area as endpoints. At the end of the 14-d growth period, they deemed Hoagland's and Modified Andrew's media to be the most suitable for growth of

this macrophyte, with Modified Andrew's showing the lowest variability (Roshon et al., 1996). Others have observed successful growth of *Elodea canadenesis* in the laboratory (Knauer et al. 2006). In this case, non-axenic segments were grown in M4 media over a 21-day period showing no visual bacterial or algal contamination. The plant length endpoint for both species showed greatest increases throughout the test (70 – 75% increases) with low CVs (3 – 14%). Wet mass showed greater variation than length endpoints with CVs of 19 – 39%. The test also measured the influence of the presence of sediment on growth. It was found that plant length increased by a factor of 2 – 3 when sediment was present with CVs for plant length of 8 – 19% (Knauer et al., 2006). Turgut and Fomin (2001) also conducted a comparison of media types on the growth of *Myriophyllum aquaticum*. Hoagland's and Andrew's media were compared in this study where Hoagland's media was found to promote strongest growth for most endpoints measured including shoot and root length, chlorophyll-a, chlorophyll-b, and carotenoids (Turgut and Fomin, 2001). This study did not attempt to analyze the statistical sensitivity of each endpoint in each media type. Aside from these three previously described studies, little attention has been given to the assessment of the best media type for submerged macrophyte toxicity testing. Also careful consideration must be taken in the addition of supplemental nitrogen, phosphorus, and sugars which are components included in most growth media (ASTM, 2005b; OECD, 2002; ASTM, 2005c; Greenberg et al., 1992). It is these components that are the excess nutrient sources for microbial and algae contaminants (Knauer et al., 2006). They can be omitted or reduced within the media formulations and strong development of macrophytes may still occur as long as all other macronutrients are present, as has been describe by Smart and Barko (1985), this

will reduce the need to make the assay fully axenic. We hypothesize that by selecting for the media with the strongest growth of *E. canadensis*, it will aid in the achievement of the other criteria around the development of a useful bioassay.

With these criteria for an effective bioassay in mind, we attempted to 1) determine which commonly used plant growth media promotes the strongest development in the aquatic macrophyte *Elodea canadensis* under controlled laboratory conditions; 2) assess the effects of two common herbicides (atrazine and diuron) on *E. canadensis* cultured in the media that promoted strongest growth, assess the statistical sensitivity, or variation, of endpoints measured, and investigate any influence supplemental nitrogen and phosphorus as well as the presence and absence of root substrate on the toxicological response, 3) compare toxicity results to those of a parallel assay of *L. gibba* and *L. minor* to determine relative sensitivities of these plants and 4) compare these data with diuron and atrazine microcosm studies using *E. canadensis*.

2.3 MATERIALS AND METHODS

2.3.1 Growth media evaluated

Culturing media selected for evaluation in this study have all been used in previous aquatic macrophyte tests and included modified Andrew's Media (And) (ASTM, 2005b), AAP (OECD, 2002), Hoagland's Solution (Hoag) (Acreman, 2001), Hutner's Media (Hut) (Greenberg et al., 1992), and a general purpose (GP) media formulated by Smart and Barko (1985) and as a 'control', redistilled deionized (DI) water. All nutrient media were prepared as stock solutions with redistilled deionized water with reagents ACS grade or better (Fisher Scientific, Whitby, ON). Media stock solutions were stored at 4°C in the dark. All media recipes are described in detail in

Appendix 4 - 8. *E. canadensis* prefers to grow at a pH range of 6.5-10 (Spicer and Catling, 1988). Therefore, all media formulations were pH adjusted to just below neutrality (pH = 6.5 ± 0.1) to meet these preferential growth conditions during the assay. Any media formulations containing sucrose were prepared without sucrose in order to prevent proliferation of bacteria and algae that may be associated with the field collected *E. canadensis*. For AAP, Modified Andrew's, Hoagland's, and Hutner's media, two formulations were prepared. One formulation included all reagents and the second omitted the reagents containing supplemental nitrogen and phosphorus sources. This was achieved by eliminating the stock solutions that contained nitrogen and phosphorus sources when preparing the growth media. The only deviation from this was for AAP media where a separate stock solution was made for stock solution 1 (Appendix 5), where only magnesium chloride and calcium chloride were included without sodium nitrate. Hoagland's and Hutner's media used to conduct toxicity tests with *Lemna minor* and *gibba,* respectively, were prepared in their standard forms with no pH adjustment (Environment Canada, 2007; USEPA, 1996).

2.3.2 Plant harvesting and preparation

Elodea canadensis shoots were harvested from local streams (Correctional Facility Recreation Area, Guelph, Ontario) as well as from dormant microcosms at the University of Guelph Microcosm Facility (Guelph, Ontario) during the months of July and August 2008. Plants were brought from the field into the laboratory and maintained in glass aquaria filled with redistilled deionized (DI) water at approximately 20°C until they were needed. Once needed plants were rinsed with deionized water three times in order to reduce contamination in testing. Segments 5 cm in length were measured and

cut from the tip of meristematic growth with any branches removed. *Lemna gibba* and *Lemna minor* were obtained from axenically grown laboratory cultures established at the University of Waterloo, Waterloo, ON (*L. gibba*) and Aquatox Consulting (*L. minor*) (Guelph, ON). *Lemna gibba* were cultured in sucrose enriched Hutner's media while *L. minor* were cultured in Hoagland's media. Prior to toxicity testing (approximately 10 days) both species were transferred axenically in a laminar flow hood to sugar free media in 2800 mL Erlenmeyer flasks so that both species are autotrophic and cultured on the laboratory bench at 20°C at under cool florescent lamps (approximate fluence rate of 20-400 μmol $m^{-2}s^{-1}$).

2.3.3 Growth media test conditions

Sterilized borosillicate glass test tubes (150 mm x 23 mm (internal), 50mL volume) were used as test chambers for the growth media test. Each tube was filled with ~15 g (approximately 3 cm depth at bottom of tube) of autoclaved inert sand as a root substrate and 45 mL of growth media. These exposure chambers were selected because they occupy a small space and use a relatively low volume of media. This allowed for more efficient allocation of space within the controlled environmental chamber, as compared to other studies like Knauer et al. (2006) that used larger 1 L glass beakers as test chambers and a greater volume of waste materials. Root substrate was play sand (Spectrum Brands, Brantford, ON) sieved through a 2 mm aperture (mesh #10) sieve (Fisher Scientific, Ottawa, ON) to remove coarse particles, then subsequently sieved through a 1mm aperture (mesh #20) sieve (CCL Instruments, Toronto, ON). Material remaining on top of the 1 mm aperture sieve was used as substrate as it contained no fine particles that would create turbid growth media solutions. This material was rinsed in DI

water several times to ensure all fine particles were removed. Evaluations of each media were performed four times with root substrate and without root substrate with ten plants (n=10) per test to compare any effects it may have on growth. A 5 cm long apical meristematic shoot, with side branches removed, of *E. canadensis* was placed into each tube and it was sealed with a translucent plastic test tube cap. Plants were gently anchored into root substrate using metal forceps. Tubes were placed onto plastic test tube racks in a random arrangement and placed into a controlled environmental chamber (Conviron, Models E8 and E15, Winnipeg, MB) at a 16/8 h photoperiod at a temperature of 25/20°C with a fluence rate of 100-150 $\mu mol\ m^{-2}\ s^{-1}$ at the base of the test tube rack for a 14-day exposure period. This time period has been shown to be sufficient enough to observe effects (ASTM, 2005b) and keeps the test relatively short in duration, though longer periods may be warranted. Endpoints measured at the end of the growth period were chlorophyll content from the method described by Nusch (1980), wet/dry biomass, root number, root mass, root length, plant length, and relative growth rate (RGR) (based on wet/dry mass). Relative growth rates (RGR) were calculated using the equation presented in (Hoffmann and Poorter, 2002):

$$RGR=(\ln (W_2) - \ln (W_1))/(t_2 - t_1)$$

Where W_2 = final endpoint measurement, W_1 = initial endpoint measurement, T_2 =final timepoint and T_1 = initial timepoint., and plant length. Strongest growth was defined as the growth media that lead to consistently higher RGRs, largest increase in biomass and significant root development. The percent survival was also calculated by dividing the total plants that exhibited a healthy state at the end of the study by the total that were planted at the beginning of the study. Plants considered non-viable clearly exhibited no

new growth or were visually decayed. Prior to commencement of the growth media study, twenty meristematic segments of *E. canadensis* were measured at day-0 for wet/dry biomass in order to obtain a mean baseline measurement. Measurements taken at day-0 were then subtracted from day-14 data in order to obtain a measure of new growth. The assessment of media type on growth was replicated four times between June and August 2008.

2.3.4 Toxicity testing

The media that promoted strongest growth (in this study we considered this to be AAP since it promoted significantly higher growth for most endpoints compared to other media) was used in the subsequent toxicity tests with *E. canadensis* with and without the addition of nitrogen and phosphorous in the media. The test chambers and conditions were the same as those described earlier for the growth media tests with the exception that all test chambers contained root substrate for toxicity testing. *E. canadensis* shoots were exposed to herbicide containing growth media for 14-d (n = 10 for control, n = 5 for six treatments), at which point chlorophyll content, wet/dry biomass, root number, root mass, root length, and relative growth rate (RGR) based on wet/dry mass and plant length, were measured. Also, twenty segments of *E. canadensis* were measured for wet/dry biomass at the start of each test and the mean of these measurements were subtracted from results at the end of the toxicity tests to obtain results for new growth only.

Lemna spp. toxicity tests were conducted according to previously described methods (Greenberg et al., 1992) in 10 mL of media in Petri dish tests chambers (60 x 15 mm). For each species, three plants with four fronds each were placed into each Petri dish

with 10 mL of the appropriate test solution (n = 6 for control, n = 3 for treatment). Test solutions were replaced on day 4 to ensure consistent exposures. Dishes were placed onto plastic trays that were then placed in the controlled environmental chamber for the 7-d exposure. At the end of 7-d, the endpoints of frond number, plant number, wet and dry biomass, chlorophyll-a content, and relative growth rate (RGR) based on plant and frond number were measured. For the endpoints of frond number and plant number, initial day-0 values of 4 and 3 respectively, were subtracted from day-7 values to obtain measurements for new growth only.

2.3.5 Model contaminants

Two commonly used herbicides, atrazine (96% active ingredient, Syngenta Crop Protection Inc., Greensboro, NC) and diuron (80% active ingredient, DuPont, Mississauga, ON, Canada), were chosen as the model contaminants for this study. Both atrazine and diuron are used for the treatment of broadleaf weeds in agricultural applications by inhibiting the electron flow to photosystem II, thus inhibiting photosynthesis (Moncada, 2008; Kamrin, 1997). The herbicides were in a solid form with atrazine being a fine white powder and diuron being coarse granules. Diuron was dissolved in 1L of re-distilled deionized water in a volumetric flask to make a 20 mg/L stock solution. Atrazine was first dissolved in 50 mL of acetone, and then an aliquot was diluted in re-distilled deionized water to make a stock solution of 20mg/L. From these stock solutions, a geometric series of dilutions using sterile plant growth media were made based on effects ranges from previous toxicity studies with aquatic macrophytes. For *E. canadensis*, expected diuron concentrations were 0, 3.125, 6.25, 12.5, 25, 50, and 100 µg/L and expected atrazine concentrations were 0, 9.375, 18.75, 37.5, 75, 150, and

300 µg/L. For both *Lemna* species, expected diuron concentrations were 0, 3.125, 6.25, 12.5, 25, 50, and 100 µg/L and expected atrazine concentrations were 0, 12.5, 25, 50, 100, 200, and 400 µg/L. Samples of each of the growth media solutions from the highest test concentration of each herbicide were taken on day-0 and the final day (day-14 for *E. canadensis*, day-7 (ie. 3 days after replacement) for *Lemna* spp.) for residue analysis.

2.3.6 Herbicide residue analysis

Residue concentrations were measured using Enzyme Linked Immunosorbent Assay (ELISA) (Abraxis Kits, Warminster, PA, USA). This was a microplate method with a detection limit of 0.03 µg/L of diuron and 0.05 µg/L of atrazine and was completed in triplicate for each concentration within 24 hr of sampling using a Bio-Rad Model 680 microplate reader (Hercules, CA, USA). Samples were diluted when necessary to ensure they fell within the range of the standard five-point curve (0.1 – 5 µg/L for atrazine and 0.03 – 3 µg/L for diuron). Specific methods of the analysis were conducted as per the ELISA kit manual. An Ohmicron RPA-1 photometric analyzer (Ohmicron Corp., Newtown, PA) was used to calculate calibration curves and concentrations.

2.3.7 Statistical analysis

2.3.7.1 Growth media test

The overall mean derived from the means of each of the four replicated tests (n=10) for each endpoint and media type was calculated. The endpoint means of each media type were then compared using a one-way ANOVA analysis ($p < 0.05$) with SigmaStat 3.5 (Systat Software 2006, Jandel, San Rafael, CA, USA). Data not

conforming to ANOVA assumptions were by ln or square root transformed. If data still did not meet ANOVA assumptions, the Fisher's Least Significant Difference method was used for this multiple-comparisons and Tukey's method used. In order to determine the influence of root substrate on growth, a Student's t-test ($p < 0.05$) was used with a Mann-Whitney Rank Sum Test used if data were not normally distributed to compare between the same media in the presence or absence of rooting substrate. This same procedure was used to determine any effect of the presence or absence of supplemental nitrogen and phosphorus on growth within media type.

2.3.7.2 Toxicity tests

For each endpoint and each test species, a one-way ANOVA was conducted using the Dunnett's test ($p < 0.05$) with SigmaStat 3.5 (Systat Software 2006, Jandel, San Rafael, CA, USA) to determine significant differences from the control groups. This was used to determine no observable effect concentrations (NOEC) and lowest observable effect concentrations (LOEC) for each endpoint. Data not conforming to ANOVA assumptions were by ln or square root transformed. If ANOVA assumptions were still not met, a Kruskal-Wallis ANOVA on Ranks was used. Data were also fit to various regressions models using SigmaPlot 10.0 (Systat Software 2006, Jandel, San Rafael CA, USA). Models used were hormetic, three parameter logistic and linear models:

Linear $f = ((-b^* 0.5)/e)^*x + b$

Hormetic $f = (t^*(1 + h^*x))/(1 + ((0.5 + h^*x)/0.5)^*(x/e)^{\wedge}b)$

Logistic $f = t/(1 + (x/e)^{\wedge}b)$

Where the variable e is the EC_{50} of the concentration-response relationship modeled, x is the actual concentration being evaluated (µg/L), f is the response or change from control

of the modeled endpoint and b, t and h are constants. The model selected depended on how well the data fit according to the adjusted r^2 value. Models that gave the highest correlation were chosen for that particular endpoint. From these models, effective concentrations (EC_x), specifically the EC_{10}, EC_{25} and EC_{50} were calculated for each endpoint.

In order to assess statistical sensitivity, coefficient of variation (CV), minimum detectable difference (MDD) and minimum significant difference (MSD) was calculated. This was done according to calculations outlined by Hanson and Solomon (2004). Since control groups had higher number of replicates than treatment groups, replicates for control groups were set equal to treatment groups (n = 5 for *E. canadensis* and n = 3 for *Lemna* species) for the calculations of MDD and MSD in order to obtain a more conservative estimate.

2.4 RESULTS

2.4.1 Residue analysis

ELISA measurements of day-0 diuron residues showed tests for all species to be within 15% of nominal concentrations (Table 2.1). For the atrazine measurements, *E. canadensis* tests had day-0 concentrations within 10% of nominal concentrations and *Lemna* spp. tests were within 40% of nominal concentrations (Table 2.1). A laboratory *Lemna* toxicity test conducted by Kirby and Sheahan (1994) showed atrazine concentrations within a mean of 40% of nominal concentration at day-0, which is similar to what was observed in this study. Over the 14-d exposure period for *E. canadensis*, approximately 50% of diuron and 5% of atrazine dissipated from test solutions (Table 2.1). For the 7-d *Lemna* spp. exposure period, approximately 90% of diuron and between

8-40% of atrazine dissipated from test solutions (Table 2.1). Dissipation of atrazine in another *Lemna* laboratory toxicity test has been shown to range from 21 – 43% over a 2-d period (Kirby and Sheahan, 1994), indicating better recovery for this study, since analyisis of atrazine took place after 3-d and showed only 5% dissipation.

2.4.2 Growth media test

ANOVA comparisons between media showed significant differences for some endpoints and some clear trends. Where significant differences were found, AAP media showed greater growth for $RGR_{wet\ mass}$, plant length, and RGR_{length} in both substrate and no substrate test systems compared to many of the other media types (Figures 2.1 – 2.6). All other media comparisons can be found in Appendix 9 – 26. For plant length, AAP media showed mean increases in plant length ranging from 0.8 – 1.4 cm, $RGR_{wet\ mass}$ of 0.02 – 0.04 $gg^{-1}day^{-1}$, and RGR_{length} of 0.01 – 0.02 $cmcm^{-1}day^{-1}$ over the 14-d duration. For all media types, root growth was generally poor, ranging from 0 – 2 roots. Most plant shoots survived in each of the tests. The only exception was with Hoagland's and Hutner's media, both of which showed a range of 1 – 10 (out of 10) shoots completely decaying due to contamination (Appendix 17 and 26). For both substrate and no substrate test systems, Hoagland's and Hutner's media showed the lowest growth consistently for most endpoints (Appendix 9 – 26, Figure 2.1 – 2.6). In some cases, other media showed significantly higher growth than AAP media. Higher growth based on RGR_{dry} and chlorophyll-a was found for Smart and Barko media (GP) and DI water compared to other media (Appendix 9 – 11, 19 - 26). Although there were no significant differences in the percent survival across the media types and formulations, it is

important to note that the few plants that showed complete decay or non-viability occurred in Hoagland's, Hutner's, and Hutner's without nitrogen and phosphorus media.

Student's *t*-tests revealed significant differences between substrate and no substrate test systems. In almost all endpoints and media types where significant differences occurred between the two test systems, test systems with substrate had significantly greater growth then without substrate. Endpoints showing significant differences between substrate and no substrate test systems were consistently RGR (wet and dry mass), length, and root endpoints (mass and length) (Table 2.2).

When comparing media types with and without supplemental nitrogen and phosphorus, significant differences were also found. These differences were found in Hutner's, Hoagland's and AAP media types for RGR (wet/dry mass and length), plant length, and root endpoints (number, mass and length), percent survival, and chlorophyll-a (Table 2.3). For Hoagland's and Hutner's media, means for formulations with nitrogen and phosphorus were consistently and significantly lower than formulations without supplemental nitrogen and phosphorus. For AAP media, endpoints showing significant differences between the two formulations showed higher growth for the formulation with N and P compared to no N and P (Table 2.3).

Upon completion of the media evaluation, AAP media was chosen to use for subsequent toxicity tests. It was chosen because it showed consistent strong growth in *E. canadensis* many endpoints compared to other media and did so across all three media test runs. From visual inspection of the test chambers, it also showed a regular ability to keep the proliferation of algal contamination to a minimum. These criteria made AAP media the best prospect for further testing in this study. In order to further investigate

effects of additional supplemental nutrients to the systems and observed toxicity, both AAP and AAP without nitrogen and phosphorus were used in subsequent toxicity testing.

2.4.3 Toxicity testing

2.4.3.1 NOEC and LOEC

Overall, due to test system variability, few NOECs and LOECs were calculated. *E. canadensis* tested in AAP with diuron showed length and RGR_{length} being the most sensitive endpoints. Tests with diuron in AAP media without nitrogen and phosphorus showed no calculable NOECs and LOECs below the highest test concentration (Table 2.4). NOEC and LOEC values could not be calculated for *E. canadensis* atrazine exposures. Both *Lemna* species exhibited lower variation and distinct responses (Table 2.4). For *Lemna* species exposed to diuron, most NOEC and LOEC values were 25 and 50 µg/L respectively, with the exception of *L. gibba* endpoints of wet mass, which was more sensitive at 12.5 and 25 (NOEC and LOEC respectively) and chlorophyll-a, which had a NOEC and LOEC greater then 100 µg/L (Table 2.4). The most sensitive endpoints for *Lemna* species exposed to atrazine were frond number for *L. gibba* and wet and dry mass for *L. minor*. According to NOEC and LOEC values, *Lemna* species were consistently more sensitive than *E. canadensis* with *L. minor* being the more sensitive of the two duckweed species.

2.4.3.2 Effective concentrations (EC_x)

Effective concentrations for *E. canadensis* exposed to diuron were generally lower in AAP media compared to AAP media without nitrogen and phosphorus. Wet mass and RGR_{wet} were the most sensitive endpoints in AAP media with EC_{50} values of

13.6 and 15.2 µg/L respectively (Table 2.4). Two other endpoints, plant length and root number, had very low EC_{50}s (< 0.1 µg/L) and correlation coefficients >0.60. In spite of good fits to regression models, this data were considered as outliers since no other EC_{50}s calculated were that low. For AAP without N and P, the most sensitive endpoints were root number and root wet mass with EC_{50}s of 75.6 and 66.1 µg/L respectively (Table 2.4). Out of all the EC_x values derived from regression models that actually fit the data (18 in total), only seven of the endpoints showed correlation coefficients (r^2) higher than 0.5 (Table 2.4) with most endpoints also showing very broad 95% confidence intervals (CI). *E. canadensis* exposed to atrazine showed no toxicological response for most endpoints in both substrate and no substrate test systems. Effective concentrations calculated for *Lemna* species showed high sensitivity to both herbicides and fit regression models very well, with almost all endpoints showing r^2 values of >0.9 (Table 2.4). For both herbicides and both *Lemna* species, wet and dry masses were the most sensitive endpoints (Table 2.4). Overall, toxicity across all endpoints showed diuron to be more toxic to all test species compared to atrazine. Trends on overall sensitivity to diuron were *E. canadensis* > *L. minor* > *L. gibba*, although differences in sensitivity to diuron between the two *Lemna* species were marginal. Trends in sensitivity for the atrazine exposure were *L. gibba* > *L. minor* > *E. canadensis*.

2.4.3.3 Coefficients of variation (CV), minimum detectable difference (MDD), and minimum significant difference (MSD)

Coefficients of variation amongst the *E. canadensis* test systems showed some distinct trends. For toxicity tests conducted using AAP media with nitrogen and phosphorus, coefficients of variation were lower with diuron compared to atrazine,

whereas the opposite occurred with AAP media without nitrogen and phosphorus (Table 2.5). Overall, AAP media with nitrogen and phosphorus had lower CVs compared to tests using AAP media without nitrogen and phosphorus. Chlorophyll-a content CV was consistently the smallest in all tests for *E. canadensis*. Overall, for all endpoints and exposures, *L. minor* exhibited the lowest CVs. Endpoints with lowest amounts of variation were RGR_{frond} for *L. gibba* for both herbicides RGR_{plant} for *L. minor* exposed to diuron, and wet mass for *L. minor* exposed to atrazine. CVs for *Lemna* species (ranging from 4-30%) were all generally lower compared to those for *E. canadensis* (ranging from 27 - >100%) (Table 2.5). Minimum detectable differences and minimum significant differences were high for *E. canadensis* tests (Table 2.5), exceeding 100% of control for many endpoints. For *E. canadensis* tested in AAP media with nitrogen and phosphorus, MSD and MDDs were lower for diuron exposures compared to atrazine exposures. The opposite trend occurred with AAP media without nitrogen and phosphorus, with atrazine exposures having lower MDDs and MSDs. Overall, AAP media with nitrogen and phosphorus had the lowest MDDs and MSDs (Table 2.5). The endpoint exhibiting lowest MSDs and MDDs for *E. canadensis* across both media formulations and both herbicide exposures was chlorophyll-a (Table 2.5). *Lemna* species showed the lowest MSDs and MDDs ranging from 7 – 78% with most in the 15-25% range (Table 2.5). MSDs and MDDs were consistently lower for *L. minor* compared to *L. gibba* across all endpoints and exposures (Table 2.5). RGR_{frond} was the endpoint that showed the lowest MSDs and MDDs for both species with the exceptions of RGR_{plant} (*L. minor*, diuron exposure) and wet mass (*L. minor*, atrazine exposure) having lower MDDs (Table 2.5).

2.5 DISCUSSION

In ANOVA comparisons where significant differences were detected, AAP media consistently promoted the strongest growth in *E. canadensis*. For this study, the typical 20X formulation was replaced with a lower concentration 5X formulation. This reduced the amount of nitrogen and phosphorus in the media compared to other media types. The excess amounts of nitrogen and phosphorus in other media likely increased the amount of algal contamination. This algal contamination attributed to non-axenic plant material is a major challenge of successful macrophyte growth in the laboratory, It is important that there is a balance between providing enough nutrients for plant growth and not providing an excess that would encourage algal and bacterial contamination (Smart and Barko, 1985). This balance has been described in freshwater environments with regards to the Redfield ratio (Townsend et al., 2008). This ratio describes the consistent concentrations of nitrogen and phosphorus in marine algal species, which is 16:1 (N:P). In freshwater systems, this value has been shown to be closer to 17:1 (N:P) (Townsend et al., 2008). AAP media components showed a N:P ratio close to 17:1 therefore, nitrogen and phosphorus were not limited. In other media, which contained much contamination, these ratios were higher, showing an excess of nitrogen. This could have caused a shift in the proliferation of certain algal species, which could explain the contamination. With the enrichment of nitrogen and phosphorus in most media, algal species can proliferate (Veraart, 2008) and can reduce growth of aquatic macrophytes (Mulligan et al., 1976; Ryan et al., 1972; Gerloff et al., 1966; Ozimek et al., 1991; Pieczynska et al., 1996) due to competition, shading, or the release of substances toxic to the macrophytes. The presence of bacterial contamination has also been shown to be an issue in laboratory

testing. The presence of supplemental carbon sources, such as sucrose, has been shown to increase bacterial growth in macrophyte tests (Knauer et al., 2006). Since this study removed sugars from all media formulations, bacterial growth was not deemed to be a serious issue, though bacteria are obviously still present in the systems.

In order to establish a rapid growth media investigation, *E. canadensis* shoots were washed thoroughly with DI water in lieu of any chemical treatment to clean the plants. This made the preparation process much faster. Although plants were cleaned thoroughly, periphytic algal species were likely to remain in most cases. This algal contamination was visually present in many of the test chambers, with more being visible in tests with media containing nitrogen and phosphorus components. The presence of algae, both living and decomposing, with aquatic macrophytes has been shown to significantly affect macrophyte growth. In a study conducted by Mohr et al. (2007), filamentous algae growing in controls showed reduced biomass of the macrophytes *M. verticillatum* and *P. natans*, postulated to be by interspecific competition. A laboratory study conducted by Ozimek et al. (1991) also found significant biomass decreases of *E. canadensis* in the presence of the filamentous algae *Cladophora glomerata* (L.) Kutz. It concluded that the density and compaction of the algae could create significant shading for the macrophyte, leading to lower growth. With the presence of filamentous algae in the test chambers, this shading effect may have lead to reduced growth of the macrophytes in these tests. Large amounts of decomposing filamentous algae (two to four times more algae than macrophyte mass) can also reduce macrophyte growth, where it is thought that the toxic chemicals released by decomposition outbalance the release of essential nutrients (Pieczynska et al., 1996). With the relatively short duration of these

tests and a clear dominance of macrophyte biomass to algal biomass, this was likely not a factor in these tests.

Aside from algal contamination, the issue of specific formulation must be addressed as a factor for growth in this experiment. The removal of nitrogen and phosphorus constituents in this study involved the absence of a certain stock solution from the final test media. What this meant was that a stock solution bottle, containing a nitrate or phosphate salt, was eliminated from the formulation. This allowed for a simple and quick way of removing nitrogen and phosphorus from the media without having to stoichiometrically adjust the entire media recipe for all components present. The nitrate and phosphate salts contained potassium, calcium, or sodium, depending on the media type (Appendix 4 - 8). With these amounts of potassium, calcium, or sodium removed, they may have caused a deficiency of those elements for the plant, which could have reduced growth. With these adjustments to the media, non-axenic plant material can be used in tests without the proliferation contamination. The possibility of achieving contamination free test systems using non-axenic test material was shown by Knauer et al. (2006). That study found M4 media to show no significant contamination in test chambers in growing non-axenic shoots of *E. canadensis* and *M. spicatum*.

Another source of variation that may have affected these growth tests may have been the sources of the plant material for the tests. *E. canadensis* plants were collected from two distinct locations, the microcosm facility at the University of Guelph and in local streams/lakes located in the city of Guelph. Each location is subject to its own sources of variability like inflows of contaminants from various locations, sediment quality, water quality parameters, and competition with other species. With this in mind,

plants from each location may have distinct genetic characteristics that lend to existence in those specific locations. Plants were taken from multiple locations in order to accommodate availability at the time of collection, but in order to reduce variability it is important for future testing that plants be obtained from one discreet location if possible to avoid these in possible inconsistencies and ensure the ability to test year round.

With using a non-axenic test method for macrophyte toxicity testing, it is clear that using a media that reduces contaminant proliferation is essential. The results from this study show that AAP media could be used to successfully conduct a non-axenic toxicity test with *E. canadensis*. An adjustment to AAP media without nitrogen and phosphorus that includes the sodium that was removed with the nitrogen constituent could be conducted to determine if growth could be improved. Further testing with other media types could also be conducted.

In comparing substrate test systems to those that lacked substrate, the substrate systems consistently showed greater growth. This suggests a physiological signaling for *E. canadensis* to anchor itself into root substrate prior to expansive growth. This was indicated by all root endpoints showing significantly higher growth in substrate systems along with plant growth in general. With roots being sent into the substrate, this provided another route of uptake of nutrient media and since *E. canadensis* obtain a large proportion of their nutrients from the substrate via the roots (Spicer and Catling, 1988), this also helps to explain the improved growth observed. Another variable to consider is the type of substrate chosen. The sand substrate used was very porous and not as coarse as the commonly used Turface (a gravel-like aggregate used to prevent compaction on sports fields) (ASTM, 2005b), which gave the substrate a lower shear strength (Handley

et al., 2002), allowing for substrate particles to shift more easily as roots passed into the matrix. In general, stronger growth in systems containing substrate has been documented in other studies. Knauer et al., (2006), measured growth of non-axenic cultures of *E. canadensis* and *M. spicatum* grown in M4 media with sediment substrate and without. Both macrophytes showed up to three times more shoot development in sediment systems compared to systems without sediment. Also, *M. spicatum* showed up to six times as many roots in sediment systems.

The problem of algal contamination also presented itself in the toxicity test results for *E. canadensis*. Visual inspection of the test chambers over the duration of the tests showed algal contamination to be present in many test chambers, especially controls. With controls competing with algae, effects of the herbicides may have been masked, as shown by the lack of a concentration response relationship for atrazine, which has also been described by Mohr et al. (2007) for a microcosm study with macrophytes. Large coefficient of variation values for most endpoints (100% or greater) indicated significant variation in control test chambers, likely due to reduced growth in the presence of algal competition. With such a high amount of variation across many endpoints, NOEC and LOEC values could not be calculated for *E. canadensis*. Due to reduced and highly variable growth of control systems, many endpoints showed no significant differences in comparing controls to highest exposure, and for exposure to atrazine, EC_x values could not be calculated. The variability of plant source location of may have also influenced these results, as described earlier for the growth media tests.

In spite of various sources of variation, diuron exposures showed reasonable concentration response relationships and relatively low CVs for some endpoints. This

was likely because concentrations were high enough to elicit a response and be toxic to algae present in the test chambers at the lowest tested concentrations, allowing for a stronger growth of *E. canadensis* in the lower test concentrations. Toxicity of diuron to various common freshwater algal species was characterized by Bednarz (1981) with *Chlorococcum* spp. and *Anabaena variabilis* being the most sensitive with 14-d LC_{50}s of 5 and 5.8 µg/L respectively for population growth rate. Studies conducted by Ma et al. (2001, 2002) found the 14-d EC_{50}s for diuron exposed to *Chlorella pyrenoidosa* and *Chlorella vulgaris* to be 1.3 and 43 µg/L respectively for population growth rate. These EC_{50}s fall within the range of concentrations used in this study and support the hypothesis that algae would have been reduced within the exposure test chambers, reducing this confounding factor in exposed plants. In the case of atrazine, the herbicide may not have been high enough to kill the algae. This could have lead to the algae surviving and outcompeting the macrophtyes, leading to weak concentration-response data for *E. canadensis*. This is supported by Ma et al. (2003) who reported a 14-d atrazine EC_{50} to the alga *Scenedesmus quadricauda* of 4300 µg/L, well over the highest concentration tested in this study. Bednarz (1981) also found algae species that were insensitive to atrazine. Bednarz (1981) found 14-d LC_{50} values ranging from 500-1500 µg/L (population growth rate) for *Chlorella* spp., *Dictyosphaerium pulchellum*, and *Anabaena variabilis*. With these high toxic concentrations, the presence of algae having the ability to affect the macrophyte across all concentrations becomes a possibility. Although there is evidence that atrazine concentrations were not high enough to affect algal species, this evidence must be cautioned since the algal species diversity was not investigated in these test systems. There could exist a variety of responses of various algal species to atrazine

that may differ from data presented here. Therefore, these EC_{50} values presented here only offer a speculation as to why algal contamination proliferated.

Atrazine may not have been tested at high enough concentrations to elicit effects in the macrophytes, even though concentrations tested fell within the range of toxicity previously described. Fairchild et al. (1998) found a 14-d EC_{50} (wet mass) for *E. canadensis* of 21 µg/L and Forney and Davis (1981) found a 14-d EC_{50} (leaf growth) for *E. canadensis* of 160 µg/L, both in a laboratory setting, for atrazine. Therefore, at concentrations tested, it was expected to observe effects. Only ~5% of the atrazine was observed to degrade over the 14-d exposure period and expected concentrations were within 10% of nominal, so the difference in toxicity is not due to lack of exposure.

Exposure to diuron lead to relatively low variation and was most toxic to *E. canadensis* relative to other plant species tested, revealing its potential as a test species in this system. For this test, wet mass was the most sensitive endpoint for AAP media and root wet mass was most sensitive of AAP without nitrogen and phosphorus, with EC_{50}s of 13.6 µg/L and 66.1 µg/L respectively. An organism's statistical sensitivity, or ability to detect ecological changes occurring from exposure, needs to be understood for toxicity data to be more relevant in risk assessment (Hanson et al., 2003). For this study, the statistical sensitivity for *E. canadensis* endpoints was very low, with CVs ranging from 27->100%. Also, MDDs ranged from 50->100%, even for toxicologically sensitive endpoints, indicating a relatively poor ability to detect small changes induced by toxicant exposure using simple univariate approaches. Hanson et al. (2003) reported statistical sensitivity of a standard laboratory toxicity bioassay using the macrophytes *Myriophyllum spicatum* and *Myriophyllum sibiricum*. Plant length and wet mass were

found to be statistically most sensitive with CVs ranging from 6 – 11% and MDDs ranging from 13 – 23%. This is consistent with another study conducted by Hanson and Solomon (2004) that showed plant length and wet mass having CVs of 7 – 11% and MDDs of 11 – 21% for *M. spicatum* and *M. sibiricum*. Hanson et al. (2003) also reported data from a laboratory toxicity test using *E. canadensis*. Endpoints of biomass and chlorophyll florescence showed CVs ranging from 0 – 13% and MDDs ranging from <1 – 40%. It has been suggested that a change of ≥20% in biological function of plants can indicate an ecologically significant change (Christman et al., 1994). With this in mind, a common threshold of ≤ 25% difference from controls has been used for endpoints in toxicity tests to determine an ecologically significant difference, which is reflective of the EC_{25} (Hanson et al., 2003). Since this study showed very high values (greater than the ≤ 25% threshold) for CV, MDD, and MSD, this laboratory methodology has difficulty in detecting ecologically significant changes in most endpoints. In some cases, *E. canadensis* showed relatively high statistical sensitivity. The chlorophyll-a endpoint showed CVs of 27% and MDD of 50%, but toxicologically, it was the least sensitive endpoint. Hanson and Solomon (2004) observed similar results for chlorophyll-a with *M. spicatum* and *M. sibiricum* where EC_{50} values were up to an order of magnitude higher than other endpoints with very respectable CVs, MSDs, and MDDs of < 20%. These types of results call into question the utility of chlorophyll-a as an informative endpoint. If toxicological sensitivity is not achieved, it is important to use other endpoints to capture the true sensitivity of a compound. In spite of being affected by many sources of variation, the *E. canadensis* tests did show promise for further use.

In contrast to the *E. canadensis* results, the *Lemna* species, as a comparative test, showed much higher sensitivity both statistically and toxicologically. Since these species were obtained from an axenic culture, algal contamination was not an issue with these tests, thus lowering variability. Statistically, *Lemna* species outperformed *E. canadensis*. CVs ranged from 4 – 30% of control, indicating low variation of control data compared to *E. canadensis* tests. Also MDD and MSDs ranged from 4 – 78% of control, with most being in the 15-30% range. These tests indicated that ecologically significant changes can be detected by these tests (ie. $\leq$ 25% change from control (Christman et al., 1994). All endpoints tested RGR (frond and plant number) and wet mass exhibited consistently low CVs as well as MDD and MSD values (all in the range of 4 – 30% of control). These endpoints would therefore be best suited for risk assessment purposes. This statistical sensitivity is comparable with results described by Hanson and Solomon (2004) where CVs ranged from 5 – 16% and MSD and MDDs ranged from 11 – 30% from control.

Although *Lemna* showed some endpoints being less sensitive to diuron than *E. canadensis*, some factors must be considered in evaluating the utility of this data. One of these factors is the different exposure duration of the *Lemna* and *Elodea* tests. The chronology of toxicological responses is important since properties of compounds, such as half-life, can lead to responses at different times (Bunce et al., 2003; Rozman, 2000). Therefore, in order to express responses for *Lemna* and *Elodea* over a similar exposure period, Haber's Rule (Concentration x Time = Constant) was used to correct toxicity data. In many toxicology studies, chronology of toxicological responses is rarely considered with most emphasis placed on final parameters like EC_{50} (Bunce et al., 2003).

The importance of Haber's Rule is it makes time a quantifyable parameter in toxicity assessement (Rozman, 2000). Therefore, in order to compare *Lemna* toxicity, which manifested over a 7-d period, to submersed macrophyte toxicity, *Lemna* data were adjusted to 14-d duration to match data of the submersed macrophyte tests. This converted data showed higher sensitivity to diuron for *E. canadensis* compared to *Lemna* spp. in some cases (Table 2.4), but this *E. canadensis* data were subject to great variation. Both the regression coefficients (which were very low) and the 95% CIs (which were very wide) for most endpoints make this data statistically insignificant. The *Lemna* spp. data, on the other hand, which did show lower sensitivity in some cases compared to *E. canadensis*, was much more consistent in its toxicological and statistical sensitivity (Tables 2.4, 2.5). It showed EC_{50}s consistently in the range of 30 – 100 µg/L with strong fitting regression models and narrow 95% CIs, which shows the higher quality of data obtained from these tests. Also, *E. canadensis* data corrected by Haber's Rule from AAP media without N and P showed lower sensitivity overall compared to *Lemna* species. Rentz and Hanson (2009) found that when 96 hr *Lemna* laboratory data were converted to 14-d EC_{50}s using Haber's Rule, it shows approximately one order of magnitude more sensitivity compared to other macrophytes. This suggests that if *Lemna* tests are extended to the same test duration as rooted submersed macrophytes, they are adequate in predicting effects of other macrophytes in the laboratory. In combining higher sensitivity and statistical quality of data, *Lemna* presents itself as a strong test species, which supports its use as the only current required species for chemical registration. (Davy et al., 2001). Overall, *Lemna* spp. is a strong model to use in risk assessment on the effects of contaminants on macrophytes. The test is rapid, being 7-d rather than the

minimum to date 14-d for changes in gross morphology of rooted macrophytes and the assays shows reasonable predictive powers for other macrophytes in the field with standard uncertainty factors (Rentz and Hanson, 2009). Any new macrophyte test species would have to perform as well or better to be considered truly useful for broad applications in ecological risk assessment.

This study has shown that the choice of growth media can lead to varying responses of various endpoints, with AAP promoting strongest development and lowest susceptibility to contamination of *E. canadensis*. Strong development is important to these tests because higher RGRs can lead to increased sensitivity and can make small biological changes induced by the toxicant more easily detected in short test durations. This media type should be further tested for its utility in not only *E. canadensis* test methods, but in the general culturing of the plant in the laboratory so that natural and likely highly variable natural populations do not have to be relied upon for source material. Future work could determine if AAP media can be used to establish a long-term stock culture of *E. canadensis* in the laboratory free of contamination that can also grow to large amounts of biomass quickly from apical shoots. The use of *E. canadensis* as a standard test species is a possibility in future risk assessment applications, but not without further development of the methods employed in this study. Although this macrophyte showed increased sensitivity to the herbicide diuron compared to the widely used *Lemna* spp. species, algal contamination reduced the sensitivity of the test and made the authors leary of the data attributing very low concentrations to significant effects. In order to further investigate the use of this macrophyte in toxicity tests, a culture free of phytoplankton and periphyton would be recommended. Without the confounding nature

of contamination present, it may be possible for *E. canadensis* to produce low MDD values much like *Myriophyllum* and *Lemna* spp. In conducting the comparative assay with *Lemna* spp. species, it is important to consider the true strength of these species as surrogates for other macrophytes since they showed consistent sensitivity in both toxicity data and statistical analysis. Also, with their small size, rapid growth rates, and short test durations, they make toxicity testing a very efficient process.

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Table 2.1 Fate of diuron and atrazine in various toxicity test systems.

Species	Media Type	Compound	Test Duration (d)	Initial Concentration			Final Concentration		Amount Dissipated (%)
				Nominal Concentration (µg/L)	Measured Concentration (µg/L)	Percent of Nominal (%)	Measured Concentration (µg/L)	Percent of Nominal (%)	
E. canadensis	AAP	Diuron	14	100	96	96	45	45	53
E. canadensis	AAP w/o NP	Diuron	14	100	84	84	43	43	49
L. gibba	Hutner's	Diuron	7	100	91	91	10	10	89
L. minor	Hoagland's	Diuron	7	100	85	85	7	7	92
E. canadensis	AAP	Atrazine	14	300	317	106	300	100	5
E. canadensis	AAP w/o NP	Atrazine	14	300	271	90	291	97	0
L. gibba	Hutner's	Atrazine	7	400	490	122	451	113	8
L. minor	Hoagland's	Atrazine	7	400	563	141	343	86	39

Table 2.2 Significant differences between *Elodea canadensis* growth with root substrate and without root substrate for various endpoints over a 14-day growth period. Reported values are the mean and standard errors (n = 4 test means). Significance determined using a t-test ($p < 0.05$) or a Mann-Whitney Rank Sum Test if data were not normally distributed.

Media type	Endpoint	Mean (w/substrate) ± SE	Mean (w/o substrate) ± SE	P-value
Hoagland's	RGR wet mass ($gg^{-1}day^{-1}$)	$9.7 \times 10^{-3} \pm 7.3 \times 10^{-4}$	$2.2 \times 10^{-2} \pm 2.6 \times 10^{-3}$	0.03
Hoagland's	RGR dry mass ($gg^{-1}day^{-1}$)	$7.5 \times 10^{-3} \pm 9.4 \times 10^{-4}$	$1.57 \times 10^{-2} \pm 2.1 \times 10^{-3}$	0.02
Hoagland's w/o N+P	Plant length (cm)	0.5±0.2	$4.8 \times 10^{-2} \pm 2.8 \times 10^{-2}$	0.04
Hoagland's w/o N+P	RGR plant length ($cmcm^{-1}day^{-1}$)	$5.9 \times 10^{-3} \pm 1.9 \times 10^{-3}$	$6.5 \times 10^{-4} \pm 7.6 \times 10^{-4}$	0.03
Hoagland's w/o N+P	Root Number	0.5±0.2	0	0.03
Hoagland's w/o N+P	Root mass wet (g)	$2.5 \times 10^{-3} \pm 7.3 \times 10^{-3}$	0	0.03
Hoagland's w/o N+P	Root length (cm)	1.9±0.8	0	0.03
Hoagland's w/o N+P	Chlorophyll-a (µg/mg)	3.9±0.4	0.6±0.4	0.001
Hoagland's w/o N+P	Percent Survival	82.5±6.3	30.0±17.8	0.03
Hutner's	RGR wet mass ($gg^{-1}day^{-1}$)	$1.6 \times 10^{-2} \pm 1.7 \times 10^{-3}$	$6.7 \times 10^{-3} \pm 2.9 \times 10^{-3}$	0.03
Hutner's	RGR dry mass ($gg^{-1}day^{-1}$)	$1.2 \times 10^{-2} \pm 2.9 \times 10^{-3}$	$1.2 \times 10^{-3} \pm 6.3 \times 10^{-4}$	0.03
Hutner's	Chlorophyll-a (µg/mg)	$0.8 \pm 4.1 \times 10^{-2}$	0.4±0.1	0.03
Hutner's w/o N+P	Root number	0.4±0.1	0	0.03
Hutner's w/o N+P	Root mass wet (g)	$1.7E\text{-}3 \pm 5.7 \times 10^{-4}$	0	0.03
Hutner's w/o N+P	Root length (cm)	1.9±0.6	0	0.03
Hutner's w/o N+P	Chlorophyll-a (µg/mg)	3.0±0.4	0.3±0.2	<0.001
Hutner's w/o N+P	% Survival	92.5±7.5	10.0±7.1	<0.001

Table 2.2 Cont.

Media type	Endpoint[a]	Mean (w/substrate) ± SE	Mean (w/o substrate) ± SE	P-value
AAP	Root number	$0.7 \pm 6.5 \times 10^{-2}$	0.1±0.1	0.005
AAP w/o N+P	Plant length (cm)	0.7±0.1	$0.3 \pm 7.9 \times 10^{-2}$	0.03
AAP w/o N+P	RGR (Plant length) ($cmcm^{-1}day^{-1}$)	$8.7 \times 10^{-3} \pm 1.4 \times 10^{-3}$	$3.7 \times 10^{-3} \pm 9.4 \times 10^{-4}$	0.03
AAP w/o N+P	Root number	0.7±0.1	$0.1 \pm 7.1 \times 10^{-2}$	0.03
AAP w/o N+P	Root mass wet (g)	$3.3E\text{-}3 \pm 4.8 \times 10^{-4}$	$8.1 \times 10^{-4} \pm 3.2 \times 10^{-4}$	0.005
AAP w/o N+P	Root length (cm)	3.2±0.3	0.7±0.2	<0.001
Andrew's w/o N+P	Plant length (cm)	0.7±0.1	0.2±0.1	0.008
Andrew's w/o N+P	RGR plant length ($cmcm^{-1}day^{-1}$)	$8.0 \times 10^{-3} \pm 9.5 \times 10^{-4}$	$2.2 \times 10^{-3} \pm 1.2 \times 10^{-3}$	0.008
Andrew's w/o N+P	Root number	0.6±0.1	0	0.03
Andrew's w/o N+P	Root mass wet (g)	$2.9 \times 10^{-3} \pm 4.9 \times 10^{-4}$	0	0.03
Andrew's w/o N+P	Root length (cm)	3.0±0.6	0	0.03
DI Water	RGR dry mass ($gg^{-1}day^{-1}$)	$2.5 \times 10^{-2} \pm 2.6 \times 10^{-3}$	$1.2 \times 10^{-2} \pm 2.1 \times 10^{-3}$	0.009
DI Water	Root number	0.5±0.1	7.8E-2±4.8E-2	0.004
DI Water	Root mass wet (g)	$2.6 \times 10^{-3} \pm 5.6 \times 10^{-4}$	$1.7 \times 10^{-4} \pm 1.6 \times 10^{-4}$	0.03
DI Water	Root length (cm)	2.3±0.6	0.2±0.2	0.01

RGR - Relative growth rate, SE – Standard error

Table 2.3 Significant differences between *Elodea canadensis* growth with normal media formulation and media without supplemental nitrogen and phosphorus for various endpoints over a 14-day growth period. Reported values are the mean and standard errors (n = 4 test means). Significance determined using a paired t-test ($p < 0.05$) or a Mann-Whitney Rank Sum Test if data were not normally distributed.

Media Type	Root Substrate Present?	Endpoint	Mean (Normal formulation) ± SE	Mean (No N+P formulation) ± SE	P value
Hoagland's	Yes	RGR dry mass ($gg^{-1}day^{-1}$)	$7.5 \times 10^{-3} \pm 9.4 \times 10^{-4}$	$1.2 \times 10^{-2} \pm 1.3 \times 10^{-3}$	0.04
Hoagland's	Yes	Root number	0	0.5±0.2	0.03
Hoagland's	Yes	Root mass wet (g)	0	$2.5 \times 10^{-3} \pm 7.3 \times 10^{-4}$	0.03
Hoagland's	Yes	Root length (cm)	0	1.9±0.8	0.03
Hoagland's	No	RGR wet mass ($gg^{-1}day^{-1}$)	$2.2 \times 10^{-2} \pm 2.6 \times 10^{-3}$	$7.4 \times 10^{-3} \pm 5.4 \times 10^{-3}$	0.046
Hoagland's	No	Length (cm)	$0.4 \pm 8.5 \times 10^{-2}$	$4.8 \times 10^{-2} \pm 2.8 \times 10^{-2}$	0.006
Hoagland's	No	RGR plant length ($cmcm^{-1}day^{-1}$)	$5.1 \times 10^{-3} \pm 9.7 \times 10^{-4}$	$6.5 \times 10^{-4} \pm 3.8 \times 10^{-4}$	0.005
Hoagland's	No	Chlorophyll-a (µg/mg)	2.1±0.2	0.6±0.4	0.02
Hoagland's	No	% Survival	95.0±2.9	30.0±17.8	0.03
Hutner's	Yes	Root number	0	0.4±0.1	0.03
Hutner's	Yes	Root mass wet (g)	0	$1.7 \times 10^{-3} \pm 5.7 \times 10^{-4}$	0.03
Hutner's	Yes	Root length (cm)	0	1.9±0.6	0.03
Hutner's	Yes	Chlorophyll-a (µg/mg)	$0.8 \pm 4.1 \times 10^{-2}$	3.0±0.4	0.03
Andrew's	Yes	Root mass wet (g)	$1.0 \times 10^{-3} \pm 4.8 \times 10^{-4}$	$2.9 \times 10^{-3} \pm 4.9 \times 10^{-4}$	0.03
AAP	Yes	Plant length (cm)	1.3±0.2	0.7±0.1	0.048
AAP	Yes	RGR plant length ($cmcm^{-1}day^{-1}$)	$1.5 \times 10^{-2} \pm 1.9 \times 10^{-3}$	$8.7 \times 10^{-3} \pm 1.4 \times 10^{-3}$	0.04
AAP	No	Plant length (cm)	1.1±0.3	$0.3 \pm 7.9 \times 10^{-2}$	0.03
AAP	No	RGR wet mass ($gg^{-1}day^{-1}$)	$2.9 \times 10^{-2} \pm 5.2 \times 10^{-3}$	$1.3 \times 10^{-2} \pm 2.9 \times 10^{-3}$	0.04
AAP	No	RGR plant length ($cmcm^{-1}day^{-1}$)	$1.3 \times 10^{-2} \pm 3.6 \times 10^{-3}$	$3.7 \times 10^{-3} \pm 9.4 \times 10^{-4}$	0.03

RGR - Relative growth rate, SE - Standard error

Table 2.4 Toxicity data for *Elodea canadensis*, *Lemna gibba*, and *L. minor* exposed to diuron and atrazine for 14 days (*E. canadensis*) and 7 days (*Lemna* spp.).

Species	Endpoint	Media Type	Compound	NOEC (µg/L)	LOEC (µg/L)	EC_{10} (µg/L) (95% CI)	EC_{25} (µg/L) (95% CI)	EC_{50} (µg/L) (95% CI)	EC_{50} (14 d from Haber's Rule) (µg/L)	Model[a]	Parameters[a]	Adjusted r^2
E. canadensis	Wet mass (g)	AAP	Diuron	12.5	25	1.5 (0, 18.5)	4.5 (0, 38.8)	13.6 (0, 79.3)	13.6	Logistic	t = 0.12; b = 1.0; e = 13.62	0.47
E. canadensis	Dry mass (g)	AAP	Diuron	> 100	> 100	1.5 (0, 27.5)	4.6 (0, 56.8)	13.8 (0, 113.7)	13.8	Logistic	$t = 8.5 \times 10^{-3}$; b = 1.0; e = 13.8	0.082
E. canadensis	Plant length (cm)	AAP	Diuron	< 3.125	3.125	4.2×10^{-6} (0, 3.0×10^{-3})	2.7×10^{-5} (0, 1.6×10^{-2})	1.7×10^{-3} (0, 0.7)	1.7×10^{-3}	Hormetic	h = 4.0; b = 0.1; t = 3.57; e = 1.7×10^{-3}	0.78
E. canadensis	Root number	AAP	Diuron	> 100	> 100	1×10^{-4} (0, 3.9×10^{-2})	9×10^{-4} (0, 0.2)	5.8×10^{-2} (0, 10.0)	0.1	Hormetic	h = 4.0; b = 0.1; t = 1.25; e = 5.77×10^{-2}	0.85
E. canadensis	Root mass wet (g)	AAP	Diuron	> 100	> 100	4×10^{-4} (0, 0.2)	6.4×10^{-6} (0, 9.8)	4.4×10^{-5} (0, 60.6)	4.4×10^{-5}	Hormetic	h = 4.0; b = 0.51; t = -1.2×10^{-3}; e = 4.4×10^{-5}	0
E. canadensis	Root length (cm)	AAP	Diuron	> 100	> 100	4×10^{-4} (0, 0.2)	2.3×10^{-3} (0, 1.1)	0.1 (0, 51.0)	0.1	Hormetic	h = 4.0; b = 0.1; t = 5.54; e = 0.15	0.29

Table 2.4 Cont.

Species	Endpoint	Media Type	Compound	NOEC (µg/L)	LOEC (µg/L)	EC_{10} (µg/L) (95% CI)	EC_{25} (µg/L) (95% CI)	EC_{50} (µg/L) (95% CI)	EC_{50} (14 d from Haber's Rule) (µg/L)	Model[a]	Parameters[a]	Adjusted r^2
E. canadensis	Chl-a (µg/mg)	AAP	Diuron	12.5	25	4.0×10^3 (0, 8.4×10^4)	6.2×10^3 (0, 1.3×10^5)	1.5×10^4 (0, 3.0×10^5)	1.5×10^4	Hormetic	h = 0.1; b = 0.27; t = 2.35; e = 1.5×10^4	0.95
E. canadensis	RGR Wet mass ($gg^{-1}day^{-1}$)	AAP	Diuron	> 100	> 100	1.7 (0, 19.5)	5.1 (0, 41.0)	15.2 (0, 83.9)	15.2	Logistic	$t = 3.7 \times 10^{-2}$; b = 1.0; e = 15.22	0.51
E. canadensis	RGR Dry mass ($gg^{-1}day^{-1}$)	AAP	Diuron	> 100	> 100	0.6 (0, 10.9)	1.7 (0, 22.3)	5.1 (0, 42.0)	5.1	Logistic	$t = 2.2 \times 10^{-2}$; b = 1.0; e = 5.13	<0.01
E. canadensis	RGR plant length ($cmcm^{-1}day^{-1}$)	AAP	Diuron	< 3.125	3.125	0.2 (0, 5.3)	0.5 (0, 11.0)	1.6 (0, 19.4)	1.6	Logistic	$t = 3.5 \times 10^{-2}$; b = 1.0; e = 1.64	0.21
E. canadensis	Wet Mass (g)	AAP w/o NP	Diuron	> 100	> 100	19.8 (0, 163.5)	49.5 (0, 407.9)	99.1 (0, 817.4)	99.1	Linear	$b = 3.8 \times 10^{-2}$; e = 99.08	0
E. canadensis	Dry mass (g)	AAP w/o NP	Diuron	> 100	> 100	22.9 (0, 230.2)	57.3 (0, 575.7)	114.6 (0, 1.2×10^3)	114.6	Linear	$b = 4.4 \times 10^{-3}$; e = 114.56	0

Table 2.4 Cont.

Species	Endpoint	Media Type	Compound	NOEC (µg/L)	LOEC (µg/L)	EC_{10} (µg/L) (95% CI)	EC_{25} (µg/L) (95% CI)	EC_{50} (µg/L) (95% CI)	EC_{50} (14 d from Haber's Rule) (µg/L)	Model[a]	Parameters[a]	Adjusted r^2
E. canadensis	Plant length (cm)	AAP w/o NP	Diuron	> 100	> 100	5.6 (0, 36.5)	8.3 (0, 53.2)	18.8 (0, 119.8)	18.8	Hormetic	h = 4.0; b = 0.51; t = 0.58; e = 18.8	0.34
E. canadensis	Root number	AAP w/o NP	Diuron	> 100	> 100	15.1 (0, 41.3)	37.8 (0, 103.2)	75.6 (0, 206.4)	75.6	Linear	b = 0.83; e = 75.6	0.55
E. canadensis	Root mass wet (g)	AAP w/o NP	Diuron	> 100	> 100	13.2 (0, 37.4)	33.1 (0, 93.6)	66.1 (0, 187.1)	66.1	Linear	b = 0.01; e = 66.1	0.51
E. canadensis	Root mass dry (g)	AAP w/o NP	Diuron	> 100	> 100	12.2(0, 109.6)	17.0 (0, 154.2)	35.4 (0, 382.8)	35.4	Hormetic	h = 4.0; b = 0.56; t = 5.0E-4; e = 35.4	0.13
E. canadensis	Root length (cm)	AAP w/o NP	Diuron	> 100	> 100	15.7 (0, 54.6)	39.3 (0, 136.6)	78.6 (0, 273.3)	78.6	Linear	b = 6.18; e = 78.59	0.34
E. canadensis	Chl-a (µg/mg)	AAP w/o NP	Diuron	> 100	> 100	35.6 (0, 137.8)	132.4 (0, 636.3)	2.2×10^3 (0, 2.2×10^4)	2.2E4	Hormetic	h = 1.80; b = 0.14; t = 2.62; e = 2.2×10^4	0.83

Table 2.4 Cont.

Species	Endpoint	Media Type	Compound	NOEC (µg/L)	LOEC (µg/L)	EC_{10} (µg/L) (95% CI)	EC_{25} (µg/L) (95% CI)	EC_{50} (µg/L) (95% CI)	EC_{50} (14 d from Haber's Rule) (µg/L)	Model[a]	Parameters[a]	Adjusted r^2
E. canadensis	RGR Dry mass ($gg^{-1}day^{-1}$)	AAP w/o NP	Diuron	> 100	> 100	21.1 (0, 204.9)	26.0 (0, 264.4)	5.7×10^{-6} (0, 3.4)	5.7×10^{-6}	Hormetic	h = 4.0; b = 0.38; t = -3.3×10^{-3}; e = 5.7×10^{-6}	0
E. canadensis	RGR plant length ($cmcm^{-1}day^{-1}$)	AAP w/o NP	Diuron	> 100	> 100	2.8 (0, 42.8)	8.4 (0, 86.6)	25.2 (0, 170.2)	25.2	Logistic	t = 7.0×10^{-3}; b = 1.0; e = 25.24	0.16
E. canadensis	Dry mass (g)	AAP w/o NP	Atrazine	> 300	> 300	21.1 (0, 420.7)	62.6 (0, 760.0)	187.8 (0, 1.4×10^{3})	187.8	Logistic	t = 6.5×10^{-3}; b = 1.0; e = 187.8	0
E. canadensis	RGR dry mass ($gg^{-1}day^{-1}$)	AAP w/o NP	Atrazine	> 300	> 300	51.5 (0, 1.3×10^{3})	147.0 (0, 1.9×10^{3})	435.9 (0, 5.3×10^{3})	435.9	Logistic	t = 1.7×10^{-2}; b = 1.0; e = 435.94	0
L. gibba	Frond number	Hutner's	Diuron	25	50	19.1 (1.9, 36.2)	24.9 (7.3, 42.6)	41.6 (12.1, 71.2)	20.8	Hormetic	h = 0.1; b = 0.9; t = 45.21; e = 41.64	0.95
L. gibba	Plant number	Hutner's	Diuron	25	50	26.7 (0, 64.4)	35.5 (0, 78.4)	61.8 (0, 145.2)	30.9	Hormetic	h = 0.1; b = 0.83; t = 8.22; e = 61.81	0.84

Table 2.4 Cont.

Species	Endpoint	Media Type	Compound	NOEC (µg/L)	LOEC (µg/L)	EC_{10} (µg/L) (95% CI)	EC_{25} (µg/L) (95% CI)	EC_{50} (µg/L) (95% CI)	EC_{50} (14 d from Haber's Rule) (µg/L)	Model[a]	Parameters[a]	Adjusted r^2
L. gibba	Wet mass (g)	Hutner's	Diuron	12.5	25	12.4 (0.9, 23.8)	17.2 (5.5, 28.9)	30.5 (13.6, 47.4)	15.2	Hormetic	h = 0.1; b = 0.9; t = 0.10; e = 30.46	0.97
L. gibba	Dry mass (g)	Hutner's	Diuron	25	50	9.9 (0.07, 19.7)	17.8 (6.3, 29.4)	32.0 (18.8, 45.2)	16.0	Logistic	$t = 5.3 \times 10^{-3}$; b = 1.88; e = 32.01	0.98
L. gibba	Chl-a (µg/mg)	Hutner's	Diuron	> 100	> 100	637.4 (0, 9.1×10^3)	4.0×10^3 (0, 8.8×10^4)	2.3×10^5 (0, 9.1×10^6)	1.2×10^5	Hormetic	h = 0.79; b = 0.1; t = 1.18; $e = 2.3 \times 10^5$	0.02
L. gibba	RGR frond number (day^{-1})	Hutner's	Diuron	25	50	22.3 (0, 45.5)	34.6 (10.5, 58.6)	74.7 (25.0, 124.3)	37.3	Hormetic	h = 0.1; b = 0.61; t = 0.27; e = 74.68	0.96
L. gibba	RGR plant number (day^{-1})	Hutner's	Diuron	25	50	26.1 (0, 59.7)	44.5 (5.8, 83.1)	115.7 (0, 255.4)	57.9	Hormetic	h = 0.12; b = 0.46; t = 0.24; e = 115.74	0.92
L. gibba	Frond number	Hutner's	Atrazine	< 12.5	12.5	41.8 (0, 113.4)	70.8 (0, 150.3)	120.0 (32.9, 207.1)	60.0	Logistic	t = 42.80; b = 2.08; e = 120.0	0.94

Table 2.4 Cont.

Species	Endpoint	Media Type	Compound	NOEC (µg/L)	LOEC (µg/L)	EC_{10} (µg/L) (95% CI)	EC_{25} (µg/L) (95% CI)	EC_{50} (µg/L) (95% CI)	EC_{50} (14 d from Haber's Rule) (µg/L)	Model[a]	Parameters[a]	Adjusted r^2
L. gibba	Plant number	Hutner's	Atrazine	100	200	18.0 (0, 103.2)	48.0 (0, 194.8)	128.4 (0, 365.6)	64.2	Logistic	t = 10.12; b = 1.12; e = 128.38	0.8
L. gibba	Wet mass (g)	Hutner's	Atrazine	100	200	7.1 (0, 36.1)	21.4 (0, 79.4)	64.3 (0, 174.9)	32.1	Logistic	$t = 9.3 \times 10^{-2}$; b = 1.0; e = 64.30	0.88
L. gibba	Dry mass (g)	Hutner's	Atrazine	50	100	13.7 (0, 86.2)	35.6 (0, 160.4)	93.0 (0, 295.9)	46.5	Logistic	$t = 3.8 \times 10^{-3}$; b = 1.16; e = 93.0	0.76
L. gibba	RGR Frond number (day^{-1})	Hutner's	Atrazine	50	100	57.8 (4.8, 110.7)	104.1 (43.5, 164.9)	187.9 (119.2, 256.7)	94.0	Logistic	t = 0.26; b = 1.86; e = 187.92	0.98
L. gibba	RGR plant number (day^{-1})	Hutner's	Atrazine	100	200	36.2 (0, 144.4)	102.9 (0, 279.9)	292.2 (0, 590.6)	146.1	Logistic	t = 0.25; b = 1.05; e = 292.20	0.89
L. minor	Frond number	Hoagland's	Diuron	25	50	22.3 (0, 54.1)	35.0 (3.3, 66.8)	54.9 (22.3, 87.5)	27.5	Logistic	t = 80.2; b = 2.43; e = 54.91	0.92

Table. 2.4 Cont.

Species	Endpoint	Media Type	Compound	NOEC (µg/L)	LOEC (µg/L)	EC_{10} (µg/L) (95% CI)	EC_{25} (µg/L) (95% CI)	EC_{50} (µg/L) (95% CI)	EC_{50} (14 d from Haber's Rule) (µg/L)	Model[a]	Parameters[a]	Adjusted r^2
L. minor	Plant number	Hoagland's	Diuron	25	50	21.6 (0, 49.0)	33.5 (6.5, 60.4)	51.7 (24.5, 78.8)	25.8	Logistic	t = 26.25; b = 2.52; e = 51.7	0.94
L. minor	Wet mass (g)	Hoagland's	Diuron	25	50	19.4 (0, 49.6)	29.2 (0, 58.7)	44.1 (15.1, 73.2)	22.1	Logistic	t = 0.13; b = 2.67; e = 44.12	0.91
L. minor	Dry mass (g)	Hoagland's	Diuron	25	50	27.9 (0, 82.4)	31.7 (0, 67.8)	41.8 (12.1, 71.4)	20.9	Logistic	t = 6.1 x 10^{-3}; b = 4.0; e = 41.78	0.89
L. minor	RGR frond number (day^{-1})	Hoagland's	Diuron	25	50	29.3 (0, 63.6)	54.7 (20.7, 88.6)	102.0 (54.2, 149.7)	51.0	Logistic	t = 0.34; b = 1.76; e = 101.96	0.94
L. minor	RGR plant number (day^{-1})	Hoagland's	Diuron	> 100	> 100	25.8 (7.5, 44.2)	50.3 (31.1, 69.5)	97.7 (70.8, 124.6)	48.9	Logistic	t = 0.38; b = 1.65; e = 97.72	0.98
L. minor	Frond number	Hoagland's	Atrazine	50	100	55.1 (7.8, 102.3)	85.8 (37.6, 134.0)	133.6 (83.5, 183.7)	66.8	Logistic	t = 80.2; b = 2.48; e = 133.59	0.98

Table 2.4 Cont.

Species	Endpoint	Media Type	Compound	NOEC (µg/L)	LOEC (µg/L)	EC_{10} (µg/L) (95% CI)	EC_{25} (µg/L) (95% CI)	EC_{50} (µg/L) (95% CI)	EC_{50} (14 d from Haber's Rule) (µg/L)	Model[a]	Parameters[a]	Adjusted r^2
L. minor	Plant number	Hoagland's	Atrazine	50	100	39.4 (0, 86.6)	76.1 (17.3, 134.9)	146.9 (76.1, 217.7)	73.5	Logistic	t = 28.62; b = 1.67; e = 146.92	0.98
L. minor	Wet mass (g)	Hoagland's	Atrazine	12.5	25	25.9 (5.3, 46.4)	46.8 (22.1, 71.4)	84.5 (55.9, 113.1)	42.3	Logistic	t = 0.15; b = 1.86; e = 84.52	0.99
L. minor	Dry mass (g)	Hoagland's	Atrazine	12.5	25	21.4 (11.1, 31.5)	41.3 (28.2, 54.4)	79.9 (63.6, 96.1)	39.9	Logistic	t = 7.4 x 10^{-3}; b = 1.67; e = 79.86	0.99
L. minor	RGR frond number (day^{-1})	Hoagland's	Atrazine	50	100	78.4 (46.4, 110.5)	130.8 (97.1, 164.6)	218.2 (182.1, 254.3)	109.1	Logistic	t = 0.34; b = 2.15; e = 218.18	0.99
L. minor	RGR plant number (day^{-1})	Hoagland's	Atrazine	50	100	68.9 (32.3, 105.6)	148.8 (103.9, 193.6)	321.0 (260.1, 381.8)	160.5	Logistic	t = 0.39; b = 1.43; e = 320.98	0.99

Chl-a – Chlorophyll-a, RGR – Relative growth rate, CI – Confidence interval

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

Table 2.5 Coefficient of variation (CV), minimum significant difference (MSD), minimum detectable difference (MDD) of various endpoints measured for *Elodea canadensis*, *Lemna gibba*, and *L. minor* exposed to diuron and atrazine for 14 days (*E. canadensis*) and 7 days (*Lemna* spp.).

Species	Endpoint	Media Type	Compound	CV (%)	MSD (%)	MDD (%)
E. canadensis	Wet mass (g)	AAP	Diuron	35	70	65
E. canadensis	Dry mass (g)	AAP	Diuron	57	90	104
E. canadensis	Plant length (cm)	AAP	Diuron	47	47	87
E. canadensis	Root number	AAP	Diuron	37	64	68
E. canadensis	Root mass wet (g)	AAP	Diuron	55	101	101
E. canadensis	Root mass dry (g)	AAP	Diuron	111	164	205
E. canadensis	Root length (cm)	AAP	Diuron	47	96	86
E. canadensis	Chl-a (µg/mg)	AAP	Diuron	27	44	50
E. canadensis	RGR wet mass ($gg^{-1}day^{-1}$)	AAP	Diuron	28	68	52
E. canadensis	RGR dry ($gg^{-1}day^{-1}$)	AAP	Diuron	51	82	94
E. canadensis	RGR Plant length ($cmcm^{-1}day^{-1}$)	AAP	Diuron	41	45	76
E. canadensis	Wet mass (g)	AAP	Atrazine	52	111	96
E. canadensis	Dry mass (g)	AAP	Atrazine	75	142	137
E. canadensis	Plant length (cm)	AAP	Atrazine	137	182	251
E. canadensis	Root number	AAP	Atrazine	86	164	158
E. canadensis	Root mass wet (g)	AAP	Atrazine	105	209	192
E. canadensis	Root mass dry (g)	AAP	Atrazine	145	288	266
E. canadensis	Root length (cm)	AAP	Atrazine	102	175	187
E. canadensis	Chl-a (µg/mg)	AAP	Atrazine	45	51	83
E. canadensis	RGR wet mass ($gg^{-1}day^{-1}$)	AAP	Atrazine	46	97	84
E. canadensis	RGR dry mass ($gg^{-1}day^{-1}$)	AAP	Atrazine	70	129	128

Table 2.5 Cont.

Species	Endpoint	Media Type	Compound	CV (%)	MSD (%)	MDD (%)
E. canadensis	RGR plant length ($cmcm^{-1}day^{-1}$)	AAP	Atrazine	122	158	224
E. canadensis	Wet mass (g)	AAP w/o NP	Diuron	132	452	242
E. canadensis	Dry mass (g)	AAP w/o NP	Diuron	110	453	202
E. canadensis	Plant length (cm)	AAP w/o NP	Diuron	121	188	221
E. canadensis	Root number	AAP w/o NP	Diuron	62	108	113
E. canadensis	Root mass wet (g)	AAP w/o NP	Diuron	80	133	148
E. canadensis	Root mass dry (g)	AAP w/o NP	Diuron	105	213	194
E. canadensis	Root length (cm)	AAP w/o NP	Diuron	82	115	150
E. canadensis	Chl-a (µg/mg)	AAP w/o NP	Diuron	27	40	50
E. canadensis	RGR wet mass ($gg^{-1}day^{-1}$)	AAP w/o NP	Diuron	154	517	283
E. canadensis	RGR dry mass ($gg^{-1}day^{-1}$)	AAP w/o NP	Diuron	158	571	291
E. canadensis	RGR plant length ($cmcm^{-1}day^{-1}$)	AAP w/o NP	Diuron	114	170	209
E. canadensis	Wet mass (g)	AAP w/o NP	Atrazine	119	184	219
E. canadensis	Dry mass (g)	AAP w/o NP	Atrazine	67	91	123
E. canadensis	Plant length (cm)	AAP w/o NP	Atrazine	136	173	250
E. canadensis	Root number	AAP w/o NP	Atrazine	53	103	97
E. canadensis	Root mass wet (g)	AAP w/o NP	Atrazine	86	127	159
E. canadensis	Root mass dry (g)	AAP w/o NP	Atrazine	99	160	182
E. canadensis	Root length (cm)	AAP w/o NP	Atrazine	87	123	160
E. canadensis	Chl-a (µg/mg)	AAP w/o NP	Atrazine	28	43	52
E. canadensis	RGR wet mass ($gg^{-1}day^{-1}$)	AAP w/o NP	Atrazine	116	176	213

Table 2.5 Cont.

Species	Endpoint	Media Type	Compound	CV (%)	MSD (%)	MDD (%)
E. canadensis	RGR dry mass ($gg^{-1}day^{-1}$)	AAP w/o NP	Atrazine	58	81	106
E. canadensis	RGR plant length ($cmcm^{-1}day^{-1}$)	AAP w/o NP	Atrazine	133	171	244
L. gibba	Frond number	Hutner's	Diuron	24	26	59
L. gibba	Plant number	Hutner's	Diuron	30	41	73
L. gibba	Wet mass (g)	Hutner's	Diuron	26	28	64
L. gibba	Dry mass (g)	Hutner's	Diuron	32	33	79
L. gibba	Chl-a (µg/mg)	Hutner's	Diuron	20	34	49
L. gibba	RGR frond number (day^{-1})	Hutner's	Diuron	11	13	28
L. gibba	RGR plant number (day^{-1})	Hutner's	Diuron	13	22	33
L. gibba	Frond number	Hutner's	Atrazine	5	13	11
L. gibba	Plant number	Hutner's	Atrazine	30	31	74
L. gibba	Wet mass (g)	Hutner's	Atrazine	11	14	27
L. gibba	Dry mass (g)	Hutner's	Atrazine	21	27	52
L. gibba	Chl-a (µg/mg)	Hutner's	Atrazine	6	21	16
L. gibba	RGR frond nubmer (day^{-1})	Hutner's	Atrazine	2	7	5
L. gibba	RGR plant number (day^{-1})	Hutner's	Atrazine	15	18	36
L. minor	Frond number	Hoagland's	Diuron	11	17	28
L. minor	Plant number	Hoagland's	Diuron	12	16	29
L. minor	Wet mass (g)	Hoagland's	Diuron	13	16	31
L. minor	Dry mass (g)	Hoagland's	Diuron	11	19	28
L. minor	Chl-a (µg/mg)	Hoagland's	Diuron	7	14	18

Table 2.5 Cont.

Species	Endpoint	Media Type	Compound	CV (%)	MSD (%)	MDD (%)
L. minor	RGR frond number (day^{-1})	Hoagland's	Diuron	4	7	11
L. minor	RGR plant number (day^{-1})	Hoagland's	Diuron	4	9	10
L. minor	Frond number	Hoagland's	Atrazine	18	18	44
L. minor	Plant number	Hoagland's	Atrazine	16	18	39
L. minor	Wet mass (g)	Hoagland's	Atrazine	4	9	10
L. minor	Dry mass (g)	Hoagland's	Atrazine	8	12	20
L. minor	Chl-a (µg/mg)	Hoagland's	Atrazine	7	9	17
L. minor	RGR frond number(day^{-1})	Hoagland's	Atrazine	7	7	16
L. minor	RGR plant number (day^{-1})	Hoagland's	Atrazine	10	12	24

Chl-a – Chlorophyll-a, RGR – Relative growth rate, CV – coefficient of variation, MSD – minimum significant difference, MDD – minimum detectable difference

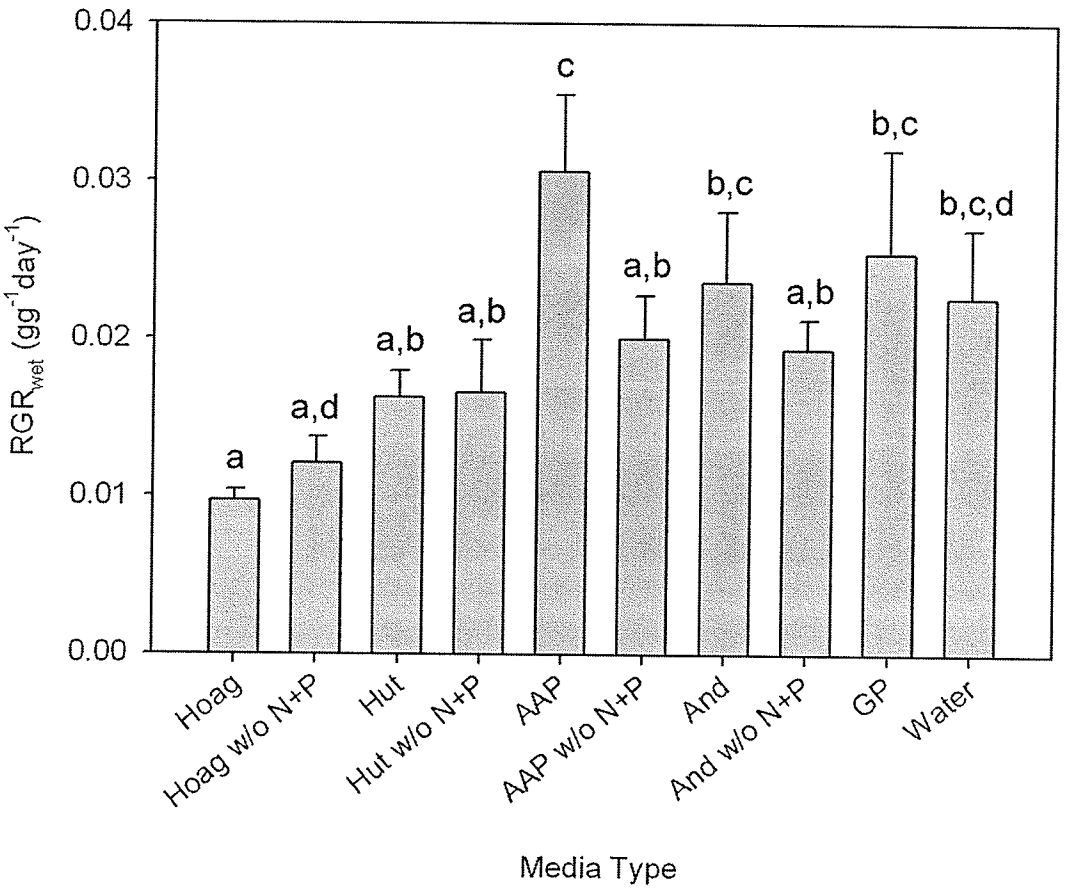

Figure 2.1 Relative growth rate (RGR) based on wet mass of *Elodea canadensis* shoots grown over a 14-day period in various culture media with root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

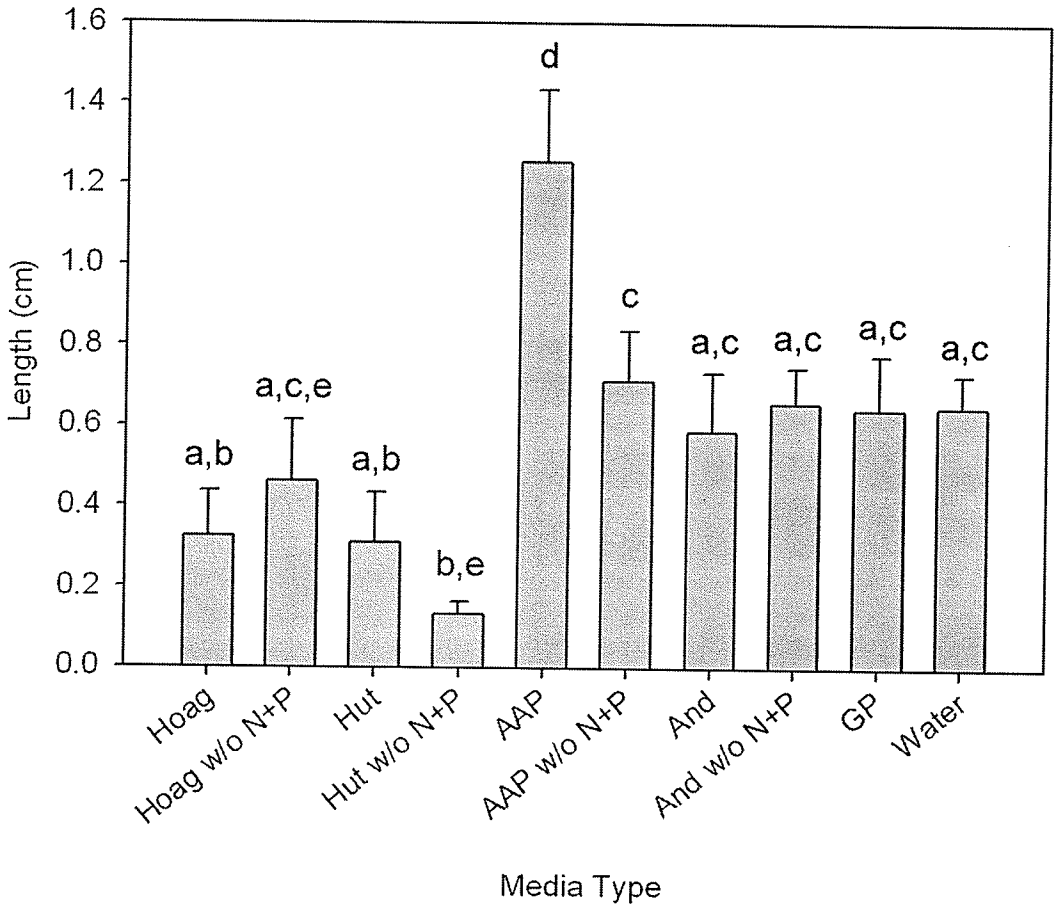

Figure 2.2 Plant length of *Elodea canadensis* shoots grown over a 14-day period in various culture media with root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

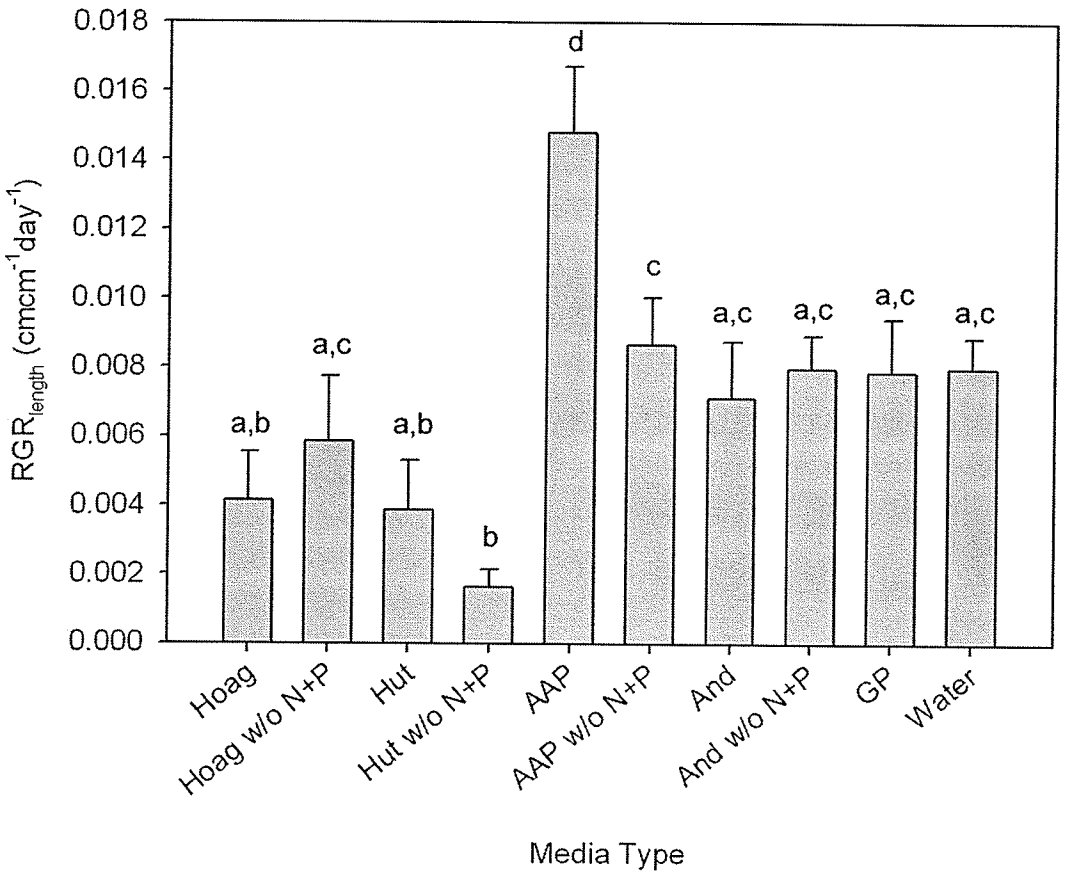

Figure 2.3 Relative growth rate (RGR) based on length of *Elodea canadensis* shoots grown over a 14-day period in various culture media with root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

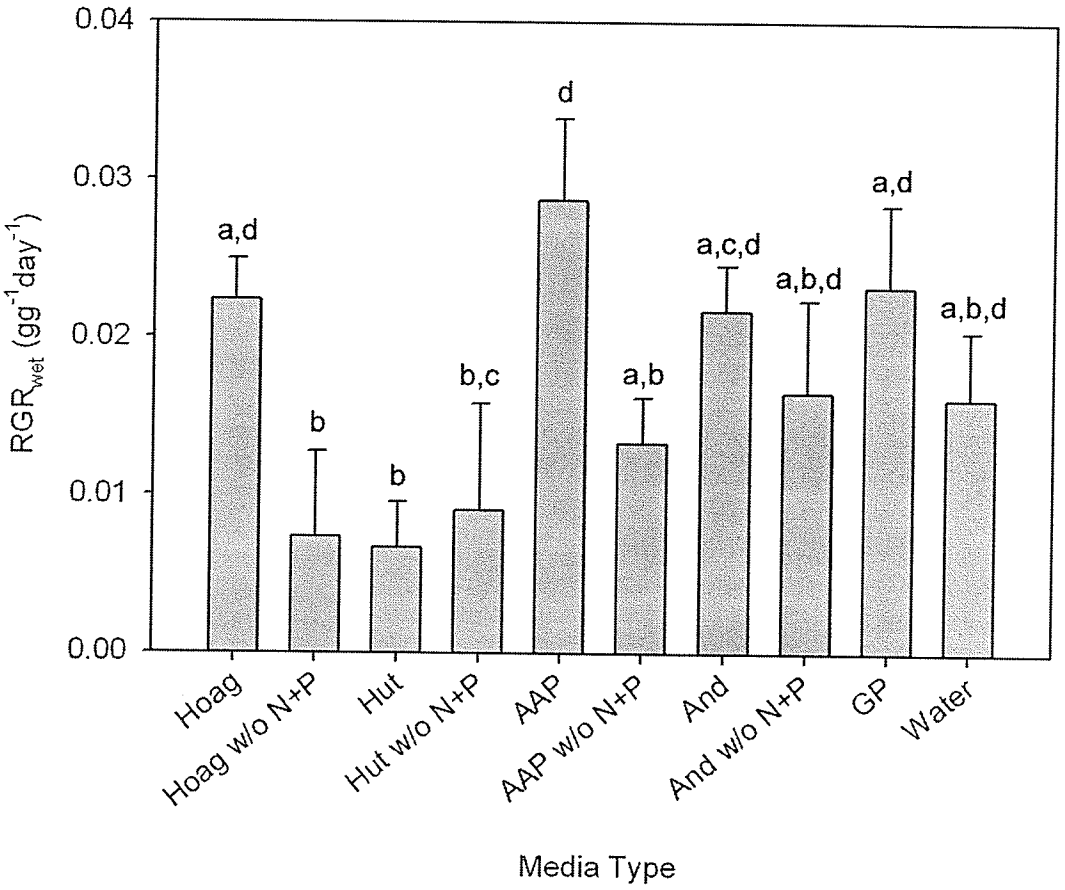

Figure 2.4 Relative growth rate (RGR) based on wet mass of *Elodea canadensis* shoots grown over a 14-day period in various culture media without root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

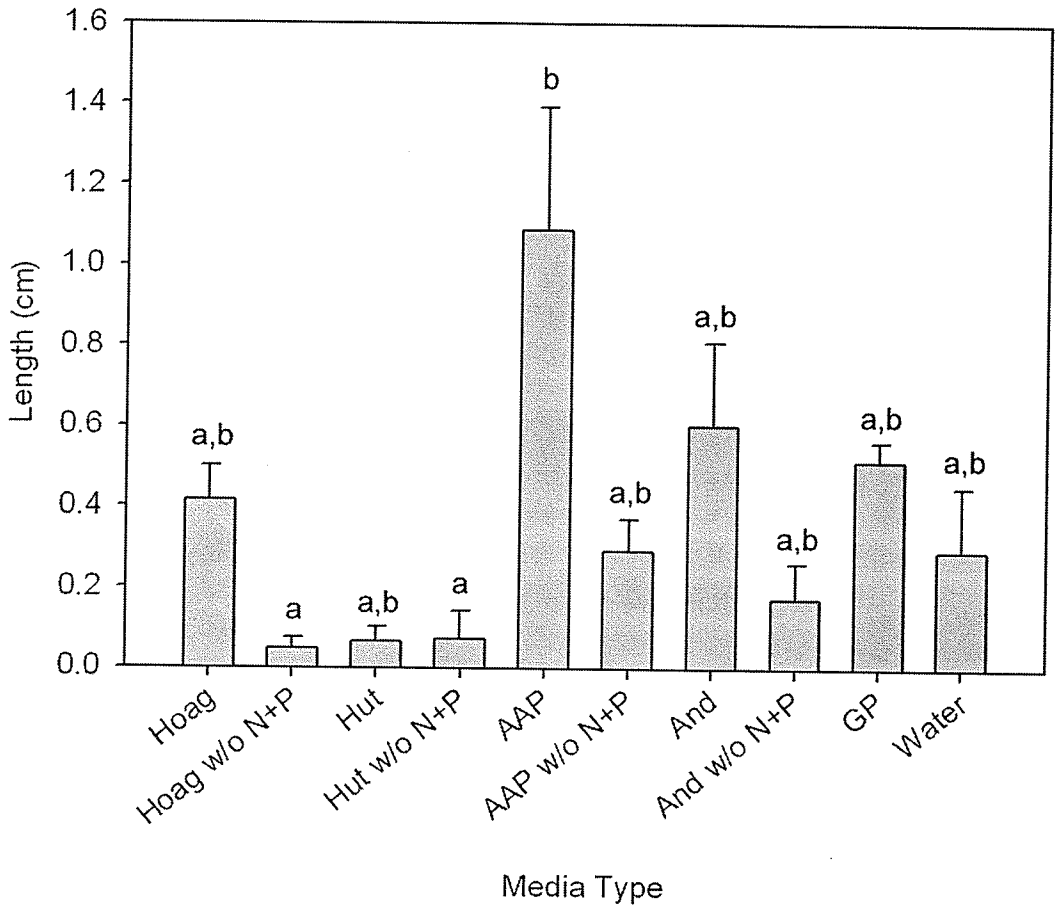

Figure 2.5 Plant length based on new growth of *Elodea canadensis* shoots grown over a 14-day period in various culture media without root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

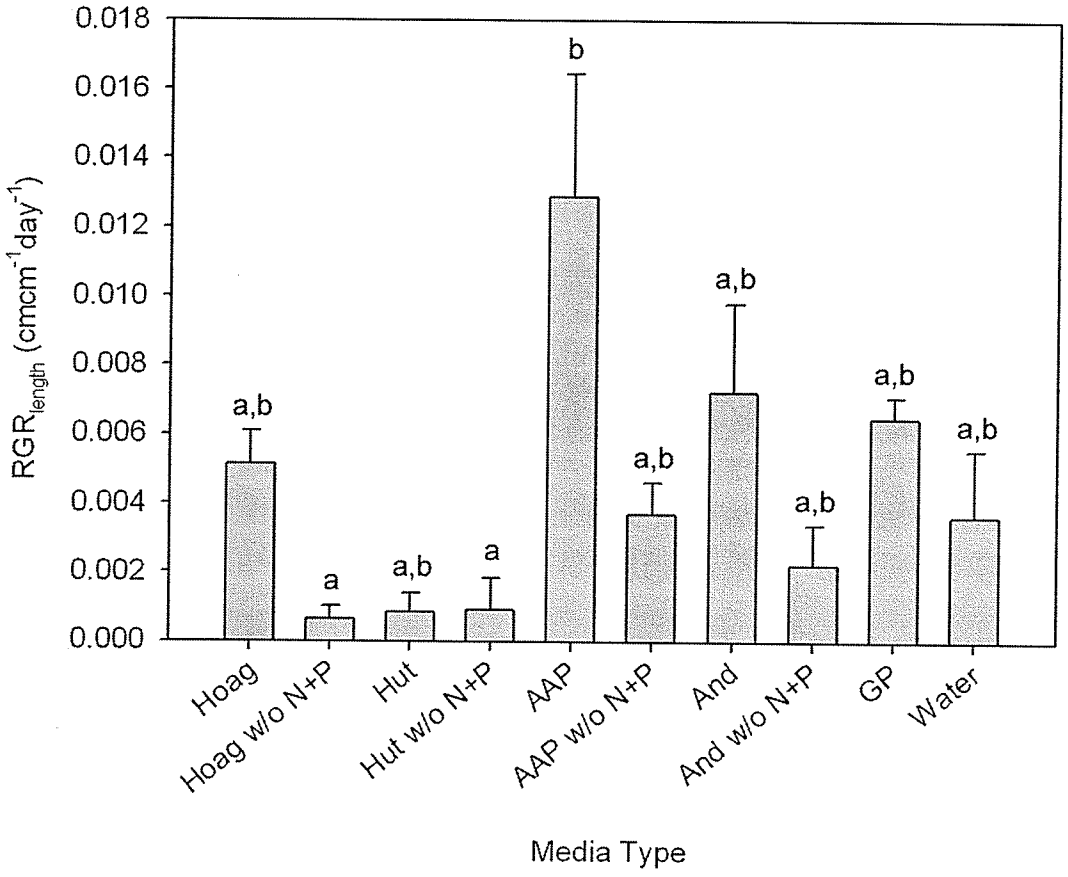

Figure 2.6 Relative growth rate (RGR) based on length of *Elodea canadensis* shoots grown over a 14-day period in various culture media without root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

3 UNDERSTANDING THE INFLUENCE OF RELATIVE GROWTH RATE, POPULATION DENSITY, AND STAND ESTABLISHMENT ON TOXICITY TO AQUATIC MACROPHYTES

3.1 ABSTRACT

The role of relative growth rate (RGR), population density and stand establishment on the toxicity of the herbicide diuron in the macrophyte *Elodea canadensis* were investigated under semi-natural field conditions. Diuron was applied at 0, 1, 10, 100, and 1000 µg/L to 12000 L outdoor aquatic microcosms for a 42-day exposure period. Newly planted model populations of *E. canadensis* at three densities, individual plants and model populations that had been established in advance of exposure were characterized for their responses to diuron. Toxicity was also assessed with *Lemna gibba* and *Myriophyllum spicatum* to compare relative sensitivities. The endpoints measured included biomass (wet/dry), RGR (for wet and dry mass), root mass (wet/dry), shoot mass (wet/dry), root number, chlorophyll-a, and shoot length. Individual plants of *E. canadensis* showed poor growth compared to those grown in populations likely due to space restrictions. Populations of macrophytes with higher densities showed lower RGRs and also showed less sensitivity to diuron. Also, established populations exhibited significantly lower RGRs than non-established populations and were, again, less sensitive to diuron. Progressing from most sensitive to least sensitive of the three macrophytes tested were *M. spicatum* > *L. gibba* > *E. canadensis* as individuals. These findings support the hypothesis that aquatic plants with higher RGRs, whether due to population density or stage of stand development, can show greater sensitivity to herbicides. The

implications for risk assessment are clear in that the developmental status (ie. establishment and population density) of the macrophyte community should be considered when conducting higher tier risk assessments, especially for herbicidal compounds.

3.2 INTRODUCTION

Aquatic macrophytes are important ecological components that produce oxygen, assist in nutrient cycling, improve water quality by assimilating heavy metals (Gouder de Beauregard and Mahy, 2002), stabilize sediments with their root structure (Wang and Freemark, 1995), and provide food for aquatic organisms (Davy et al., 2001). Despite their importance in ecosystems, they are considered as under-represented in the ecological risk assessment process of pesticides (Wang and Freemark, 1995; Davy et al., 2001). In the past, toxicity data for algae have been considered representative of macrophyte toxicity, but studies have shown that macrophytes can be significantly more sensitive than algae in some cases (Fletcher, 1990; Roshon et al., 1999), demonstrating the risks of ignoring macrophyte responses, especially for herbicides. In order to accurately characterize toxicity in aquatic plants, we need to consider the ecological relevance of the methodologies and the test systems in which they are applied, the species tested, and the exposure scenario modeled.

One approach for increasing the understanding of risk to aquatic macrophytes is the application of microcosms in order to validate the results of single species laboratory assays (Breitholtz et al., 2006). More complex field based tests can give insight to the relevance of results found in simple laboratory based studies. Included in these are

interactions between and within species that may modify responses at the population and community level (Mohr et al., 2007; McGregor et al., 2007; Hanson et al., 2009).

Current test methods, both in the field and the laboratory, rely on a small selection of macrophyte species. Currently only two species, *Lemna* spp. and *Myriophyllum* spp., have been widely used in toxicity testing for which a standard method has been developed (Davy et al., 2001). At the moment, only *Lemna spp.* is required for testing from a regulatory perspective (Davy et al., 2001; Breitholtz et al., 2006). Yet, it is well known that one species will never be consistently the most responsive or sensitive. For example, in an interspecies comparison of toxicity of sulfonylurea and imazolidinone herbicides, Roshon et al., (1999) showed that rooted macrophytes often showed higher sensitivity than floating macrophytes. Also, Fairchild et al. (1998) showed three other species of aquatic macrophytes (*Ceratophyllum sp.*, *Najas sp.*, and *Elodea sp.*) to be more sensitive to four herbicides than *L. minor.*

Laboratory investigations of population-level effects of toxicants suggest differences in sensitivity compared to effects measured on individuals (Forbes et al., 2001; Forbes and Calow, 2001). It has been noted that individual plants exhibit lower relative growth rates (RGRs) compared to model populations of the same macrophyte in microcosms (McGregor et al., 2007). Macrophytes exhibiting higher RGRs have been shown to be more sensitive to herbicide exposure under laboratory conditions (Huebert and Shay, 1993; Cedergreen et al., 2004b). In addition, no work appears to have been conducted to characterize the effect of macrophyte stand establishment on observed toxicity, i.e. do established populations respond differently than newly introduced or developing plants? Current literature tends to describe methods that use individual plants

exclusively for both laboratory and field based studies (McGregor et al., 2007; Brain et al., 2005; Hanson et al., 2001), though there are microcosm studies that do examine effects at the population or community level, the interactions between species or the ecological mechanisms behind the impacts are not explicitly examined (Mohr et al., 2007; Van Wijngaarden et al., 2004).

The primary test organism for this study was Canadian Pondweed, *Elodea canadensis* Michx, a rooted, dioecious, monocotyledonous, submersed aquatic macrophyte with characteristic dichotomously branched shoots with whorls of three leaves 6 – 17 mm long and 1 – 5 mm wide (Spicer and Catling, 1988). *Elodea* tends to grow well in communities with *Ceratophyllum demersum*, *Myriophyllum spicatum*, *Myriophyllum exalbescens*, *Ultricularia vulgaris*, and *Potamogeton* spp (Spicer and Catling, 1988). *E. canadensis* has been used in studies regarding the effects of metals on macrophytes (Stoyanova and Tchakalova, 1993), the use of macrophytes for phytoremediation of environmental contaminants (Rice et al., 1997), as well as the effects of herbicides (Fairchild et al., 1998; Cedergreen et al., 2004b; Cedergreen et al., 2004a).

The model stressor used was the common herbicide diuron (N'(3,4-dichlorophenyl)-N'N-dimethylurea), used to treat broadleaf weeds and grasses in crops such as alfalfa, small grains, and a variety of fruit crops (Ware and Whitacre, 2004). It is moderately sorbed to soil/sediment with a K_{oc} of 418-560 ml/g and an aerobic soil half-life of 372 days (Moncada, 2008). Diuron is also persistent in water with hydrolysis and photolysis half-lives ranging from 43 – 2180 days (Moncada, 2008). Therefore, diuron is considered persistent in the environment. Agricultural application rates range from 0.22 – 7.2 kg active ingredient/hectare, while non-agricultural (roadside/railroad defoliating) is

about 0.9 – 13.5 kg active ingredient/hectare (USEPA, 2003). The mode of action of diuron is via the inhibition of photosynthesis (Ware and Whitacre, 2004). Diuron binds to the D1 protein of photosystem II blocking electron transport, thus preventing carbon dioxide fixation, halting ATP production for plant growth (Moncada, 2008). The toxicity of diuron to aquatic macrophytes has been characterized for a limited number of species. Teisseire et al. (1999) reported the diuron 7-d EC_{50} for *L. minor* relative growth rate inhibition for fronds was 25±3 µg/L. Chevre et al. (2006) also reported a 7-d EC_{50} of 25 µg/L for *Lemna minor*, though the specific endpoint was not reported. A study conducted by Liu and Cendeno-Maldonado (1974) found EC_{50}s of population growth inhibition for *Spirodela polyrhiza* and *Lemna perpusilla* to be 41 and 15 µg/L respectively. The macrophyte *Halophila ovalis* exhibited toxicity to diuron with an EC_{50} of 100 µg/L for chlorophyll-a (Ralph, 2000). All toxicity data presented in literature have been in a laboratory setting, with no toxicity characterization occurring in the field.

Environmental levels of diuron have also been reported in a variety of agricultural areas. The South Florida Water Management District monitors 32 surface water sampling sites in areas used extensively for sugar cane and citrus fruit production. During the December 1998 sampling period, only two sites contained diuron above the detection limit of 0.4 – 0.8 µg/L. These sites showed concentrations of 0.64 and 1.2 µg/L (Pfeuffer, 1998). A review of data reported by the United States Geological Survey (USGS) found that out of 942 samples from agricultural applications (with 7.96% positive detections) and 315 samples from urban application (with 13.02% positive detections), mean concentrations of diuron were below levels of detection (0.05 µg/L) for both urban and agriculture use patterns (APVMA, 2005). A monitoring study in

Australia over a 5 year period (1990 – 1995) covered an area of >7.0 x 10^5 ha mainly used for citrus crops. It was found that surface runoff samples, although showing ~40% detections of diuron, reached maximums of only 20 µg/L with creeks draining large irrigation areas reaching maximums of 7.5 µg/L (APVMA, 2005). Collections of runoff from one particular citrus farm with 4.5 kg ai/ha applied showed diuron concentrations ranging from 1.2 – 20 µg/L with a mean of 10.9 µg/L after the first rainfall event (APVMA, 2005). It is clear from these collected data that diuron from agricultural applications like citrus farming leads to much lower concentrations in surface water compared to more intensive applications like roadside ditches or grass seed farms.

Very few regulatory levels of diuron have been established. The Pesticide Management Regulatory Agency of Canada has not set a maximum residue limit for diuron, but states that under the Food and Drug Regulations subsection B.15.002(1) residues cannot exceed 0.1 mg/L on food products (PMRA, 2006). Since diuron is consistently found in the surface waters of the United Kingdom, a non-statutory Environmental Quality Standard for diuron of 0.1 µg/L was established (PAN-UK, 2005). In Australia the ADI (acceptable daily intake) is 6.0 x 10^{-3} mg/kg bw/day (APVMA, 2005). Other than these few guidelines, no others are published for diuron.

In regards to this study, our objectives were to; 1) assess the toxicity of diuron to *E. canadensis*, *L. gibba*, and *M. spicatum* under field conditions, 2) characterize the effect of population density on toxicity of *E. canadensis* by exposing the macrophyte grown at three different population densities, 3) characterize the effects of stand establishment on toxicity by contrasting newly introduced populations with established *E. canadensis* populations and, 4) determine the relationship between relative growth rate

(RGR) and toxicological sensitivity, specifically within newly developing populations of different densities and individual plants.

3.3 MATERIALS AND METHODS

3.3.1 The microcosms

The study was conducted at the University of Guelph Microcosm Facility located at the Guelph Turfgrass Institute. Fifteen of thirty outdoor experimental microcosms (diameter: 4 m, depth: 1.2m, water depth: 1.0 m, surface area = 11.95 m^2) were used for the study, each holding a volume of approximately 12,000 L (Figure 3.1D). Microcosms were cleaned of all residual material and sediment, filled with water from an adjacent well-fed pond (62 x 62 x 4 m), and allowed to circulate prior to treatment with diuron for approximately three weeks. Circulation was halted three days prior to the start of the study (June 25, 2007) in order to make each microcosm a self-contained unit. Unlike previous studies at this facility (i.e., McGregor et al., 2007), sediment in large plastic trays at the bottom of the microcosms was not added. This was done to ensure that the majority of the diuron remained in the water column due to its potential to partition into sediment (Harino et al., 2007) thereby easing the analytical measurements and modeling of diuron exposure in the macrophytes. The only sediment present was the sediment in each population tray or cone-tainer used to grow *E. canadensis* shoots.

3.3.2 Diuron exposure

After a preliminary laboratory range-finding test exposing *E. canadensis* to a range of diuron concentrations based on calculated expected environmental concentrations from typical agricultural application rates, a range of effective diuron

concentrations was determined. Concentrations of 1, 10, 100, and 1000 μg/L of diuron (80% active ingredient, DuPont, Mississauga, ON, Canada), plus controls (0 μg/L) were applied to randomly selected microcosms in an ANOVA design (n=3) on June 28, 2007. The diuron was in a granular form and appropriate masses were placed into amber glass bottles (250 mL), which were then filled with microcosm water, and shaken to a slurry and then poured slowly into the microcosm and mixed with a paint-mixing rod powered by an electric drill as previously described (Brain et al., 2004).

3.3.3 Water sampling and analysis

Using a depth-integrated water sampler (Solomon et al., 1982), approximately 4 L of water was collected from random locations within each microcosm on day -1, 0, 7, 14, 28, and 42. Aliquots were transferred to a 1L amber glass bottle for diuron residue analysis, to a 500 mL nalgene bottle for pH/conductivity/hardness/alkalinity analysis, and to a 500 mL nalgene bottle for nutrient analysis. Samples for nutrients (total phosphorus and total nitrogen) were kept frozen at -20°C while diuron residue and hardness/alkalinity aliquots were kept in a refrigerator at 4°C until analysis. The pH of each sample was taken using the Accumet Research AR20 pH/conductivity meter (Fisher Scientific, Waltham, MA, USA) on the day of sampling. Hardness and alkalinity were measured using kits from Hach (Hach Company, Ames, Iowa, USA). Nutrient analysis (total nitrogen and phosphorus) was conducted using persulfate oxidation with the QuikChem FIA+ 8000 System (Lachat Instruments, Loveland, CO) according to Qualls (1989).

Diuron residue analysis was conducted using ELISA (enzyme linked immunosorbent assay) within 24 hours of sampling (Abraxis Kits, Warminster, PA, USA). Prior to ELISA analysis, 10 mL aliquots of the residue samples were centrifuged

for 10 minutes at 805 g. This was a microplate method with a detection limit of 0.03 μg diuron /L and quantification was in triplicate using a Bio-Rad Model 680 microplate reader (Hercules, CA, USA). Samples were diluted when necessary to ensure they fell within the range of the standard five-point curve (0.03 – 3 μg/L). Specific methods of the analysis were conducted as per the ELISA kit manual. Results obtained were then used to calculate relevant time weighted averages for diuron concentrations occurring over the 42-d exposure period.

Along with the biweekly sampling, daily dissolved oxygen (YSI Model 85, YSI Incorporated, Yellow Springs, OH, USA) and maximum/minimum temperature measurements were taken in each microcosm before 9:00 am at a depth of 30 cm. On the biweekly sampling days, a dissolved oxygen profile was taken to determine the differences in dissolved oxygen at different depths in the microcosms. This was conducted at sunrise and sunset on that day at both 30 cm and 60 cm. On day 0, 7, 14, 28, and 42 the photosynthetically active radiation was determined using the Li-Cor Quantum/Radiometer/Photometer Model LI-185A (Lincoln, NB, USA) at a depth of 60 cm. Readings were taken between noon and 2 pm when the sun was at its maximum intensity.

3.3.4 Macrophyte introduction, sampling, and analysis

3.3.4.1 Individual plant studies (*Elodea* canadensis and *Myriophyllum spicatum*)

Elodea canadensis Michx. were harvested from existing populations in control microcosms from previous studies, artificial lakes located at the Correctional Facility Recreation Area in Guelph, Ontario, and the Eramosa River in Guelph, Ontario.

Myriophyllum spicatum was harvested from the same areas. Cone-tainers (Steuwe and Sons, Corvallis, OR, USA) were used to grow individual plant segments as in previous studies (Hanson et al., 2001). Cone-tainers (14 cm long with an internal diameter of 3.8 cm) were filled with an organic rich sediment (1:1:1 mixture of topsoil:manure:compost) (Waterdown Garden Supply, Troy, ON). Total organic and inorganic carbon content of the sediment was analyzed on a Leco SC444 (Leco, St. Joseph, MI, USA) and found to be 13.3% carbon (1.4% inorganic and 11.9% organic). The filled containers were soaked in their respective microcosms for 24-h prior to the planting and introduction of the plants into the microcosms, which took place on June 27, 2007 (day -1). Individual apical meristematic segments without sideshoots (5 cm) were planted into each cone-tainer. Each plant segment was planted approximately 1-2 cm deep and surrounded by approximately 0.5 cm (depth) of Turface to provide a visual contrast between the plant and the sediment as well as to help secure the plant in the sediment. Six *E. canadensis* and six *M. spicatum* cone-tainers were placed in their respective microcosms. The cone-tainers were evenly spaced in a 96 well plastic plant rack (Steuwe and Sons, Corvallis, OR, USA) to keep them upright (Figure 3.1B). The rack was placed in the center of each microcosm to provide optimal light conditions for growth and reduce possible edge effects. On the day of planting, 20 shoots of both *E. canadensis* and *M. spicatum* were measured for total wet and dry mass. These measurements were then averaged for each plant species to give a mean pre-treatment measurement of each endpoint.

Sampling of individual plants occurred on 14-d, 28-d and 42-d. *E. canadensis* and *M. spicatum* were removed from their cone-tainers, rinsed clean of sediment using microcosm water, and placed into individual labeled container for transport to the

laboratory. Endpoints analyzed for both species included; biomass (wet/dry weight) of roots and shoots, and chlorophyll-a content. Number of roots and plant length were determined for *M. spicatum* only, since *Elodea* exhibits a high degree of branching and sends out few roots. All plants were further washed with distilled water and spun dry in a salad spinner. Wet and dry masses of individual plants were taken with Sartorius CP124S analytical balance (Elk Grove, IL, USA). Dry mass was taken after 24-h of drying at 60°C. Chlorophyll-a was calculated using standard equations (Nusch, 1980) from extractions made with a weighed section (~ 50 mg, not macerated) of an apical meristematic shoot in 10 mL of 90% ethanol extracted for 24 h. Absorption was measured on a Ultrospec 3100 pro UV/visible Spectrophotometer (Amersham Biosciences, Piscataway, NJ, USA).

3.3.4.2 *E. canadensis* population density studies

Plastic rectangular trays ((l x w x d) 33.02 x 17.78 x 10.16 cm) were filled with the same soil as the cone-tainers and left to soak in their respective microcosms for at least two days (Figure 3.1A). Each microcosm contained nine trays total, which were planted with 5 cm apical meristematic segments of *E. canadensis*. Trays were planted with 9, 18, and 27 plants (0.015, 0.031 and 0.0467 plants/cm^2), based on natural densities of the *M. spicatum* (0.016 plants/cm^2) observed in Fish Lake, Wisconsin (Lillie et al., 1997). This density could reflect those of *E. canadensis* since these two species grow co-dominantly in similar conditions, are ecologically similar (Abernethy et al., 1996) and similar densities were successfully used in other microcosm studies with *E. canadensis* with good growth observed at these densities (McGregor et al., 2007; McGregor et al., 2008). All populations were planted and submerged on June 26, 2007. The three trays

for each density were arranged around the rack containing the cone-tainers with one tray sampled at 14, 28 and 42 d. The endpoints monitored were total biomass (wet/dry). The same data used to determine a time zero biomass for individual plants were used for population studies to derive a mean pre-treatment measurement for each population of *E. canadensis*. The individual plant mean was multiplied by 9, 18, and 27 in order to derive estimates of pre-treatment biomass for each population density studied.

3.3.4.3 Established population studies (*E. canadensis*)

Methods for planting the established cultures were similar to those previously described for population density studies. Three plant trays were placed into each microcosm at random on May 14, 2007 to allow sediment to soak and settle in the microcosm water. On May 16, 2007, the trays were planted with eighteen 5 cm apical meristematic shoots of *E. canadensis* (a density of 0.031 plants/cm^2) which was chosen since it was reflective of a natural moderate macrophyte density and low enough to ensure that the amount of available plant material was not exceeded. The plants were left to grow for 43 days in order to become established populations. Established population trays were arranged around the cone-tainer rack along with the newly established population trays (Figure 3.1D). After a 43-d establishment period, populations exhibited very dense growth to the point where original individual plants were no longer distinguishable. Measurements of wet mass and dry mass per tray were taken on June 27, 2008 by removing all biomass from three trays and rinsing off sediment attached to roots. The three masses were then averaged to derive a mean pre-treatment total biomass measurement.

3.3.4.4 *Lemna gibba* studies

Lemna gibba were obtained from an axenic laboratory culture established at the University of Waterloo, Waterloo, ON maintained in 250 mL Erlenmeyer flasks according to Marwood et al. (2001). Two weeks prior to the start of the study, *L. gibba* were transferred to a 2800-mL flask with 1 L of sugarless Hunters' media (Greenberg et al., 1992). Immediately after microcosm treatment with diuron, *L. gibba* were placed into floating corrals (38 x 14 cm) containing 3 separate cells (Figure 3.1C). Two *L. gibba* plants with a minimum of three fronds per plant were placed into each cell. All *L. gibba* were collected from each cell of their respective corrals after 7 days of exposure (July 5, 2007) and analyzed for biomass (wet/dry), chlorophyll-a, and plant and frond number. Chlorophyll-a was analyzed as described above.

3.3.5 Statistical analysis

3.3.5.1 NOEC and LOEC

A one-way analysis of variance (ANOVA) design was used to determine the significance of the effect of diuron concentration on the various endpoints tested. Data were first tested with the Kolmogorov-Smirnov test and a Levene Median test to determine if the ANOVA assumptions of normally distributed residuals and equal variance were satisfied. Data not conforming to ANOVA assumptions were by ln or square root transformed. If assumptions were still not met, data were compared with a Kruski-Wallis one-way ANOVA in SigmaStat 3.5 (Systat Software 2006, Jandel, San Rafael, CA, USA). If significance was found ($p<0.05$), means were compared to controls using the Dunnett's test, which was used to derive the no observable effect concentrations (NOEC) and lowest observable effect concentrations (LOEC).

3.3.5.2 Effective concentrations (EC_x)

Before regression analysis was conducted, all pre-treatment endpoint measurements were subtracted from all other time point measurements in order to assess new plant growth (shoot length and biomass) only. This provides a more sensitive measure of response. Time-weighted averages of diuron in the microcosms were determined after 14, 28 and 42 days and used in all calculations to represent exposure for that particular time point and were used for the subsequent regression analysis. Data were analyzed in proc NLIN procedure in SAS v9.1 (SAS Institute, Cary, NC, USA) and Sigmaplot 2000 (SPSS Inc. Chicago, IL, USA) in order to determine EC_{10}, EC_{25}, and EC_{50} values (McGregor et al., 2007; Stephenson et al., 2000) from a three-parameter logistic model with the equation:

$$f = t/(1 + (x/e)^b)$$

Where the variable e is the EC_{50} of the concentration-response relationship modeled, x is the actual concentration being evaluated (µg/L), f is the response or change from control of the modeled endpoint and b, t and h are constants. Correlation coefficients were calculated by inputting response data and diuron concentrations into SigmaPlot 2000 and fitting the data to a three- parameter logistic model.

3.3.5.3 Relative growth rate (RGR)

Relative growth rates (RGR) for wet and dry biomass were calculated using the equation presented in (Hoffmann and Poorter, 2002):

$$RGR=(\ln (W_2) - \ln (W_1))/(t_2 - t_1)$$

Where W_2 = final endpoint measurement, W_1 = initial endpoint measurement, T_2 =final timepoint and T_1 = initial timepoint.

RGR data for control microcosms were compared to determine if population density influenced RGR. Relative growth rates of control microcosms for individuals and populations were analyzed using a one-way ANOVA in SigmaStat 3.5 (Systat Software 2006, Jandel, San Rafael, CA, USA) with non-parametric methods if ANOVA assumptions were not met ($\alpha = 0.05$). Control microcosm data for established and non-established populations were also compared. A t-test ($\alpha = 0.05$) was used to determine if RGRs were significantly different in established and non-established populations at the same density (ie. 0.031 plants/cm^2). Relative growth rate data for individuals and populations at all treatment levels were also used as an endpoint for toxicity with statistical methods described in the two previous sections.

3.3.5.4 Interactions between density and observed toxicity

A two-way ANOVA was also conducted on the various endpoints measured in the population density study to determine if there were any significant interactions between density and observed response due to exposure to diuron. This was conducted in SigmaStat 3.5 (Systat Software 2006, Jandel, San Rafael, CA, USA). The factors of diuron concentration and population density were used to determine if there were significant ($p < 0.05$) interactions between the two factors. Data not conforming to the assumptions of ANOVA were analyzed with non-parametric methods in a two-way ANOVA on ranks.

3.4 RESULTS

3.4.1 General parameters

Physical and chemical parameters measured in microcosms are summarized in Table 3.1. All parameters were measured over the 42-day exposure period along with pre-exposure measurements starting at day -3. Results presented are averages of the entire study period for each treatment. Most parameters showed little variation amongst treatments with no significant differences between treatments with the exception of alkalinity where significant differences ($p<0.05$) occurred between the control means and all other treatments. The 1000 µg/L treatment was also significantly different from the 100 and the 10 µg/L treatments. In addition, daily dissolved oxygen measurement showed significant differences amongst the treatment levels with data summarized in Figure 3.2. A concentration response relationship was observed in the dissolved oxygen and alkalinity measurements in the microcosms. Higher exposure concentrations showed higher alkalinity and lower dissolved oxygen measurements compared to lower exposure concentrations.

3.4.2 Fate of diuron

Mean dissipation of diuron from the water column over the entire 42 days was 76% (Figure 3.3). Time weighted averages were calculated for each sampling day and each treatment concentration (Table 3.2). Half-lives of diuron were calculated for each treatment (Table 3.2) assuming pseudo first order kinetics using SigmaPlot 2000 (SPSS Inc. Chicago, IL, USA). Half-lives ranged from 12 – 33 days depending on the exposure concentration (Table 3.2).

3.4.3 Macrophyte growth and RGRs

Differences in RGR between population densities in control microcosms were compared to determine if this factor affected growth (Figures 3.4 and 3.5). Although most results were not statistically significant, there seemed to be a trend towards generally decreasing RGR as population density increased. Individual shoots of *E. canadensis* had statistically significantly ($p<0.05$) lower RGRs than the population grown at 0.015 plants/cm^2. When established and non-established populations were compared, established cultures had statistically significantly ($p<0.05$) lower RGRs than non-established cultures on days 28 and 42 (Figure 3.6 and 3.7).

3.4.4 Toxicity

3.4.4.1 NOEC and LOEC

For *E. canadensis* (Table 3.4), total wet mass, total dry mass, RGR_{wet}, and RGR_{dry} all showed significant differences ($p< 0.05$) from controls across all time points. There were some trends in NOEC or LOEC indicating differential sensitivity between population densities. These were mainly evident between the highest (0.042 plants/cm^2) and the lowest density (0.015 plants/cm^2) for the endpoints RGR_{dry} (day 28), total wet mass (day 28, 42), and RGR_{wet} (day 14). Still, this was inconsistent since the opposite trend was also observed with the endpoints of RGR_{dry} (day 14) and total dry mass (day 28, 42) comparing the highest and lowest densities. Clearer trends were observed when comparing established and non-established populations. The total wet and dry biomass and RGR_{wet} endpoints showed lower NOECs and LOECs for non-established populations. Generally, both NOEC and LOEC values were lower for individual *M. spicatum* compared to *E. candensis* on days 14 and 28 for biomass endpoints (Tables 3.5

and 3.6). *L. gibba* showed lower NOEC and LOEC values compared to *E. canadensis* for wet and dry mass and wet mass compared to *M. spicatum* (Tables 3.5, 3.6 and 3.7).

3.4.4.2 Effective concentrations (EC_x)

Non-linear regression analysis of *E. canadensis* populations exhibited somewhat inconsistent trends in effective concentration values amongst different population densities. The logistic model used in the analysis represented the concentration-response relationships well with r^2 values ranging from 0.66 – 0.98 with narrow 95% confidence intervals. Effective concentrations for RGR_{wet} on day 14 and 28 showed increases with increasing population density (Table 3.4). The EC_{50} values for the RGR_{dry} endpoint on day 42 also showed an increase with increasing population density. Effective concentrations for *M. spicatum* individuals were generally lower than those for *E. canadensis* individuals (Tables 3.5 and 3.6). Endpoints of length and chlorophyll-a tended to be less sensitive for both species compared to biomass and RGR endpoints. Effect concentrations for *L. gibba* at the end of the 7-d exposure for total wet and dry mass were lower than *E. canadensis* (at day 14) yet higher than those for *M. spicatum* (at day 14) (Tables 3.5, 3.6, and 3.7).

3.4.4.3 Interactions between density and observed toxicity

Two-way analysis of variance showed significant main effects ($\alpha = 0.05$) of diuron concentration and population density for all four endpoints analyzed (RGR_{wet}, RGR_{dry}, wet mass, dry mass) and for all time points except for day 14 (Table 3.3). Interaction between the two main factors was also significant for all time points except

for day 14 (Table 3.3). This indicates that population density had a significant effect on observed toxicity in this study.

3.5 DISCUSSION

Diuron underwent significant dissipation in the microcosms over the course of the 42-day study. Aside from dissipation from the water column due to uptake by plants and other organisms as well as sorption to sediment in the plant population trays, various processes may have degraded diuron. In aqueous environments, the two main modes of diuron degradation are typically hydrolysis and photolysis (USEPA, 2003). Of these two, photolysis represents the largest route of degradation of diuron. Half-lives calculated for this study were comparable with other diuron degradation studies under field conditions. A pilot scale wetland study determined a half-life of 21.3 days via photodegradation (Rose et al., 2006). The aqueous half-life of diuron in one laboratory study ranged from 7.8 – 16.9 hours (APVMA, 2005). Based on the quantum yield of diuron, the laboratory derived half-lives of 7.8-16.9 hours corresponded to 7-22 days under natural sunlight and in another study was found to be 9.0 hours which would correspond to 43 days under natural sunlight (APVMA, 2005). The half-life of diuron has been shown to be dependent on other factors such as pH and nitrite concentration (Shankar et al., 2007). As pH increased, half-life decreased and with increasing concentrations of nitrite, half-life decreases since the nitrite provides a degradation pathway coupled with UV light. These factors can clearly affect the degradation of diuron in the natural environment. In this study, total nitrogen concentrations were fairly consistent across all system and test concentrations, so it was likely to not have influenced diuron fate. With increasing concentrations of diuron, pH showed a decrease due to the increase of acidic carbonate

species accumulated from reduced photosynthesis. With this reduction in pH, the calculated half-lives did show an increase consistent with the results obtained by Shankar et al. (2007).

Water quality parameters exhibited a clear concentration response in this study, especially comparing the two highest concentrations (100 and 1000 µg/L) to the three lowest concentrations (0, 1, and 10 µg/L). At the two highest concentrations, dissolved oxygen remained approximately 7 mg/L lower than the three lowest concentrations over the 42 days. Alkalinity was approximately 50 mg/L higher in the two highest exposure concentrations compared to all other concentrations. This is a clear indication diuron inhibiting photosynthesis within the primary producer community, leading to a reduction of oxygen produced and an accumulation of dissolved inorganic carbon.

Exposure to diuron resulted in clear toxicity to all of the macrophyte species tested (Tables 3.4 - 3.7). Non-linear regression showed strong concentration-response relationships for *E. canadensis* populations at all densities as demonstrated by the high correlation coefficients and relatively tight confidence intervals (Table 3.4). This was not the case for individually grown plants of *M. spicatum* and *E. canadensis* (Tables 3.5 and 3.6), as non-linear models did not fit this data as well. This was likely due to the overall poor growth exhibited by plants grown in the cone-tainers. Over time, there appeared to be a general decrease in toxicity as evident from the no observable effect concentration (NOEC) and lowest observable effect concentration (LOEC) data and an overall trend of increasing toxicity from the EC_x data (Table 3.4 and 3.5), with the exception of *M. spicatum* EC_x data, which showed a general decrease in toxicity (Table 3.6). This difference in observed toxicity over time between NOEC/LOEC and EC_x may be due to

the nature of variability associated with the NOEC/LOEC. Since observable effect concentrations are based solely on a statistical comparison to controls rather than biological responses, these values are subject to great variation.

Concentration ranges over which toxicity occurred depended on the data set used. According to the calculated observable effect concentration data, the range of concentrations over which toxicity was observed for *E. canadensis* populations was 10 to >1000 µg/L for LOEC data (with only total wet biomass at day 42 for the 0.031 plants/cm^2 population showing a LOEC of 1 µg/L) and 1 to > 1000 µg/L for NOEC data (Table 3.4). For individual plants of *E. canadensis,* toxicity ranged from <1 to >1000 µg/L for NOEC data and 100 to >1000 µg/L for the LOEC with two exceptions being root wet mass and root dry mass at day 14 with LOECs of <1 and 10 µg/L respectively (Table 3.5). For *M. spicatum*, toxicity was observed in the range of 1 to >1000 µg/L for NOEC data and 10 to 1000 µg/L for the LOEC with a majority of measured endpoints and timepoints being in the range of 100 to 1000 µg/L (Table 3.6). Toxicity in *L. gibba* was observed in the range of 1 to >1000 µg/L for NOEC data and 10 to >1000 µg/L for LOEC data. Ranges of toxicity from EC_{50} data for *E. canadensis* populations were 0.01 (0, 0.1) µg/L (total dry mass) to 218 (0, 449) µg/L (RGR_{wet}). EC_{50}s for individual *E. canadensis* plants ranged from 0.1 (0, 0.7) (root dry mass) to 438 µg/L (0, 995) (chlorophyll-a). EC_{50} values for individual plants of *M. spicatum* showed a toxicity range of 0.01 (0, 0.1) (chlorophyll-a) to 141 (0, 378) µg/L.

Sensitivity of endpoints varied throughout the experiment. For *E. canadensis* population studies, wet and dry mass endpoints were consistently more sensitive than RGR (wet and dry) endpoints for all three sampling events. Overall, the pattern of

sensitivity of endpoints was dry mass > wet mass > RGR_{dry} > RGR_{wet}. For individual studies of *E. canadensis*, the most sensitive endpoint was dry root mass and the least sensitive endpoint was chlorophyll-a. This result was consistent with a field study that exposed *E. canadensis* to metsulfuron methyl (Wendt-Rasch and Woin, 2003). In this study, no significant differences between control and herbicide treatments were found for dry biomass, but at the 5 and 20 µg/L treatments, no roots formed while controls grew long roots (Wendt-Rasch and Woin, 2003). A microcosm study of the effects of atrazine on *E. canadensis* found wet shoot mass to be the most sensitive endpoint for individually grown plants with chlorophyll-a being the least sensitive at all time points over the 42 day study (McGregor et al., 2008). For *M. spicatum*, the most sensitive endpoints were chlorophyll-a and total dry mass and the least sensitive was plant length. Two microcosm studies, one analyzing the effects of perfluoroocatanoic acid (PFOA), the other analyzing the effects of the antibiotic tylosin on *M. spicatum* also showed dry mass as being one of the most sensitive endpoints and plant length being one of the least sensitive endpoints for this species (Brain et al., 2005; Hanson et al., 2005). Another study that investigated the effects of atrazine on *M. spicatum* in microcosms found wet and dry root mass to be the most sensitive for the first 28 days of the study with dry shoot mass being the most sensitive at the end of 42 days (McGregor et al., 2008). The results of these two studies were fairly consistent with this study, where root mass was shown to be one of the most sensitive endpoints. For *L. gibba*, dry mass was the most sensitive endpoint and plant number and frond number were the least sensitive (Table 3.7). This contrasted to results obtained by Brain et al. (2004) in a microcosm study of the effects of

a pharmaceutical mixture on *L. gibba* which showed frond number as being one of the most sensitive endpoints.

Comparing total wet and dry mass between species, it was evident that there were differential sensitivities between the test species. When comparing the results at the end of the 7-day *L. gibba* study to the earliest sampling point for *M. spicatum* and *E. canadensis* (day 14), overall sensitivity was *M. spicatum* > *L. gibba* > *E. canadensis* . The only other macrophyte toxicity data existing for diuron is for *L. minor*. Teisseire and Vernet (1999) reported the 7-d EC_{50} for diuron in *L. minor* of 25±3 µg/L for frond number under laboratory conditions which is consistent with our study where we observed an EC_{50} of 17 (0, 130) µg/L for frond number was observed.

Conducting a basic hazard quotient assessment can provide a quick and simple evaluation of the risk posed by a chemical to the environment (Suter, 1995). This method involves dividing an actual or predicted environmental concentration of the contaminant by a toxicological benchmark concentration. If the quotient is greater than one, a risk is present. Since the lowest NOEC observed in this study was 1 µg/L, it was used as the benchmark concentration in order to provide the most conservative risk quotient. As diuron is used for a variety of applications, different hazard quotients were calculated for each use pattern. For runoff into ditches from roadside application, maximum concentrations of 2800 µg/L have been found (Powell et al., 1996). This would give a hazard quotient of 2800, which indicates great potential of toxicity under these unique conditions. For agricultural application, average concentrations of 10.9 µg/L have been found in surface runoff (APVMA, 2005). Again, this would lead to a hazard quotient of 10.9, indicating significant risk. Both of these environmental concentrations were found

in close proximity to the application site where dilution of the chemical would not have occurred to a great extent. A review of data reported by the United States Geological Survey (USGS) found that out of 942 samples from agricultural applications (with 7.96% positive detections) and 315 samples from urban application (with 13.02% positive detections), mean concentrations of diuron were below levels of detection (0.05 μg/L) for both urban and agriculture use patterns (APVMA, 2005). If that limit of detection is used as the environmental concentration, a hazard quotient of 0.05 is found. This would indicate a very low risk to macrophytes generally from diuron. Overall, in areas where diuron is used at high concentrations like roadside ditches, greater risk will be present than with typical agricultural application but, generally, the risk from all diuron use appears to be small.

Population density appeared to have an effect on the observed toxicity of diuron, especially for the endpoint of RGR (Figure 3.4 and 3.5). For all three sampling events, EC_x values for RGR increased with increasing density. This pattern was not consistently present in the wet mass and dry mass endpoints. The lower sensitivity observed for higher density populations may be due to differences in RGR as influenced by restrictions for resources. Results for this study showed a general, yet not statistically significant, trend of higher growth rates for individuals within lower population densities. Higher densities will mean that each plant will have less space in which to grow and may lead to reductions in growth rate. This is supported by population dynamics theory, where at low densities, the population will grow exponentially at a particular rate (known as the per capita rate of increase, r), but as density increases, growth rate will show a decrease until the carrying capacity of that system is reached (Forbes et al., 2001). In this

study, the growing area provided by the plastic trays limited the carrying capacity. Limitation of light resources may have also influenced the growth of high-density populations as individual plants may shade each other. Abernethy et al. (1996) showed minor, yet not statistically significant, decreases in biomass in high shade compared to low and unshaded areas for monocultures of *E. canadensis* and monocultures of *M. spicatum*, implying that high population density may have caused some shading of other plants in the population, leading to a reduction in growth. This could explain reductions in growth rate for the established populations as well as the highest population density trays in this study, where this type of shading would occur. Effects of shading were described in inter-specific competition as well. The free floating macrophytes *Eichhornia crassipes* and *Pistia stratiotes* were grown together in outdoor tanks (Agami and Reddy, 1990). Within 30 days, *E. crassipes* was able to grow above *P. stratiotes* and grew horizontal leaves that shaded *P. stratiotes*, leading to a reduction of *P. stratiotes* biomass. This further illustrates the influence of shading on plant growth. Restrictions of spatial resources may also account for the differences in growth between individual plants in 'cone-tainers' compared to those grown as populations. Overall, individually grown plants showed lower RGRs in comparison to those grown in populations, possibly due to the space allotted for growth by each type of growth container. Individual plants grown in 'cone-tainers' had 13.9 cm^2 in which to grow whereas the lowest population density individual plants had approximately 65 cm^2, the intermediate density had 32.6 cm^2, and the highest density had 21.7 cm^2 in which to grow. The higher surface area provided for each plant in the population containers may have lead to better growth than the individuals planted in 'cone-tainers'. Reduced growth due to potential restricted growth

space has been reported in other studies as well (McGregor et al., 2007; Tag El Seed, 1978).

Population stand establishment showed a clear effect on the ability of diuron to induce toxicity. For all four measured endpoints and all three sampling events, established populations were consistently less sensitive than non-established stands (Table 3.4). Again, the established stands contained a very densely packed population that could have lead to light and spatial resource limitation for some members of the population, but the population likely reached its carrying capacity prior to the start of the study, while non-established stands were still in the exponential growth phase (Forbes et al., 2001). This was exhibited by the lower RGRs shown by established stands relative to controls (Figures 3.6 and 3.7). Since diuron is a photosynthetic inhibitor, any populations that are progressing in their rapid exponential growth phase, with higher associated RGRs, (Figures 3.6 and 3.7) will clearly be affected the greatest. Since photosynthesis in the rapidly growing non-established stands would be inhibited by diuron, they showed the greatest sensitivity compared to established stands, which had essentially stopped growing, leaving little ability for the chemical to exert toxicity.

In summary, all of the previously discussed trends in sensitivity can be tied to RGR. The RGRs of each test system differed in some way. In control treatments, higher density populations and established populations showed the lowest RGR. In these systems, greater intra-specific competition for resources may have lead to some plants being at a disadvantage, thus reaching the carrying capacity of the population. A similar situation was present in the established populations, where again, competition for resources may have reduced RGR compared to non-established populations, though the

population may also have reached its carrying capacity. Also, plants individually grown in cone-tainers had significantly less growth surface area compared to populations, which may have lead to their lower RGRs. This difference in RGR between individually grown plants and populations was also observed in a study of the effects of the pharmaceutical monensin on aquatic macrophytes conducted by McGregor et al. (2007). The low density in this experiment (0.015 plants/cm^2) is comparable to the 0.025 plants/cm^2 density used for planting *E. canadensis* populations by McGregor et al. (2007) and showed similar RGRs to this experiment in the range of 0.1-0.15 $gg^{-1}day^{-1}$. These RGRs were significantly different that those for individually grown plants. The trend of higher RGRs leading to greater sensitivity has been documented in other studies. A review of toxicity assessment using duckweeds by Huebert and Shay (1993) found that faster control group RGRs resulted in a lower concentration of the toxicant needed to produce an EC_{50}. A study conducted by Cedergreen et al. (2004b) found a negative correlation between EC_{50} and RGR for macrophytes exposed to metsulfuron-methyl, indicating higher sensitivity with higher RGR. These results suggest that higher RGRs can lead to higher sensitivity and it presents a significant consideration in the choice of a test system that should maximize RGR for the chosen species.

The results of this study, as well as others like it (McGregor et al., 2007; Huebert and Shay, 1993; Cedergreen et al., 2004b), state the importance of incorporating different test systems in aquatic macrophyte toxicity testing. Since individual plants grown in cone-tainers showed significantly lower growth rates and lower sensitivities than plants grown in populations, individual plants may underestimate toxicity (McGregor et al., 2007). Also, plants grown at higher densities or at a progressed state of establishment

with their lower RGRs and associated lower sensitivities may also underestimate toxicity. In only using established populations for toxicity tests, the entire range of responses may not be represented (Hanson et al., 2009). The results presented here show that in capturing a more comprehensive picture of toxicity of aquatic plants in semi-natural conditions both individual and population/community arrangements should be used together as well as established and non-established populations/communities. In applying this suite of growth arrangements in a risk assessment context a more accurate understanding of toxicity can be obtained.

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Table 3.1 Physical and chemical parameters in microcosms exposed to diuron averaged over a 42 day period plus pre-treatment measurements (± std dev).

Treatment (µg/L)	Minimum temperature (°C) (n = 36)	Maximum temperature (°C) (n = 36)	pH (n = 5)	Hardness[a] (mg/L) (n = 5)	Alkalinity[a] (mg/L) (n = 5)	Conductivity (µS/cm) (n = 5)	PAR[b] (µE/m^2/s) (n = 5)	TKN[c] (mg/L) (n = 5)	TP[d] (mg/L) (n = 5)
Control	21 ± 2	25 ± 2	8.3	254 ± 52	116 ± 34	715 ± 171	1134 ± 700	0.17 ± 0.06	0.24 ± 0.29
1	21 ± 2	25 ± 2	8.4	251 ± 48	109 ± 36	710 ± 175	1152 ± 650	0.14 ± 0.03	0.10 ± 0.04
10	22 ± 2	25 ± 2	8.3	268 ± 52	128 ± 36	731 ± 171	1038 ± 450	0.11 ± 0.01	0.09 ± 0.03
100	22 ± 2	25 ± 2	7.8	343 ± 23	188 ± 15	830 ± 259	1124 ± 350	0.18± 0.05	0.16 ± 0.07
1000	21 ± 2	25 ± 2	7.7	347 ± 24	193 ± 19	853 ± 279	1538 ± 700	0.15 ± 0.05	0.13 ± 0.04

[a]Measured as mg $CaCO_3$/L
[b]Photosynthetically active radiation
[c]Total kinetic nitrogen
[d]Total phosphorus

Table 3.2 Initial concentrations, time weighted averages, disspation slope (k), and half lives with 95% confidence intervals for diuron residues in aquatic microcosms over a 42-day study period.

Parameter	Treatment concentration (µg/L)			
	1	**10**	**100**	**1000**
Initial concentration - Day 0 ± std dev (µg/L)	$1.3 \pm 3.0 \times 10^{-2}$	12.1 ± 0.9	157.5 ± 17.7	1336.1 ± 122.9
TWA - Day 14 (µg/L)	0.8	8.6	121.1	1090.4
TWA - Day 28 (µg/L)	0.6	6.1	113.3	1008.3
TWA - Day 42 (µg/L)	0.4	4.6	101.9	900.1
k (d^{-1})	-0.06	-0.06	-0.02	-0.02
Half-life (95% CI) (days)	13.0 (0, 23.6)	12.1 (3.6, 20.0)	36.1 (9.9, 55.4)	33.0 (12.1, 54.0)

Table 3.3 Main and interactive effects of diuron concentration and population density on wet mass, dry mass, and relative growth rate (RGR) of *Elodea canadensis* grown in microcosm conditions, determined using a fixed model two way ANOVA ($\alpha = 0.05$) in SAS v. 9.1.

		Main effects		Interaction
Day	**Endpoint**	**Diuron concentration (p-value)**	**Population density (p-value)**	**Diuron concentration * Population density (p-value)**
14	Wet Mass	<0.001*	<0.001*	0.046*
	Dry Mass[n]	<0.001*	0.07	0.14
	RGR Wet[n]	<0.001*	<0.001*	0.12
	RGR Dry[n]	<0.001*	0.01*	0.06
28	Wet Mass[n]	<0.001*	<0.001*	<0.001*
	Dry Mass[n]	<0.001*	<0.001*	<0.001*
	RGR Wet[n]	<0.001*	<0.001*	<0.001*
	RGR Dry[n]	<0.001*	<0.001*	<0.001*
42	Wet Mass[v]	<0.001*	<0.001*	<0.001*
	Dry Mass[n]	<0.001*	<0.001*	<0.001*
	RGR Wet[n]	<0.001*	<0.001*	<0.001*
	RGR Dry[n]	<0.001*	<0.001*	<0.001*

* Statistically significant effect
[n] Normality test failed
[v] Equal variance test failed

Table 3.4. Effective concentrations of diuron (µg/L) using a logistic model and no observable effect concentrations (NOEC) for *Elodea canadensis* populations over a 42 day exposure period for a variety of endpoints.

Endpoint	Density (plants/cm^2)	Day	NOEC (µg/L)	EC_{10} (95% CI) (µg/L)	EC_{25} (95% CI) (µg/L)	EC_{50} (95% CI) (µg/L)	Parameters[a]	Adjusted r^2
Wet mass (g)	0.015	14	1	0.7 (0, 2.7)	1.6 (0, 5.0)	4.0 (0, 9.4)	b = 1.21; t = 1.16; x = 4.0	0.77
Wet mass (g)	0.031	14	1	0.4 (0, 1.2)	1.6 (0, 4.0)	7.0 (0, 14.2)	b = 0.73; t = 0.9; x = 7.0	0.88
Wet mass (g)	0.031 (Established)	14	10	2.8 (0, 11.8)	13.9 (0, 43.8)	69.3 (0, 163.7)	b = 0.68; t = 51.19; x = 69.27	0.78
Wet mass (g)	0.046	14	1	0.04 (0, 0.2)	0.4 (0, 1.5)	4.6 (0, 11.8)	b = 0.47; t= 0.92; x = 4.64	0.84
Dry mass (g)	0.015	14	1	0.9 (0, 3.3)	1.8 (0, 5.5)	3.9 (0, 8.8)	b = 1.46; t = 0.09; x = 3.91	0.84
Dry mass (g)	0.031	14	10	2.5 (0, 12.9)	4.9 (0, 15.3)	9.8 (1.8, 17.9)	b = 1.59; t = 0.06; x = 9.82	0.8
Dry mass (g)	0.031 (Established)	14	10	2.8 (0, 8.6)	8.9 (0, 21.6)	28.3 (0, 57.7)	b = 0.95; t = 7.3; x = 28.29	0.86
Dry mass (g)	0.046	14	1	0.1 (0, 0.4)	0.6 (0, 1.7)	3.5 (0, 7.9)	b = 0.62; t = 0.08; x = 3.45	0.86

Table 3.4 Cont.

Endpoint	Density (plants/cm^2)	Day	NOEC (µg/L)	EC_{10} (95% CI) (µg/L)	EC_{25} (95% CI) (µg/L)	EC_{50} (95% CI) (µg/L)	Parameters[a]	Adjusted r^2
RGR_{wet} ($gg^{-1}day^{-1}$)	0.015	14	1	0.4 (0, 1.2)	2.5 (0, 6.5)	17.5 (0, 36.1)	b = 0.57; t = 0.17; x = 17.46	0.89
RGR_{wet} ($gg^{-1}day^{-1}$)	0.031	14	1	0.8 (0, 2.4)	5.0 (0, 12.0)	33.0 (2.8, 63.3)	b = 0.58; t = 0.16; x = 33.04	0.91
RGR_{wet} ($gg^{-1}day^{-1}$)	0.031 (Established)	14	10	7.7 (0, 29.1)	40.9 (0, 113.1)	218.3 (0, 448.5)	b = 0.66; t = 0.12; x = 218.3	0.79
RGR_{wet} ($gg^{-1}day^{-1}$)	0.046	14	10	0.7 (0, 2.6)	5.3 (0, 14.6)	39.6 (0, 85.2)	b = 0.55; t = 0.15; x = 39.64	0.87
RGR_{dry} ($gg^{-1}day^{-1}$)	0.015	14	100	1.2 (0, 3.4)	2.9 (0, 6.0)	7.0 (3.5, 10.5)	b = 1.26; t = 0.13; x = 7.04	0.95
RGR_{dry} ($gg^{-1}day^{-1}$)	0.031	14	>1000	5.5 (0, 26.7)	8.3 (4.4, 12.2)	12.6 (0, 53.5)	b = 2.63; t = 0.1; x = 12.59	0.93
RGR_{dry} ($gg^{-1}day^{-1}$)	0.031 (Established)	14	10	7.7 (0, 18.7)	24.3 (1.3, 47.4)	77.1 (33.2, 120.9)	b = 0.95; t = 0.13; x = 77.05	0.94
RGR_{wet} ($gg^{-1}day^{-1}$)	0.046	14	10	0.7 (0, 2.6)	5.3 (0, 14.6)	39.6 (0, 85.2)	b = 0.55; t = 0.15; x = 39.64	0.87

Table 3.4 Cont.

Endpoint	Density (plants/cm^2)	Day	NOEC (µg/L)	EC_{10} (95% CI) (µg/L)	EC_{25} (95% CI) (µg/L)	EC_{50} (95% CI) (µg/L)	Parameters[a]	Adjusted r^2
Wet mass (g)	0.015	28	1	3.3 (0, 1.7 x 10^3)	4.4 (0, 1.2 x 10^3)	5.7 (0, 300.8)	b = 4.0; t = 3.57; x = 5.73	0.81
Wet mass (g)	0.031	28	1	0.3 (0,1.1)	1.3 (0, 3.4)	5.4 (0.2, 10.6)	b = 0.80; t = 2.51; x = 5.35	0.9
Wet mass (g)	0.031 (Established)	28	100	12.9 (0, 86.4)	29.4 (0, 137.7)	66.8 (0, 191.1)	b = 1.34; t = 47.05; x = 66.8	0.69
Wet mass (g)	0.046	28	10	5.0 (0, 1.3 x 10^3)	6.6 (0, 776.9)	9.2 (0, 1.1 x 10^3)	b = 3.4; t = 1.39; x = 9.21	0.76
Dry mass (g)	0.015	28	100	3.6 (0, 2.4 x 10^3)	4.6 (0, 1.1 x 10^3)	6.2 (0, 109.2)	b = 4.0; t = 0.44; x = 6.17	0.81
Dry mass (g)	0.031	28	1	0.03 (0, 0.2)	0.2 (0, 0.9)	1.7 (0, 4.5)	b = 0.56; t = 0.44; x = 1.66	0.8
Dry mass (g)	0.031 (Established)	28	10	2.3 (0, 6.8)	5.9 (0, 13.8)	15.2 (0, 33.6)	b = 1.16; t = 12.44; x = 15.19	0.84
Dry mass (g)	0.046	28	10	4.2 (0, 2.3 x 10^3)	5.6 (0, 748.4)	7.3 (0, 2.0 x 10^3)	b = 4.0; t = 0.19; x = 7.32	0.85

Table 3.4 Cont.

Endpoint	Density (plants/cm^2)	Day	NOEC (µg/L)	EC_{10} (95% CI) (µg/L)	EC_{25} (95% CI) (µg/L)	EC_{50} (95% CI) (µg/L)	Parameters[a]	Adjusted r^2
RGR_{wet} ($gg^{-1}day^{-1}$)	0.015	28	>1000	3.2 (1.4, 5.1)	6.9 (4.2, 9.5)	14.5 (7.7, 21.3)	b = 1.47; t = 0.12; x = 14.49	0.98
RGR_{wet} ($gg^{-1}day^{-1}$)	0.031	28	1	4.7 (2.3, 7.1)	7.8 (4.3, 11.3)	12.9 (0, 26.9)	b = 2.18; t = 0.11; x = 12.92	0.98
RGR_{wet} ($gg^{-1}day^{-1}$)	0.031 (Established)	28	10	37.4 (0, 198.2)	66.0 (0, 209.4)	116.3 (54.7, 178.0)	b = 1.94; t = 5.4 x 10^{-2}; x = 116.3	0.81
RGR_{wet} ($gg^{-1}day^{-1}$)	0.046 plants/cm^2	28	>1000	6.6 (0.9, 12.3)	10.9 (0.6, 21.3)	18.1 (0, 41.9)	b = 2.17; t = 0.09; x = 18.13	0.96
RGR_{dry} ($gg^{-1}day^{-1}$)	0.015	28	10	4.4 (1.1, 7.7)	7.2 (4.1, 10.3)	11.9 (0, 27.7)	b = 2.21; t = 0.11; x = 11.86	0.98
RGR_{dry} ($gg^{-1}day^{-1}$)	0.031	28	>1000	4.0 (0.5, 7.5)	6.9 (3.8, 10.0)	12.1 (0, 26.3)	b = 1.96; t = 0.11; x = 12.11	0.96
RGR_{dry} ($gg^{-1}day^{-1}$)	0.031 (Established)	28	10	4.6 (0, 12.8)	12.3 (0, 28.3)	33.1 (2.4, 63.8)	b = 1.11; t = 8.2 x 10^{-2}; x = 33.1	0.91
RGR_{dry} ($gg^{-1}day^{-1}$)	0.046	28	>1000	5.5 (0, 121.3)	7.3 (0, 205.2)	10.5 (0, 228.2)	b = 3.24; t = 8.6 x 10^{-2}; x = 10.5	0.98

Table 3.4 Cont.

Endpoint	Density (plants/cm^2)	Day	NOEC (µg/L)	EC_{10} (95% CI)	EC_{25} (95% CI)	EC_{50} (95% CI)	Parameters[a]	Adjusted r^2
Wet mass (g)	0.015	42	10	0.2 (0, 1.0)	1.0 (0, 3.6)	5.1 (0, 13.8)	b = 0.69; t = 7.97; x = 5.14	0.78
Wet mass (g)	0.031	42	<1	0.04 (0, 0.2)	0.4 (0, 1.4)	3.1 (0, 9.4)	b = 0.51; t = 6.02; x = 3.1	0.76
Wet mass (g)	0.031 (Established)	42	100	28 (0, 2.6 x 10^4)	36.9 (0, 2.8 x 10^4)	48.6 (0, 2.6 x 10^4)	b = 4.0; t = 15.0; x = 48.55	0.76
Wet mass (g)	0.046	42	100	5.0 (0, 24.4)	7.1 (0, 159.9)	10.2 (0, 413.1)	b = 3.06; t = 2.61; x = 10.18	0.68
Dry mass (g)	0.015	42	100	0.05 (0, 0.3)	0.3 (0, 1.2)	2.3 (0, 6.2)	b = 0.58; t = 1.35; x = 2.28	0.79
Dry mass (g)	0.031	42	10	0.01 (0, 0.1)	0.2 (0, 0.8)	1.8 (0, 6.5)	b = 0.45; t = 1.1; x = 1.78	0.66
Dry mass (g)	0.031 (Established)	42	10	FTC	FTC	FTC	FTC	FTC
Dry mass (g)	0.046	42	10	2.9 (0, 10.6)	5.4 (0, 13.1)	9.9 (0, 43.1)	b = 1.8; t = 0.46; x = 9.88	0.74
RGR_{wet} ($gg^{-1}day^{-1}$)	0.015	42	>1000	4.9 (2.1, 7.6)	7.3 (0, 23.4)	11.0 (0, 55.9)	b = 2.7; t = 9.7 x 10^{-2}; x = 11.01	0.98

Table 3.4 Cont.

Endpoint	Density (plants/cm^2)	Day	NOEC (µg/L)	EC_{10} (95% CI)	EC_{25} (95% CI)	EC_{50} (95% CI)	Parameters[a]	Adjusted r^2
RGR_{wet} ($gg^{-1}day^{-1}$)	0.031	42	100	6.7 (0, 15.2)	12.7 (0.01, 25.5)	24.1 (4.5, 43.6)	b = 1.73; t = 8.8 x 10^{-2}; x = 24.08	0.97
RGR_{wet} ($gg^{-1}day^{-1}$)	0.031 (Established)	42	10	46.9 (0, 7.6 x 10^3)	64.7 (0, 7.5 x 10^3)	88.2 (0, 4.4 x 10^3)	b = 3.75; t = 4.1 x 10^{-2}; x = 88.21	0.81
RGR_{wet} ($gg^{-1}day^{-1}$)	0.046	42	10	6.3 (0, 14.8)	10.4 (0, 24.5)	17.2 (0, 44.4)	b = 2.17; t = 7.4 x 10^{-2}; x = 17.2	0.95
RGR_{dry} ($gg^{-1}day^{-1}$)	0.015	42	10	4.2 (1.7, 6.7)	6.7 (0, 14.2)	10.7 (0, 36.5)	b = 2.34; t = 9.7 x 10^{-2}; x = 10.66	0.97
RGR_{dry} ($gg^{-1}day^{-1}$)	0.031	42	>1000	5.6 (0, 14.0)	8.1 (0, 40.4)	11.9 (0, 89.6)	b = 2.91; t = 9.0 x 10^{-2}; x = 11.87	0.97
RGR_{dry} ($gg^{-1}day^{-1}$)	0.031 (Established)	42	10	38.0 (0, 3.3 x 10^4)	49.0 (0, 2.4 x 10^4)	65.2 (0, 2.0 x 10^4)	b = 3.89; t = 5.8 x 10^{-2}; x = 65.22	0.92
RGR_{dry} ($gg^{-1}day^{-1}$)	0.046	42	10	4.8 (0.5, 9.1)	8.1 (0, 17.2)	13.5 (0, 37.4)	b = 2.13; t = 7.7 x 10^{-2}; x = 13.5	0.95

RGR – Relative growth rate, NOEC – No observable effect concentration, CI – Confidence interval, EC – Effective concentration, FTC – Failed to converge logistic model

[a]Reparameterized logistic equation to fit concentration-responses of diuron exposed *E. canadensis* populations: f = t/(1 + (x_o/x)^b). The variable x is the EC_{50} of the concentration-response relationship modeled, x_o is the actual concentration being evaluated (µg/L), f is the response or change from control of the modeled endpoint and b and t are constants.

Table 3.5 Effective concentrations of diuron (µg/L) using a logistic model and no observable effect concentrations (NOEC) for individual plants of *Elodea canadensis* grown in 'cone-tainers' over a 42 day exposure period for a variety of endpoints.

Endpoint	Day	NOEC (µg/L)	EC_{10} (95% CI) (µg/L)	EC_{25} (95% CI) (µg/L)	EC_{50} (95% CI) (µg/L)	Parameters[a]	Adjusted r^2
Chlorophyll - a (µg/mg)	14	100	81.1 (0, 290.7)	188.4 (0, 525.5)	438.1 (0, 995.2)	b = 1.3; t = 1.66; x = 438.1	0.54
Root number	14	100	0.4 (0, 2.6)	2.9 (0, 14.0)	21.3 (0, 75.9)	b = 0.55; t = 3.37; x = 21.28	0.57
Shoot wet mass (g)	14	100	40.2 (0, 1.5×10^4)	49.8 (0, 8.5×10^3)	67.5 (0, 7.2×10^3)	b = 3.53; t = 0.21; x = 67.53	0.53
Root wet mass (g)	14	<1	0.13 (0, 0.8)	0.38 (0, 1.6)	1.2 (0, 3.6)	b = 1.00; t = 7.3 x 10^{-2}, x - 1.15	0.75
Shoot dry mass (g)	14	10	19.5 (0, 1.0×10^3)	25.5 (0, 1.2×10^3)	33.5 (0, 1.4×10^3)	b = 4.0; t = 3.5 x 10^{-2}; x = 33.55	0.53
Root dry mass (g)	14	1	0.02 (0, 0.17)	0.1 (0, 0.7)	0.8 (0, 2.7)	b = 0.61; t = 7.0 x 10^{-3}; x = 0.83	0.68
Total wet mass (g)	14	10	7.7 (0, 32.1)	11.1 (0, 81.9)	16.2 (0, 259.6)	b = 2.95; t = 0.26; x = 16.16	0.59

Table 3.5 Cont.

Endpoint	Day	NOEC (µg/L)	EC_{10} (95% CI) (µg/L)	EC_{25} (95% CI) (µg/L)	EC_{50} (95% CI) (µg/L)	Parameters[a]	Adjusted r^2
Total dry mass (g)	14	10	9.6 (0, 50.5)	13.4 (0, 184.4)	18.8 (0, 431.7)	b = 3.26; t = 0.039; x = 18.84	0.53
RGR wet mass ($gg^{-1}day^{-1}$)	14	>1000	7.9 (0, 27.4)	11.2 (0, 88.4)	15.8 (0, 266.5)	b = 3.16; t = 7.5 x 10^{-2}; x = 15.82	0.75
RGR dry mass ($gg^{-1}day^{-1}$)	14	>1000	10.2 (0, 103.4)	13.7 (0, 339.4)	18.4 (0, 728.2)	b = 3.74; t = 7.0 x 10^{-2}; x = 18.35	0.66
Chlorophyll - a (µg/mg)	28	>1000	5.5 (0, 2.1 x 10^3)	7.2 (0, 4.3 x 10^3)	10.2 (0, 3.1 x 10^3)	b = 3.28; t = 0.61; x = 10.18	0.11
Root number	28	>1000	3.3 (0, 2.1 x 10^3)	4.6 (0, 3.2 x 10^3)	6.1 (0, 13.8)	b = 4.0; t = 4.0; x = 6.09	0.36
Shoot wet mass (g)	28	>1000	7.6 (0, 2.2 x 10^3)	9.7 (0, 5.8 x 10^3)	12.4 (0, 1.1 x 10^4)	b = 4.52; t = 0.32; x = 12.37	0.35
Root wet mass (g)	28	>1000	2.4 (0, 191.0)	3.7 (0, 128.4)	6.1 (0, 19.3)	b = 2.68; t = 0.1; x = 6.08	0.35

Table 3.5 Cont.

Endpoint	Day	NOEC (µg/L)	EC_{10} (95% CI) (µg/L)	EC_{25} (95% CI) (µg/L)	EC_{50} (95% CI) (µg/L)	Parameters[a]	Adjusted r^2
Shoot dry mass (g)	28	100	4.3 (0, 23.6)	7.6 (0, 33.2)	13.7 (0, 102.6)	b = 1.89; t = 7.2×10^{-2}; x = 13.66	0.34
Root dry mass (g)	28	>1000	0.5 (0, 5.1)	1.4 (0, 8.9)	3.5 (0, 14.6)	b = 1.17; t = 0.01; x = 3.54	0.35
Total wet mass (g)	28	>1000	5.2 (0, 154.4)	7.3 (0, 262.7)	10.3 (0, 1.0×10^3)	b = 3.25; t = 0.42; x = 10.3	0.35
Total dry mass (g)	28	100	3.1 (0, 19.1)	6.2 (0, 24.4)	12.6 (0, 66.5)	b = 1.56; t = 8.3×10^{-2}; x = 12.55	0.34
RGR wet mass ($gg^{-1}day^{-1}$)	28	>1000	4.5 (0, 3.8×10^3)	5.8 (0, 1.0×10^3)	9.3 (0, 396.6)	b = 2.54; t = 3.7×10^{-2}; x = 9.25	0.33
RGR dry mass ($gg^{-1}day^{-1}$)	28	>1000	2.8 (0, 27.2)	5.2 (0, 20.6)	9.7 (0, 57.3)	b = 1.76; t = 4.1×10^{-2}; x = 9.68	0.35
Chlorophyll - a (µg/mg)	42	>1000	27.6 (0, 4.4×10^4)	36.3 (0, 4.6×10^4)	47.8 (0, 4.4×10^4)	b = 4.0; t = 7.33; x = 47.78	0.47

Table 3.5 Cont.

Endpoint	Day	NOEC (µg/L)	EC_{10} (95% CI) (µg/L)	EC_{25} (95% CI) (µg/L)	EC_{50} (95% CI) (µg/L)	Parameters[a]	Adjusted r^2
Root number	42	>1000	18 (0, 1.3 x 10^4)	29.2 (0, 3.3 x 10^4)	34.5 (0, 2.2 x 10^4)	b = 4.0; t = 0.65; x = 34.54	0.17
Shoot wet mass (g)	42	100	19.2 (0, 1.0 x 10^4)	24.9 (0, 1.0 x 10^4)	33.2 (0, 1.2 x 10^4)	b = 4.0; t = 0.67; x = 33.21	0.43
Root wet mass (g)	42	>1000	19.1 (0, 9.5 x 10^3)	25.1 (0, 1.0 x 10^4)	33.2 (0, 1.1 x 10^4)	b = 4.0; t = 0.19; x = 33.21	0.45
Shoot dry mass (g)	42	>1000	19.4 (0, 1.2 x 10^4)	25.4 (0, 1.3 x 10^4)	33.5 (0, 1.3 x 10^4)	b = 4.0; t = 0.15; x = 33.5	0.37
Root dry mass (g)	42	>1000	18.3 (0, 7.7 x 10^3)	23.9 (0, 8.3 x 10^3)	31.9 (0, 9.4 x 10^3)	b = 4.0; t = 1.9 x 10^{-2}; x = 31.93	0.46

Table 3.5 Cont.

Endpoint	Day	NOEC (µg/L)	EC_{10} (95% CI) (µg/L)	EC_{25} (95% CI) (µg/L)	EC_{50} (95% CI) (µg/L)	Parameters[a]	Adjusted r^2
Total wet mass (g)	42	>1000	18.3 (0, 8.3 x 10^3)	24.6 (0, 1.0 x 10^4)	34.8 (0, 1.4 x 10^4)	b = 4.0; t = 0.79; x = 34.81	0.42
Total dry mass (g)	42	>1000	19.5 (0, 1.2 x 10^4)	25.3 (0, 1.2 x 10^4)	33.4 (0, 1.3 x 10^4)	b = 4.0; t = 0.17; x = 33.38	0.38
RGR wet mass ($gg^{-1}day^{-1}$)	42	>1000	17.8 (0, 7.3 x 10^3)	23.5 (0, 8.2 x 10^3)	33.8 (0, 1.3 x 10^4)	b = 4.0; t = 3.7 x 10^{-2}; x = 33.81	0.42
RGR dry mass ($gg^{-1}day^{-1}$)	42	>1000	19.4 (0, 1.0 x 10^4)	23.3 (0, 7.8 x 10^3)	33.5 (0, 1.2 x 10^4)	b = 4.0; t = 4.2 x 10^{-2}; x = 33.51	0.43

RGR – Relative growth rate, NOEC – No observable effect concentration, EC – Effective concentration, CI – Confidence interval
[a]Reparameterized logistic equation to fit concentration-responses of diuron exposed *E. canadensis* individuals: f = t/(1 + (x_o/x)^b). The variable x is the EC_{50} of the concentration-response relationship modeled, x_o is the actual concentration being evaluated (µg/L), f is the response or change from control of the modeled endpoint and b and t are constants.

Table 3.6 Effective concentrations of diuron (µg/L) using a logistic model and no observable effect concentrations (NOEC) for individual plants of *Myriophyllum spicatum* grown in 'cone-tainers' over a 42 day exposure period for a variety of endpoints.

Endpoint	Day	NOEC (µg/L)	EC_{10} (95% CI) (µg/L)	EC_{25} (95% CI) (µg/L)	EC_{50} (95% CI) (µg/L)	Parameters[a]	Adjusted r^2
Chlorophyll - a (µg/mg)	14	>1000	475.4 (0, 6.5 x 10^4)	625 (0, 5.7 x 10^4)	822.5 (0, 3.8 x 10^4)	b = 4.0; t = 1.2; x = 822.49	0.57
Plant length (cm)	14	10	5.9 (0, 31.3)	28.9 (0, 109.0)	141.2 (0, 378.4)	b = 0.69; t = 23.89; x = 141.2	0.61
Root number	14	10	2.6 (0, 9.1)	8.9 (0, 23.9)	29.8 (0, 65.9)	b = 0.91; t = 29.03; x = 29.81	0.83
Shoot wet mass (g)	14	10	0.02 (0, 0.2)	0.3 (0, 1.7)	3.6 (0, 14.8)	b = 0.43; t = 1.67; x = 3.56	0.58
Root wet mass (g)	14	100	0.1 (0, 0.5)	0.6 (0, 2.1)	3.2 (0, 8.5)	b = 0.65; t = 1.1; x = 3.17	0.77
Shoot dry mass (g)	14	1	0.1 (0, 0.7)	0.8 (0, 3.0)	4.4 (0, 13.2)	b = 0.62; t = 0.19; x = 4.36	0.71
Root dry mass (g)	14	100	0.2 (0, 0.7)	0.7 (0, 2.4)	3.5 (0, 8.7)	b = 0.71; t = 6.4 x 10^{-2}; x = 3.5	0.8

Table 3.6 Cont.

Endpoint	Day	NOEC (µg/L)	EC_{10} (95% CI) (µg/L)	EC_{25} (95% CI) (µg/L)	EC_{50} (95% CI) (µg/L)	Parameters[a]	Adjusted r^2
Total wet mass (g)	14	10	0.04 (0, 0.2)	0.3 (0, 1.7)	3.4 (0, 12.0)	b = 0.48; t = 2.77; x = 3.45	0.67
Total dry mass (g)	14	1	0.04 (0, 0.2)	0.3 (0, 1.1)	2.1 (0, 5.8)	b = 0.55; t = 0.29; x = 2.05	0.78
RGR wet mass ($gg^{-1}day^{-1}$)	14	10	1.6 (0, 6.3)	5.8 (0, 17.1)	21.0 (0, 50.0)	b = 0.86; t = 0.13; x = 21.04	0.79
RGR dry mass ($gg^{-1}day^{-1}$)	14	10	0.4 (0, 1.7)	1.8 (0, 5.3)	7.4 (0, 17.2)	b = 0.77; t = 0.11; x = 7.38	0.83
Chlorophyll - a (µg/mg)	28	>1000	4.7 (0, 2.4 x 10^3)	6.1 (0, 107.6)	8.4 (0, 970.1)	b = 3.33; t = 0.91; x = 8.4	0.72
Plant length (cm)	28	10	39.7 (0, 1.3 x 10^3)	58.7 (0, 1.2 x 10^3)	86.6 (0, 777.3)	b = 2.82; t = 29.87; x = 86.65	0.85
Root number	28	10	4.8 (0, 14.7)	11.2 (0, 28.4)	25.8 (0, 58.4)	b = 1.31; t = 30.60; x = 25.76	0.86

Table 3.6 Cont.

Endpoint	Day	NOEC (µg/L)	EC_{10} (95% CI) (µg/L)	EC_{25} (95% CI) (µg/L)	EC_{50} (95% CI) (µg/L)	Parameters[a]	Adjusted r^2
Shoot wet mass (g)	28	10	20.9 (0, 5.4 x 10^3)	29.1 (0, 7.5 x 10^3)	38.1 (0, 7.8 x 10^3)	b = 4.0; t = 1.36; x = 38.07	0.45
Root wet mass (g)	28	10	3.7 (0, 14.1)	7.3 (0, 21.7)	6.1 (0, 22.0)	b = 0.59; t = 2.04; x = 6.13	0.62
Shoot dry mass (g)	28	10	19.3 (0, 4.2 x 10^3)	26.3 (0, 5.5 x 10^3)	36.2 (0, 8.0 x 10^3)	b = 4.0; t = 0.22; x = 36.21	0.38
Root dry mass (g)	28	>1000	1.3 (0, 11.8)	6.3 (0, 30.3)	13.8 (0, 73.4)	b = 1.39; t = 0.12; x = 13.77	0.59
Total wet mass (g)	28	10	18.8 (0, 2.8 x 10^3)	22.7 (0, 2.2 x 10^3)	29.9 (0, 2.5 x 10^3)	b = 4.0; t = 2.99; x = 29.88	0.56
Total dry mass (g)	28	10	17.8 (0, 2.7 x 10^3)	22.8 (0, 2.7 x 10^3)	30.1 (0, 3.1 x 10^3)	b = 4.0; t = 0.33; x = 30.1	0.45
RGR wet mass ($gg^{-1}day^{-1}$)	28	>1000	9.0 (0, 163.7)	12.0 (0, 362.6)	16.0 (0, 677.6)	b = 3.84; t = 6.9 x 10^{-2}; x = 16.02	0.85

Table 3.6 Cont.

Endpoint	Day	NOEC (µg/L)	EC_{10} (95% CI) (µg/L)	EC_{25} (95% CI) (µg/L)	EC_{50} (95% CI) (µg/L)	Parameters[a]	Adjusted r^2
RGR dry mass ($gg^{-1}day^{-1}$)	28	>1000	6.9 (0, 24.7)	10.3 (0, 71.8)	15.4 (0, 170.0)	b = 2.76; t = 5.8 x 10^{-2}; x = 15.38	0.76
Chlorophyll - a (µg/mg)	42	>1000	0.01 (0, 0.1)	0.1 (0, 0.7)	1.0 (0, 3.9)	b = 0.5; t = 0.71; x = 1.04	0.63
Plant length (cm)	42	100	32.7 (0, 5.8 x 10^4)	43.0 (0, 5.8 x 10^4)	56.6 (0, 5.2 x 10^4)	b = 4.0; t = 24.96; x = 56.63	0.66
Root number	42	10	6.9 (0, 37.8)	14.1 (0, 61.7)	28.7 (0, 99.4)	b = 1.55; t = 32.4; x = 28.74	0.74
Shoot wet mass (g)	42	>1000	16.8 (0, 5.0 x 10^3)	21.8 (0, 5.2 x 10^3)	29.8 (0, 6.7 x 10^3)	b = 4.0; t = 1.59; x = 29.85	0.5
Root wet mass (g)	42	100	0.8 (0, 4.4)	2.1 (0, 7.7)	5.8 (0, 14.6)	b = 1.1; t = 2.67; x = 5.8	0.69
Shoot dry mass (g)	42	>1000	5.4 (0, 1.2 x 10^3)	7.9 (0, 536.9)	11.7 (0, 964.8)	b = 2.96; t = 0.32; x = 11.7	0.36

Table 3.6 Cont.

Endpoint	Day	NOEC (µg/L)	EC_{10} (95% CI) (µg/L)	EC_{25} (95% CI) (µg/L)	EC_{50} (95% CI) (µg/L)	Parameters[a]	Adjusted r^2
Root dry mass (g)	42	>1000	0.9 (0, 5.2)	2.4 (0, 8.4)	6.1 (0, 15.8)	b = 1.15; t = 0.24; x = 6.14	0.67
Total wet mass (g)	42	>1000	3.4 (0, 105.6)	5.1 (0, 108.9)	8.4 (0, 170.3)	b = 2.26; t = 4.1; x = 8.41	0.62
Total dry mass (g)	42	>1000	3.7 (0, 3.6×10^3)	4.9 (0, 1.3×10^3)	7.4 (0, 507.8)	b = 2.68; t = 0.54; x = 7.42	0.44
RGR wet mass ($gg^{-1}day^{-1}$)	42	>1000	8.4 (0, 1.3×10^3)	10.8 (0, 2.3×10^3)	13.7 (0, 3.7×10^3)	b = 4.48; t = 4.8×10^{-2}; x = 13.75	0.67
RGR dry mass ($gg^{-1}day^{-1}$)	42	>1000	3.0 (0, 15.3)	5.5 (0, 18.8)	10.0 (0, 66.9)	b = 1.85; t = 4.5×10^{-2}; x = 10.02	0.51

RGR – Relative growth rate, NOEC – No observable effect concentration, EC – Effective concentration, CI – Confidence interval
[a]Reparameterized logistic equation to fit concentration-responses of diuron exposed *M. spicatum* individuals: f = t/(1 + (x$_o$/x)^b). The variable x is the EC_{50} of the concentration-response relationship modeled, x_o is the actual concentration being evaluated (µg/L), f is the response or change from control of the modeled endpoint and b and t are constants.

Table 3.7. Effective concentrations of diuron (µg/L) using a logistic model and no observable effect concentrations (NOEC) for *Lemna gibba* over a 7 day exposure period for a variety of endpoints.

Endpoint	NOEC (µg/L)	EC_{10} (95% CI) (µg/L)	EC_{25} (95% CI) (µg/L)	EC_{50} (95% CI) (µg/L)	Parameters[a]	Adjusted r^2
Plant number	>1000	16.3 (0, 98.6)	48.4 (0, 201.4)	143.5 (0, 416.4)	b = 1.01; t = 2.04; x = 143.5	0.42
Frond number	>1000	8.2 (0, 33.8)	11.7 (0, 34.1)	16.6 (0, 129.7)	b = 3.13; t = 23.17; x = 16.63	0.91
Wet mass (g)	1	2.4 (0, 5.9)	5.5 (1.1, 10.0)	12.7 (6.8, 18.6)	b = 1.32; t = 6.6 x 10^{-2}; x = 12.72	0.94
Dry mass (g)	1	1.2 (0, 3.8)	4.0 (0, 9.3)	12.8 (1.4, 24.2)	b = 0.93; t = 3.0 x 10^{-3}; x = 12.81	0.88
Chlorophyll - a (µg/mg)	>1000	699.7 (0, 4.2 x 10^5)	920.9 (0, 2.6 x 10^5)	1211.9 (0, 5.2 x 10^4)	b = 4.0; t = 0.66; x = 1.2 x 10^3	< 0.01

NOEC – No observable effect concentration, EC – Effective concentration, CI – Confidence interval

[a]Reparameterized logistic equation to fit concentration-responses of diuron exposed *L. gibba*: $f = t/(1 + (x_o/x)^b)$. The variable x is the EC_{50} of the concentration-response relationship modeled, x_o is the actual concentration being evaluated (µg/L), f is the response or change from control of the modeled endpoint and b and t are constants.

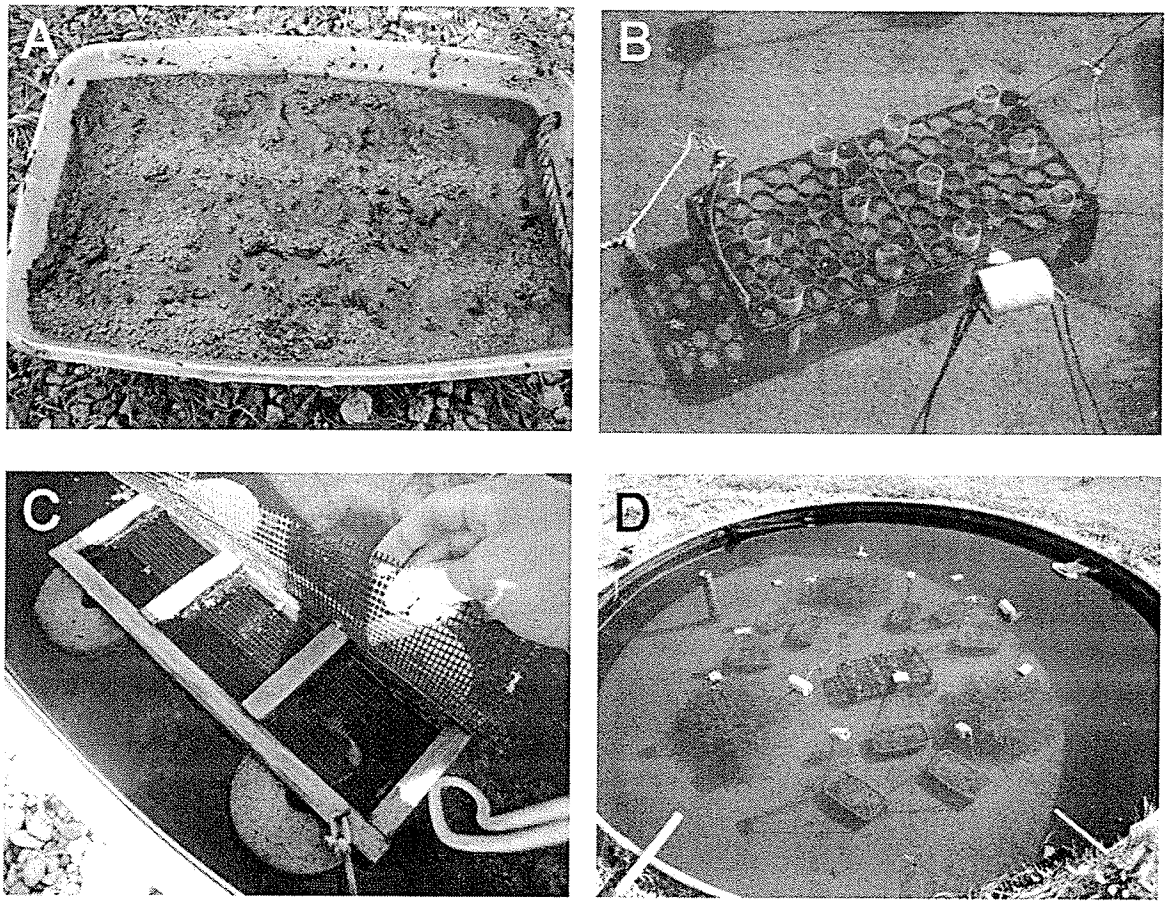

Figure 3.1 (A) Plastic trays used for E. canadensis populations (B) Cone-tainers used for individual shoots of *E. canadensis* and *M. spicatum* (C) Wooden corrals for *Lemna gibba* tests (D) Layout of various tests in one microcosm.

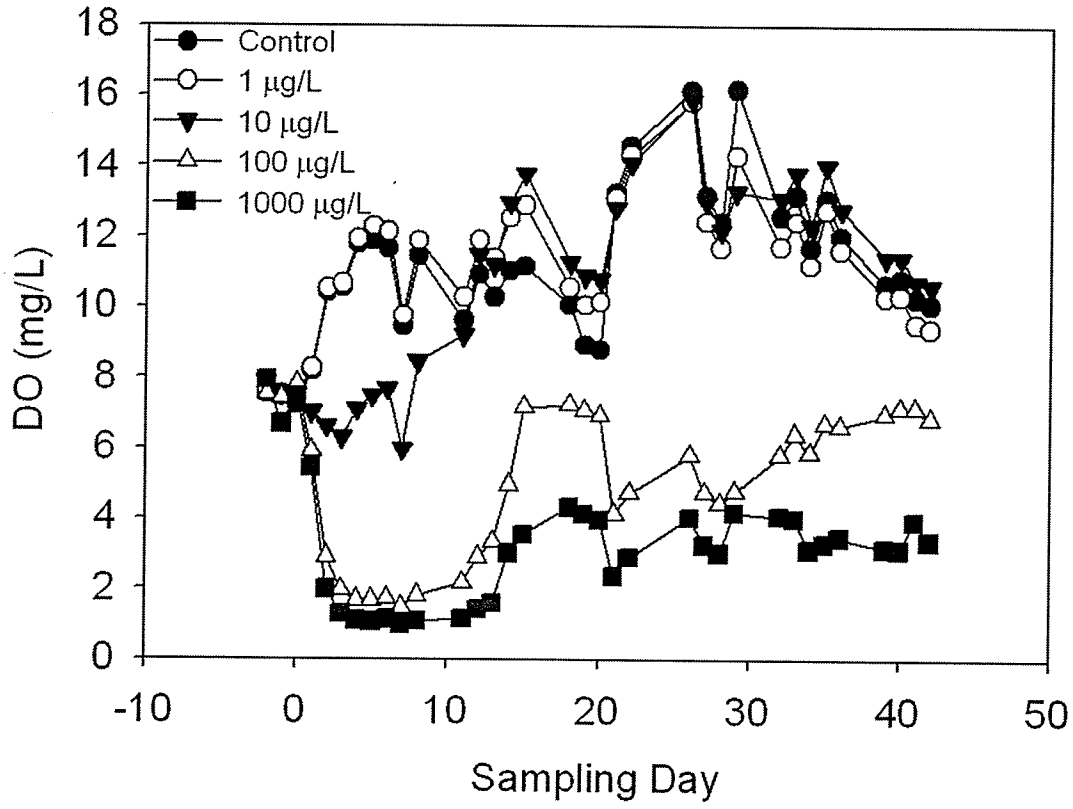

Figure 3.2 Average daily dissolved oxygen (DO) profiles for aquatic microcosms exposed to diuron over a 42-day study period.

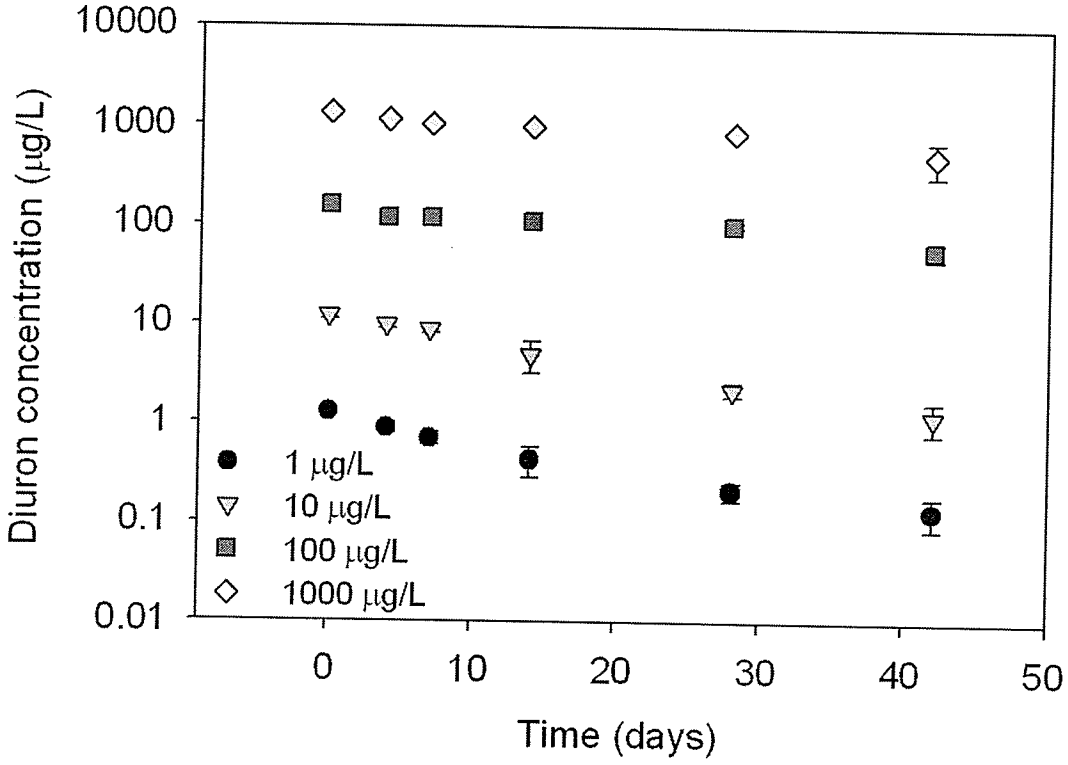

Figure 3.3 Dissipation of diuron in aquatic microcosms over a 42-day study period. Error bars represent standard deviation about the mean (n=3).

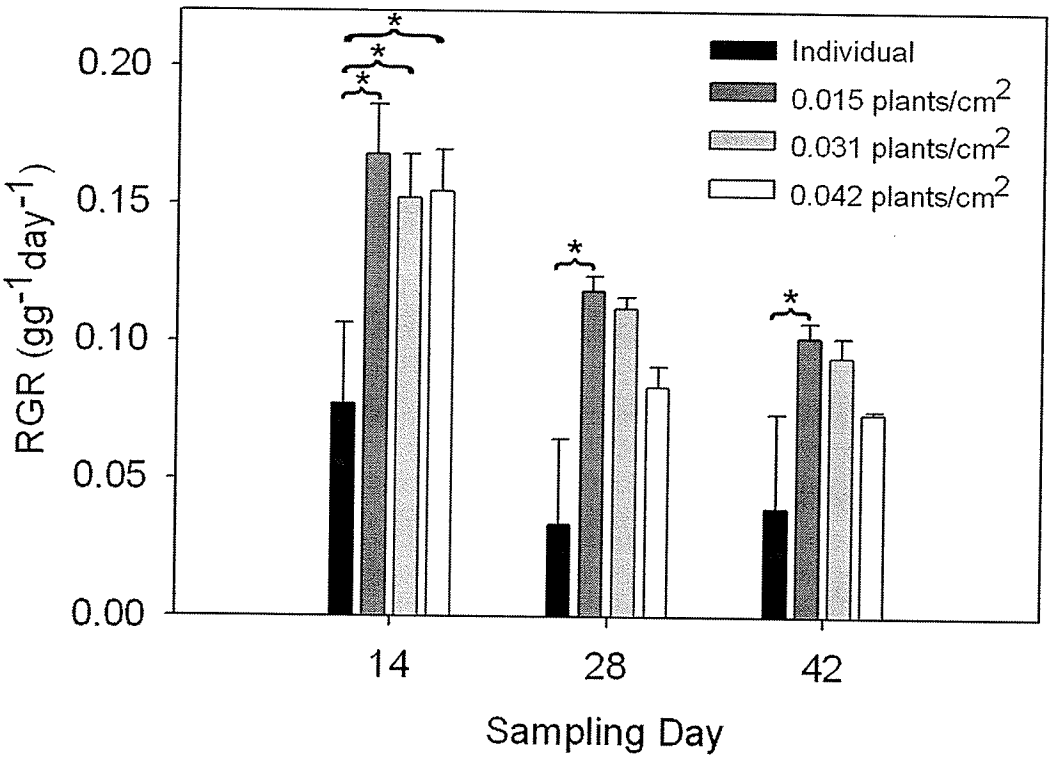

Figure 3.4 Relative growth rates (RGR) of *Elodea canadensis* populations and individual (cone-trainer) plants based on total wet mass in control microcosms. An asterisk (*) represents statistically significant differences between the two bracketed population densities ($p<0.05$). Error bars represents standard deviation about the mean (n=3).

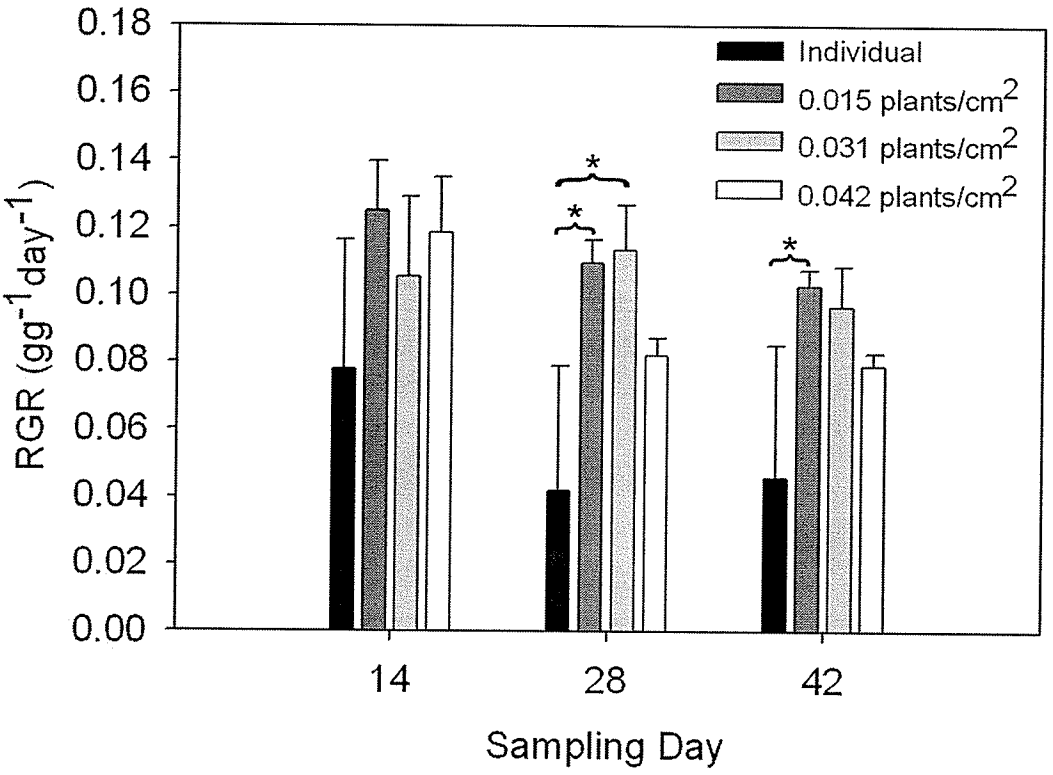

Figure 3.5 Relative growth rates (RGR) of *Elodea canadensis* populations and individual (cone-tainer) plants based on total dry mass in control microcosms. An asterisk (*) represents statistically significant differences between the two bracketed population densities ($p<0.05$). Error bars represents standard deviation about the mean (n=3).

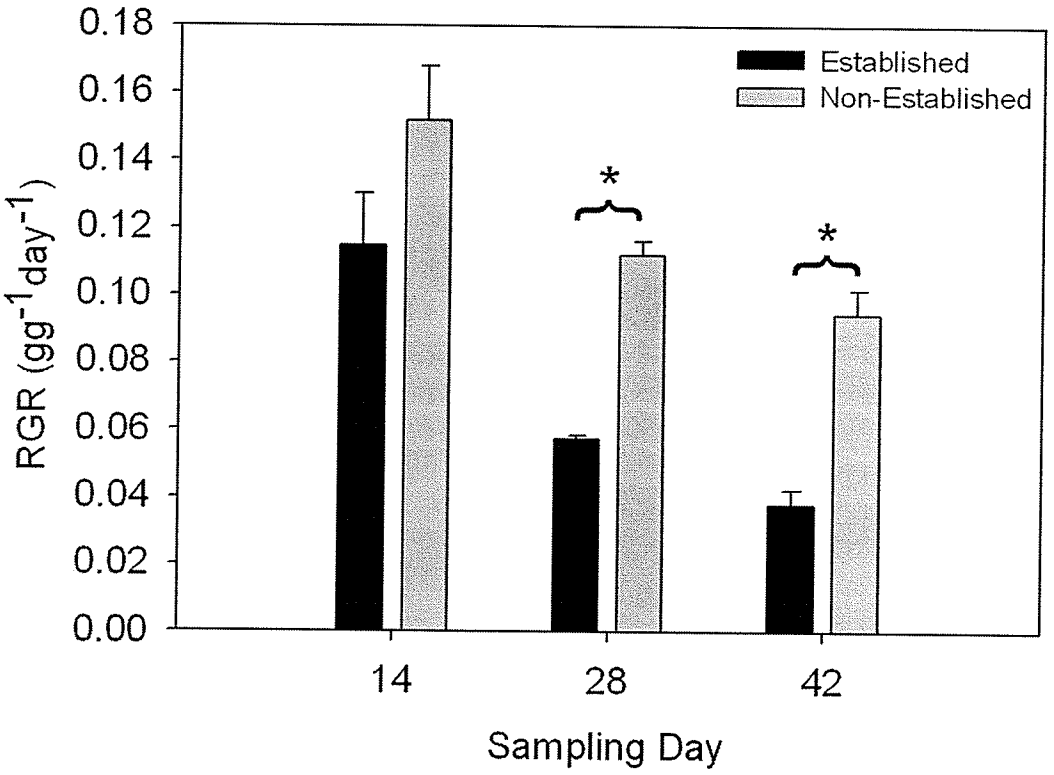

Figure 3.6 Relative growth rates (RGR) of established and non-established *Elodea canadensis* populations planted at 0.031 plants/cm^2 based on total wet mass in control microcosms. An asterisk (*) represents statistically significant differences between the two bracketed population densities using a t-test ($p<0.05$). Error bars represents standard deviation about the mean (n=3).

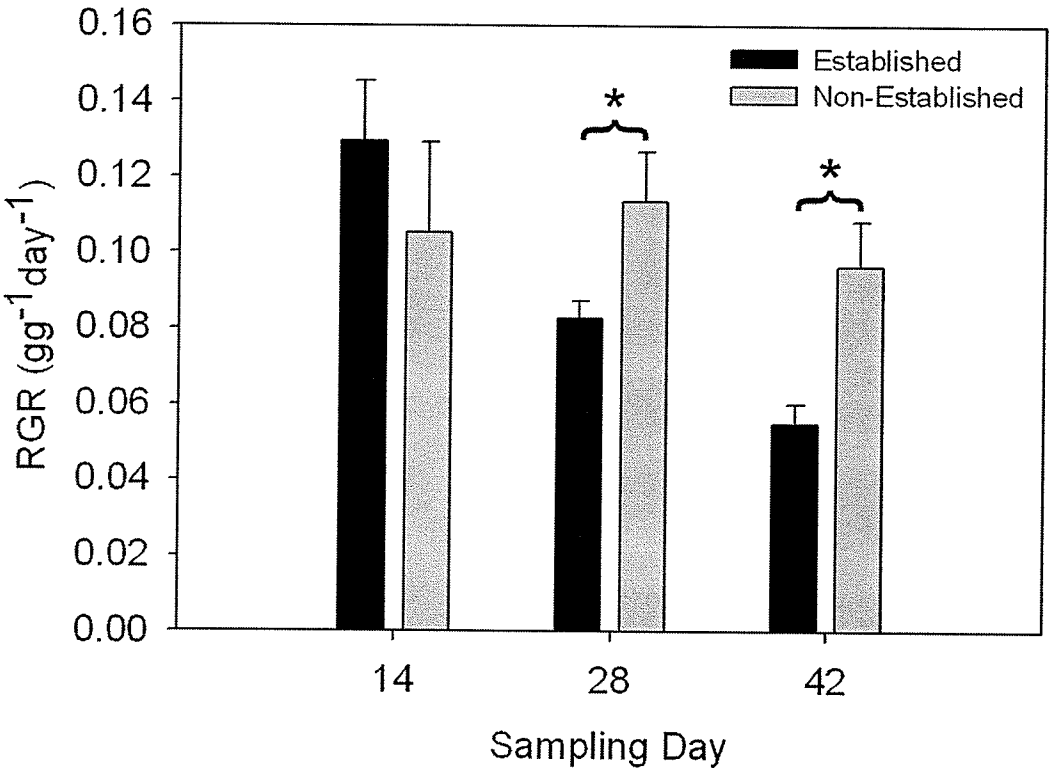

Figure 3.7 Relative growth rates (RGR) of established and non-established *Elodea canadensis* populations planted at 0.031 plants/cm^2 based on total dry mass in control microcosms. An asterisk (*) represents statistically significant differences between the two bracketed population densities using a t-test ($p<0.05$). Error bars represents standard deviation about the mean.

4 SUMMARY AND OVERALL CONCLUSIONS

4.1 CONCLUSIONS

The first objective was to assess which commonly used plant growth media promoted the strongest development in aquatic macrophytes under controlled laboratory conditions. In addition, the influence of supplemental nitrogen and phosphorus and the presence and absence of root substrate in the test systems wanted to be clearly understood. It was hypothesized that Modified Andrew's media would provide strongest growth in laboratory assays with the presence of substrate and supplemental N and P promoting strongest growth as well based on previous results in the literature.

Overall, comparisons between media for various endpoints showed that AAP media with supplemental nitrogen and phosphorus promoted the strongest and most consistent macrophyte development. Strongest development was also observed in systems containing root substrate compared to those without substrate. The presence of algal contamination was also minimal in AAP media. This was likely due to a N:P ratio close to the ratio of 17:1, which indicates no limitation of nitrogen and phosphorus that could cause proliferation of certain algal species (Townsend et al., 2008). Therefore, AAP media could potentially be used for further toxicity testing with *E. canadensis*. If non-axenic plant material is to be used in a laboratory test, it is crucial that the growth media used will promote strong growth and help keep contamination at a minimum. The N:P ratio of AAP appeared to fulfill both of these criteria for these tests. These findings also show the importance of media formulation in the success of laboratory-based tests. If other media are used for other species, it is important that variations in formulation are

tested. This can be done by adjusting the pH and the N:P ratios, as well as concentrations of other nutrients, to determine an optimal formulation that will promote strong growth and reduce contamination.

The next objective was to characterize the effects of two common herbicides (atrazine and diuron) on the aquatic macrophyte *Elodea canadensis* grown in the growth media that promoted strongest growth and assess the statistical sensitivity, or variation, of endpoints. Based on laboratory culturing methods, it was anticipated that *E. canadensis* would show a toxicological response to diuron and atrazine with RGR endpoints being most sensitive, with diuron showing the highest sensitivity and that under controlled laboratory conditions, variation for endpoints would be low.

Diuron was found to elicit a concentration-response in *E. canadensis* in the tested range, while no response was observed with atrazine. This may have been due to algal contamination out-competing the macrophytes for resources and eventually leading to a reduced growth rate in the macrophytes. This has been observed in other systems (Ozimek et al. 1991; Pieczynska et al. 1996) and could partially explain our failure to observe clear responses. Since non-axenic conditions were chosen for this methodology, which made testing more rapid, the presence of algal contamination was unavoidable and likely the source of significant variation in toxicity data. Variation in *E. canadensis* toxicity tests was large, with wide confidence intervals and coefficients of variation and minimum detectable differences in the range of >50-100% of controls. This indicated poor ability for the tests to detect ecologically significant changes, which are usually determined by the benchmark of < 25% different from controls (Christman et al., 1994). Also, since the plants were extracted from natural populations, variation caused by the

source location may have also contributed to variation in the tests. If these tests are to be developed into a standard method for *E. canadensis*, it is important that sources of variation, statistical and toxicological, are considered and reduced. Plants taken from natural sources should be taken from a single location if possible to reduce variation attributed to using multiple harvesting locations. Also, a stable laboratory culture needs to be established from naturally obtained plant material. This will provide sufficient and timely material prior to a test, which will help to reduce variation caused by algal contamination. In doing this, a more accurate and precise description of toxicological responses can be achieved.

The next objective was to compare our *E. canadensis* results to those of a parallel assay of *Lemna gibba* and *Lemna minor* to determine relative sensitivities of these plants. It was predicted that *Lemna* species would show greater sensitivity than *E. canadensis* due to their rapid growth rate and high capacity for assimilation and bioconcentration of chemicals.

In the laboratory, *L. gibba* and *L. minor* exposed to diuron were less sensitive to diuron as compared to *E. canadensis*. Overall, sensitivity to diuron was *E. canadensis* > *L. minor* > *L. gibba*. The greater sensitivity to diuron compared to *Lemna* species indicates the possible inclusion of *E. canadensis* as a commonly tested species in risk assessments. On the other hand, these results need to be viewed with caution since *E. canadensis* tests exhibited low statistical resolution compared to *Lemna* species, which had narrow confidence intervals, correlation coefficients in the > 0.9 range, and most CV and MDDs in the 15 – 25% range. These results showed that *E. canadensis* have higher sensitivity to herbicides compared to *Lemna* species, indicating promise for its further use

in risk assessment. In order to confirm its validity as a standard test organism, it is crucial establish a low level of variation in these tests so they will have both toxicological and statistical sensitivity. Until this is done, *Lemna* still shows a relative high sensitivity to herbicides both toxicologically and statistically, thus supporting their use in current risk assessment applications.

The next objective was to determine the relationship between relative growth rate (RGR) and toxicological sensitivity under field conditions, specifically within newly developing populations of different densities, individual plants, and between established and non-established stands and to determine if these various planting designs influenced toxicity. Based on previous work in these systems and bioassays in general, it was hypothesized that plants with higher RGRs will exhibit higher toxicological sensitivity compared to plants with lower RGRs and that low-density populations and non-established stands would exhibit the highest toxicological sensitivity.

Our work found that with increasing population density, RGR tended to decrease slightly. Also, established stands, which had approximately 30 days more growth time prior to exposure and sampling than non-established stands, showed significantly lower RGR than non-established stands. Individuals grown in cone-tainers showed significantly lower RGRs compared to populations of *E. canadensis*. Established stands and populations of high density showed very reduced spatial resources per plant. With a reduction in spatial resources, plants tend to exit the exponential stage of growth as it reaches its carrying capacity, leading to a slower growth rate (Forbes et al., 2001). Also, since cone-tainers provided a very reduced growth area compared to population assemblages, the reduction of spatial resources could have been a factor in these plants as

well. With such high population densities and dense established stands, shading of some plants by others may have reduced RGR (Agami and Reddy, 1990). This reduction in available resources lead to these populations reaching carrying capacity, thus showing a reduce relative growth rate compared to non-established and low-density populations. Associated with these results was a decreased sensitivity to diuron with highest population densities and with established stands. This relationship between RGR and toxicological sensitivity has been previously described. Cedergreen et al. (2004) and Huebert and Shay (1993) have both shown that higher relative growth rates can lead to greater sensitivity to chemicals.

These results signify the importance of representing natural macrophyte assemblages in toxicity testing. Populations of macrophytes that are established or at higher densities may underestimate toxicity of a compound compared to a new population or low-density population still in its exponential growth phase. Since both new and established populations exist in the environment, it is important to include these assemblages in toxicity testing. The reliance on individual plants in toxicity tests does not represent the population and community structures found in the environment. Standard testing methods must incorporate these various assemblages in order to obtain ecologically relevant results.

Another objective was to assess the toxicity of diuron to *E. canadensis*, *L. gibba*, and *M. spicatum* under field conditions. It was hypothesized that *Lemna* species will be the most sensitive to diuron based on previous observations in these systems followed by *M. spicatum* and *E. canadensis.*

In comparing the common endpoints among the three species of wet and dry mass, overall sensitivity was *M. spicatum* > *L. gibba* > *E. canadensis*. This indicates that, although the effects of population density and stand establishment on toxicity were explained well by *E. canadensis*, its overall sensitivity in field based testing is quite low compared to other commonly used species. These results support the utility of *Myriophyllum* and *Lemna* in risk assessment and also explain why they both have standard methods currently developed for them. More work needs to be done in characterizing toxicity of *E. canadensis* under field conditions to determine if it may be more sensitive than other commonly used species for other chemicals.

The final objective was to compare laboratory toxicity results to microcosm studies using atrazine and diuron to determine if laboratory results can predict toxicity of field conditions. It was hypothesized that laboratory studies will be more sensitive than field-based studies for diuron and atrazine as this has been the general observation for many plant-based bioassays.

When all data were adjusted to a 14-d duration with Haber's Rule, laboratory data for both endpoints was more sensitive than individual plants exposed in the field and within one order of magnitude of each other. Comparing laboratory data to field data of *E. canadensis* population data, it was found that for RGRwet on a per plant basis, laboratory data were more sensitive than field data, again within one order of magnitude. For the wet mass endpoint, plants grown in populations at the low and intermediate densities, showed higher sensitivities than laboratory results, again within one order of magnitude. These data show that, it is still difficult to make a direct linkage to the laboratory since sensitivities were not drastically different between the laboratory and the

field. The prevalence of contamination in the laboratory studies also reduces the confidence in establishing links between laboratory and field results. If a laboratory assay with non-axenic *E. canadensis* shoots can be conducted in a way that no contamination occurs, the exptrapolation of the laboratory to field can be better characterized. These results do show consistent sensitivity in the laboratory compared to the field and therefore indicate that through further testing, submersed macrophyte tests may be protective and used as field surrogates. Individual plants grown in populations being more sensitive than individuals grown in the laboratory also shows that significance of simulating natural assemblages in toxicity testing.

4.2 FUTURE RESEARCH DIRECTIONS

Population and stand establishment studies indicated the importance of considering model populations in toxicity testing. Since these systems more accurately reflect the natural growth of macrophytes, it is important to use them in further testing. I would propose that if *E. canadensis* be considered as a standard test organism that population and stand establishment be tested with a variety of other compounds to determine if these factors influence toxicity of other compounds. Another avenue of research in this area would be to conduct population tests with *E. canadensis* in a laboratory setting. Sediment filled aquaria with field water planted with various population densities could be used to assess toxicity of populations under more controlled conditions. This may elicit the differential population responses with higher resolution due to less sources of variation that are found in a field setting. Also, parallel assays with other macrophytes grown in populations could also be conducted to determine if population effects occur with other species. This was investigated by McGregor et al.

(2008) where *M. spicatum* and *E. canadensis*, exposed to atrazine, were planted as individuals and in population assemblages. Although no differences in toxicity were observed between planting designs in this study, only one density was used. Different results may be observed if ranges of population densities are used. An interesting aspect of McGregor et al. (2008) as well as McGregor et al. (2007) is the planting of *E. canadensis* and *M. spicatum* in community assemblages. My study focused solely on monocultures of *E. canadensis*. Since macrophytes grow in communities, specifically, *E. canadensis* grows well with *Myriophyllum* species (Spicer and Catling, 1988), this type of design is more representative of natural assemblages. No significant effect of community assemblage on toxicity was found is either study, but toxicity was also not found for individually grown plants. Since diuron showed a definite toxic response in this study, these community type assemblages may yield different results than these previous studies.

The toxicological sensitivity of *E. canadensis* to diuron grown in AAP media in the laboratory showed some promise for its continued use. In order to increase statistical resolution of these tests, much more work needs to be done. One avenue for further research would be to determine if AAP media could be used to maintain a long-term laboratory culture of *E. canadensis* from non-axenic plant material. This would allow for much more rapid methodologies since the laborious plant location and cleaning step is eliminated. In keeping non-axenic test methods, the exact amounts of nitrogen and phosphorus that encourage contamination to proliferate can also be investigated. A range of nitrogen and phosphorus concentrations can be used in the media formulations. Each concentration can be compared to determine a threshold of algal contamination growth.

If non-axenic methods cannot be used due to high variability, further media tests could be conducted axenically to determine if AAP media would still provide strongest development. This could be made easier by obtaining plant material from a single natural source. By using multiple sources, more natural variation in the macrophytes will be present. The efficacy of AAP as a standard test media could also be further researched. Performing further media tests with various media using other rooted submersed macrophyte species, could determine if it is just as useful for these species.

If *E. canadensis* is to be used further as a laboratory test species, more testing in both field and laboratory settings needs to be conducted. Also, it will be crucial to run parallel assays with *Lemna* species. Results from these tests can determine if A) *E. canadensis* is consistently more sensitive than *Lemna* in the field and in the laboratory and B) *E. canadensis* in the laboratory can predict the toxicity in the field. If *E. canadensis* is not found to be consistently more sensitive than Lemna, or within a factor of 10, the status quo of *Lemna* spp. being the only required test species could remain in place. If this is not the case, then *E. canadensis* may be a useful species in further risk assessment applications.

4.3 REFERENCES

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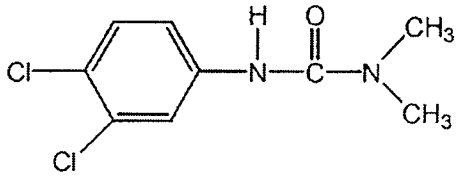
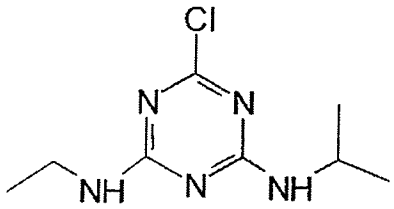
5 APPENDICES

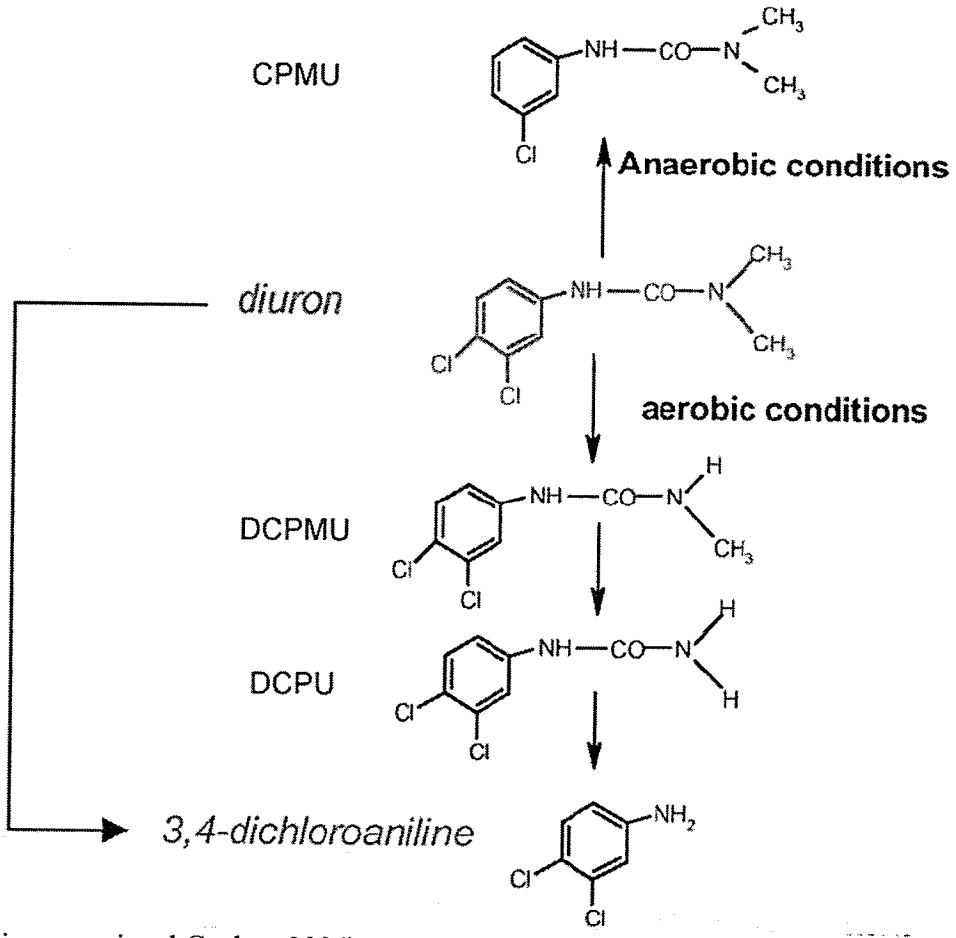
5.1 Appendix 1 – Physical and chemical properties of diuron

Property	Value	Source
Chemical Structure		
Chemical Name	N'(3,4-dichlorophenyl)-N'N-dimethylurea	Vencill, 2002
CAS Number	330-54-1	Vencill, 2002
Molecular Weight	233.1	Vencill, 2002
Molecular Formula	$C_9H_{10}Cl_2N_2O$	Vencill, 2002
Melting Point	158-159°C	Vencill, 2002
Vapor Pressure	9.2 x 10-9 kPa @ 25°C	Vencill, 2002
Solubility	42 mg/L @ 25°C	Vencill, 2002
K_{oc}	485 ml/g	PAN-UK, 2005
Log K_{ow}	2.8 @ 25°C	Vencill, 2002
Half life (aerobic, soil)	90 - 365 days	Vencill, 2002; PAN-UK, 2005

5.2 Appendix 2 – Physical and chemical properties of atrazine

Property	Value	Source
Chemical Structure		
Chemical Name	2-chloro-4-ethylamino-6-isopropyl-amino-1-*s*-triazine	Solomon et al., 1996
CAS Number	1912-24-9	Solomon et al., 1996
Molecular Weight	215.7	Solomon et al., 1996
Molecular Formula	$C_8H_{14}N_5Cl$	Solomon et al., 1996
Melting Point	175-177°C	Solomon et al., 1996
Vapor Pressure	2.9 x 10-7 mm Hg @ 25°C	Solomon et al., 1996
Solubility	33 mg/L @ 22°C	Solomon et al., 1996
K_{oc}	25.3 - 155 (depending on soil type)	Solomon et al., 1996
Log K_{ow}	2.7 @ 25°C	Solomon et al., 1996
Half Life (Aq. photolysis)	335 days (natural light)	Solomon et al., 1996
Half Life (Soil photolysis)	12 days (natural light)	Solomon et al., 1996
Half Life (Aerobic soil metabolism)	146 days (CA loam)	Solomon et al., 1996
Half Life (Anaerobic soil metabolism)	77 days (CA sandy loam)	Solomon et al., 1996
Half Life (Anaerobic soil metabolism)	159 days (CA loam)	Solomon et al., 1996
Half Life (Anaerobic aqueous metabolism)	608 days (GA sandy clay)	Solomon et al., 1996

5.3 Appendix 3 – Bacterial biodegradation pathway of diuron



(Giacomazzi and Cochet, 2004)

5.4 Appendix 4 – Modified Andrew's media

Solution Number	Salt	Weight of salt per 0.5L stock solution (g)	mL stock solution per 2L final volume
1	KNO_3	8.1	5
	$Ca(NO_3)_2 \cdot 4H_2O$	18.9	
2	$MgSO_4 \cdot 7H_2O$	9.85	5
3	KH_2PO_4	2.72	5
4	KCl	0.373	2
	H_3BO_3	0.0775	
	$MnSO_4 \cdot H_2O$	0.0845	
	$ZnSO_4 \cdot 7H_2O$	0.0575	
	$CuSO_4 \cdot 5H_2O$	0.0063	
	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.0019	
5	$FeSO_4 \cdot 7H_2O$	0.278	5
	Na_2EDTA	0.372	

To media, add 30 g/L of sucrose and
adjust to pH 5.8±0.1

5.5 Appendix 5 – 5X AAP media

Solution Number	Compound	Concentration in stock solution (g/500 mL)	Concentration in medium (mg/L)	Prepared medium	
				Element	Concentration (mg/L)
1	$NaNO_3$	13	510	Na; N	190; 84
	$MgCl_2 \cdot 6H_2O$	6	240	Mg	58.08
	$CaCl_2 \cdot 2H_2O$	2.2	90	Ca	24.04
2	$MgSO_4 \cdot 7H_2O$	7.5	290	S	38.22
3	$K_2HPO_4 \cdot 3H_2O$	0.7	30	K; P	9.4; 3.7
4	H_3BO_3	0.095	3.7	B	0.65
	$MnCl_2 \cdot 4H_2O$	0.21	8.3	Mn	2.3
	$FeCl_3 \cdot 6H_2O$	0.08	3.2	Fe	0.66
	$Na_2EDTA \cdot 2H_2O$	0.15	6	-	-
	$ZnCl_2$	0.00165	0.066	Zn	0.031
	$CoCl_2 \cdot 6H_2O$	0.0007	0.029	Co	0.0071
	$Na_2MoO_4 \cdot 2H_2O$	0.00365	0.145	Mo	0.058
	$CuCl_2 \cdot 2H_2O$	0.000006	0.00024	Cu	0.00008
5	$NaHCO_3$	7.5	300	Na; C	220; 43

5 mL of each stock solution is added to approximately 850 mL deionized water with pH adjusted to 7.5 ± 0.1 with 0.1 or 1 mol HCL or NaOH.

5.6 Appendix 6 – Hoagland's media

Solution Number	Compound	Mass of salt (g)/500 mL stock solution	Stock Solution/Liter Water
1	KH_2PO_4	68 g/500 mL	0.5 mL
2	KNO_3	50.5 g/500 mL	2.5 mL
3	$Ca(NO_3)_2 \cdot H_2O$	118 g/500 mL	2.3 mL
4	$MgSO_4 \cdot 7H_2O$	123.5 g/500 mL	1.0 mL
5	$Na_2EDTA \cdot 2H_2O$	0.75 g/500 mL	20 mL
	$FeCl_3 \cdot 6H_2O$	0.242 g/500 mL	
6	Trace Elements		0.5 mL
	H_3BO_3	1.43 g/500 mL	
	$MnCl_2 \cdot 4H_2O$	0.91 g/500 mL	
	$ZnSO_4 \cdot 7H_2O$	0.11 g/500 mL	
	$CuSO_4 \cdot 5H_2O$	0.04 g/500 mL	
	$Na_2MoO_4 \cdot 2H_2O$	0.01 g/500 mL	

Stock solutions are made by dissolving the compounds listed into deionized water. Trace elements can be combined into stock solution 6. To make one-half strength Hoagland's solution for use in testing, add specified amount off each of the stock solutions to approximately 900 mL of the dilution water. Bring the volume to 1L. Adjust to pH 6.5 with 1N KOH or 1N HCl.

5.7 Appendix 7 – Hutner's media

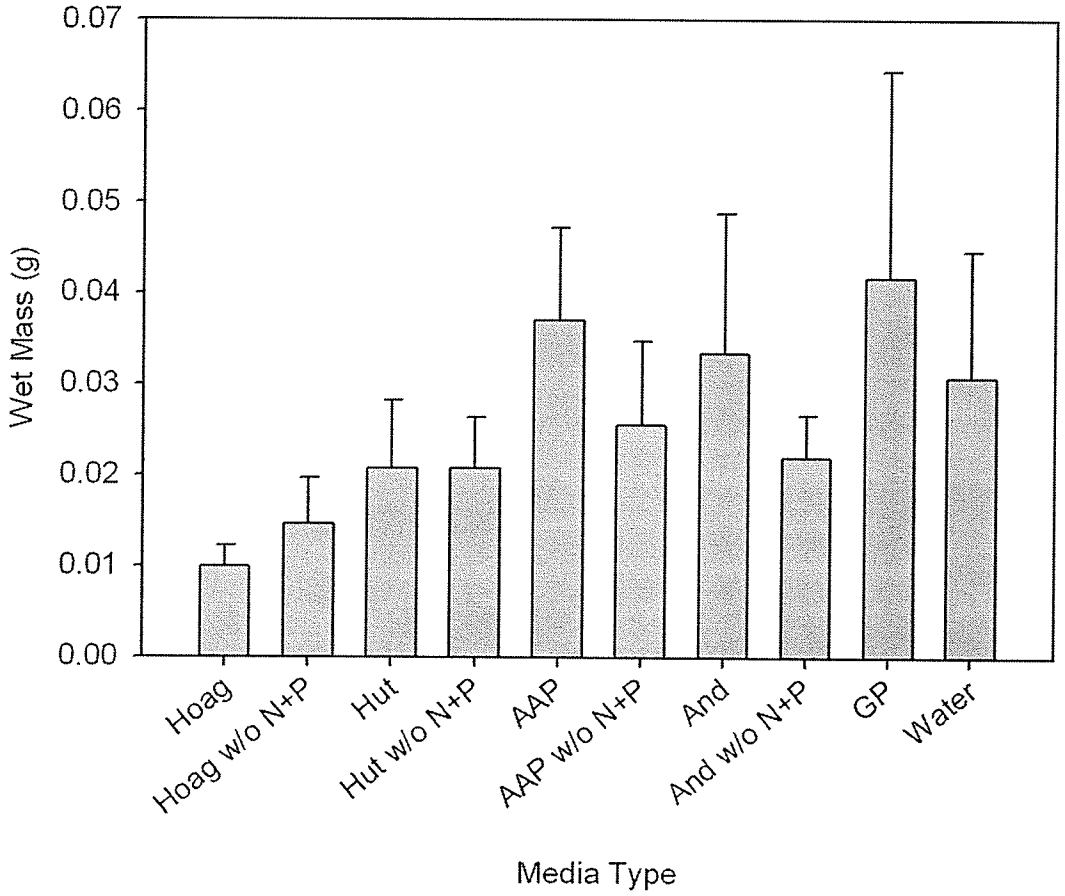
Solution Number	Compound	Mass of salt (g)/500 mL stock solution	Stock solution (mL)/liter of water
1	KNO_3	50	3
	$Ca(NO_3)_2 \cdot H_2O$	120	
2	$MgSO_4 \cdot 7H_2O$	123	3
3	KH_2PO_4	68	3
4	Ferric Citrate*	0.5	1
	Na_2EDTA	1.45	
	H_3BO_3	0.5	
	$MnSO_4 \cdot H_2O$	0.05	
	$ZnSO_4 \cdot 7H_2O$	0.5	
	$CuSO_4 \cdot 5H_2O$	0.015	
	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.05	

*Add first, heat to dissolve, then add remaining reagents
Once mixed, add 10g/L sucrose to stock solution

5.8 Appendix 8 – General purpose culture media

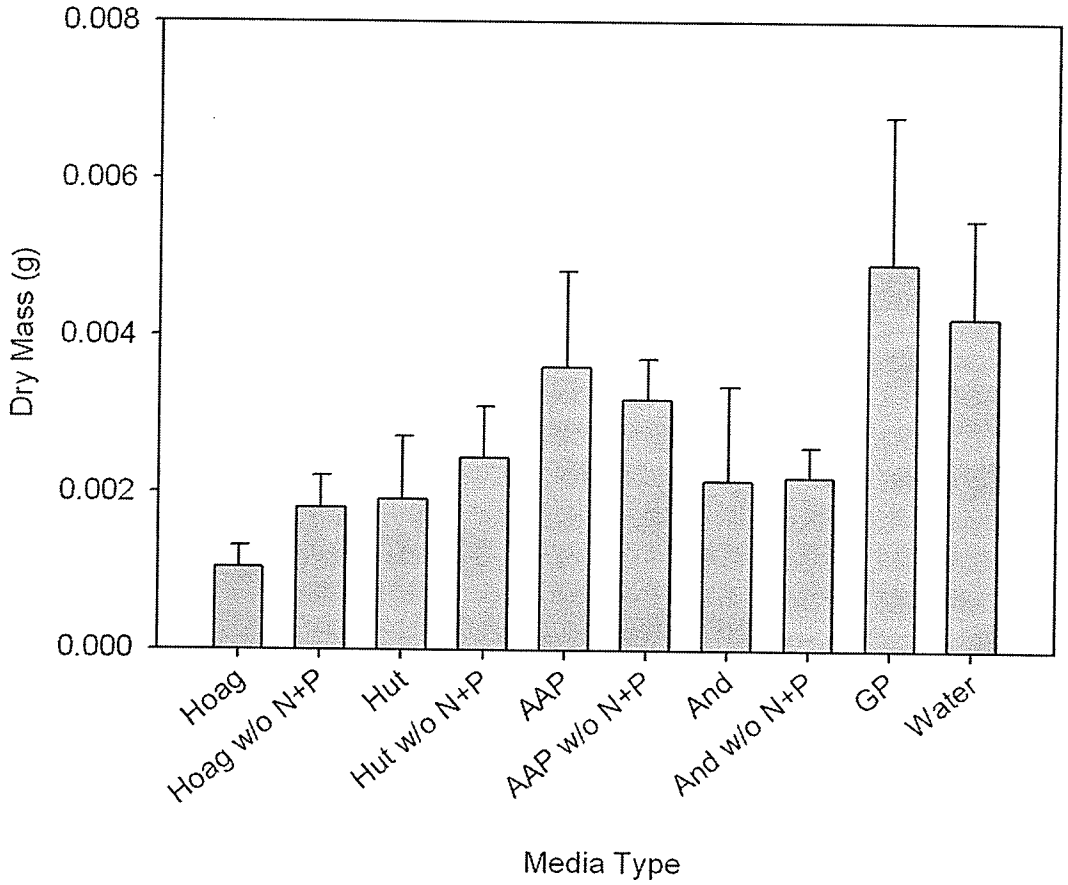
Compound	Mass of salt (g)/500 mL stock solution	Stock solution (mL)/liter of water
$CaCl_2 \cdot 2H_2O$	4.585	10
$MgSO_4 \cdot 7H_2O$	3.45	10
$NaHCO_3$	2.92	10
$KHCO_3$	0.77	10

5.9 Appendix 9 - Wet mass of *Elodea canadensis* shoots grown over a 14-day period in various culture media with root substrate



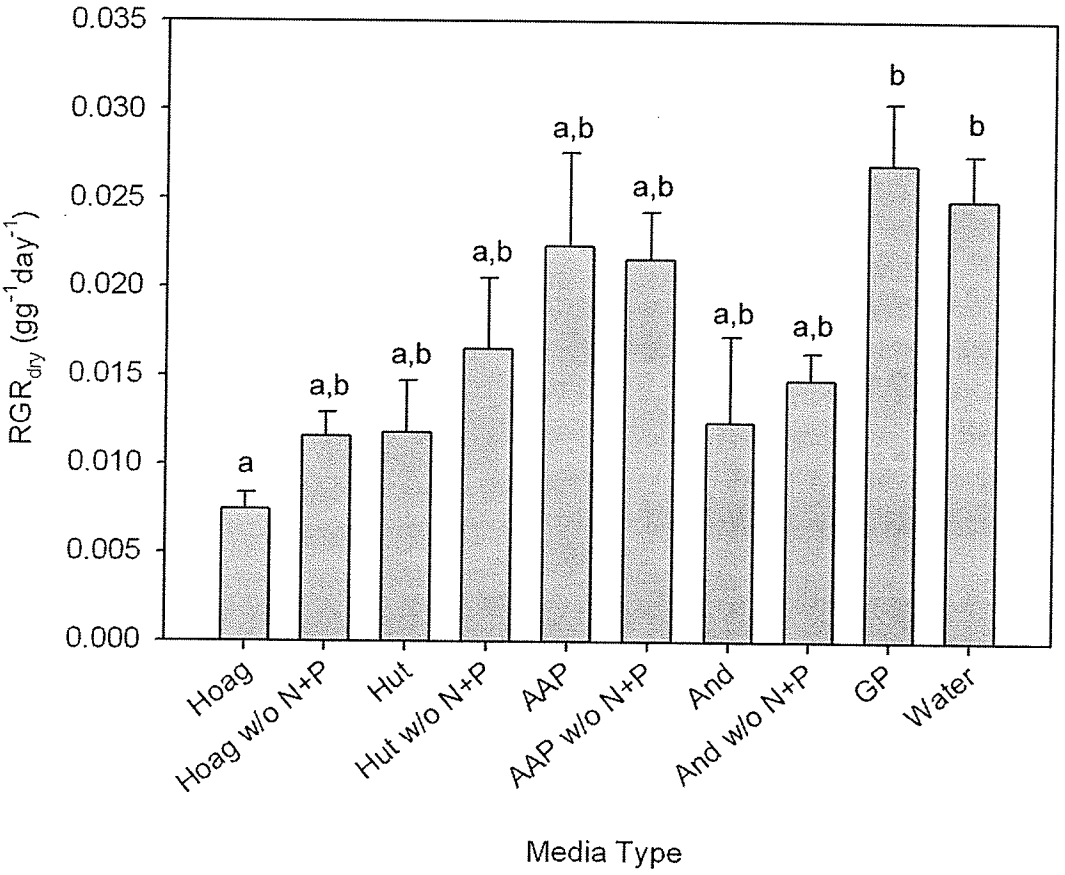
Wet mass of *Elodea canadensis* shoots grown over a 14-day period in various culture media with root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

5.10 Appendix 10 - Dry mass of *Elodea canadensis* shoots grown over a 14-day period in various culture media with root substrate



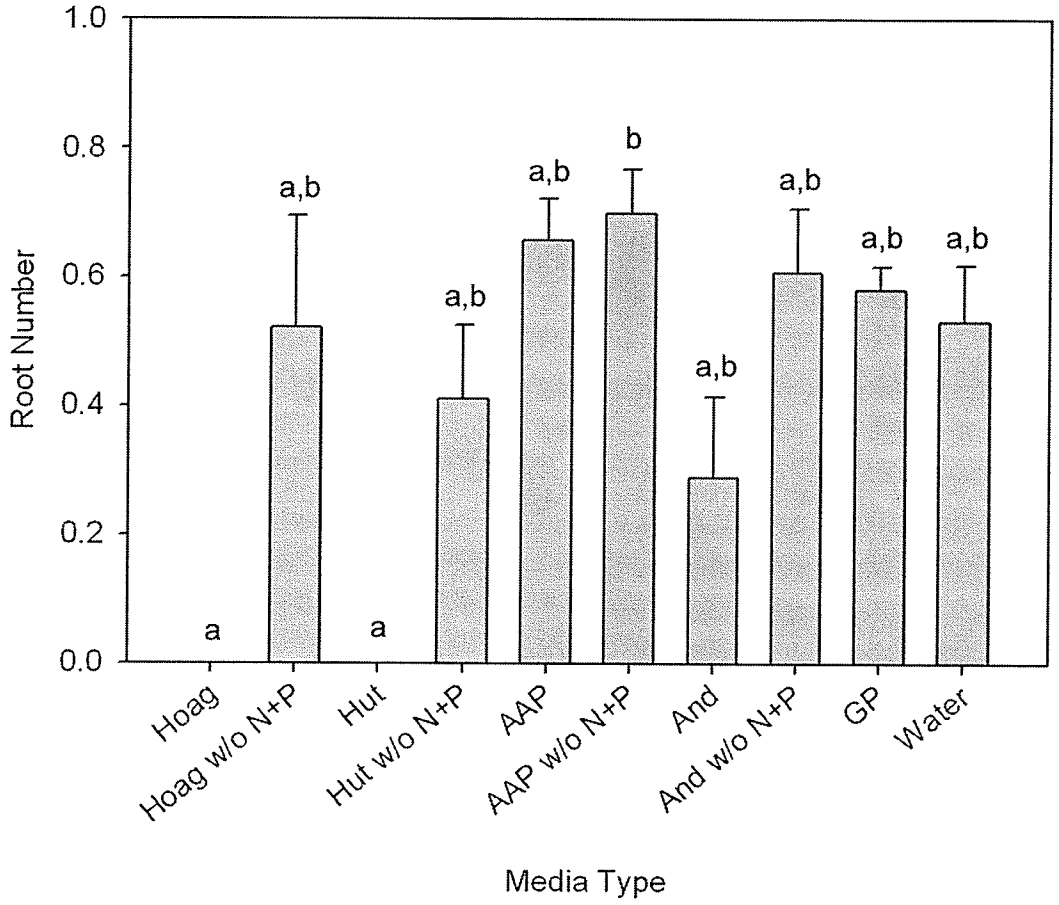
Dry mass of *Elodea canadensis* shoots grown over a 14-day period in various culture media with root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

5.11 Appendix 11 - Relative growth rate (RGR) based on dry mass of *Elodea canadensis* shoots grown over a 14-day period in various culture media with root substrate



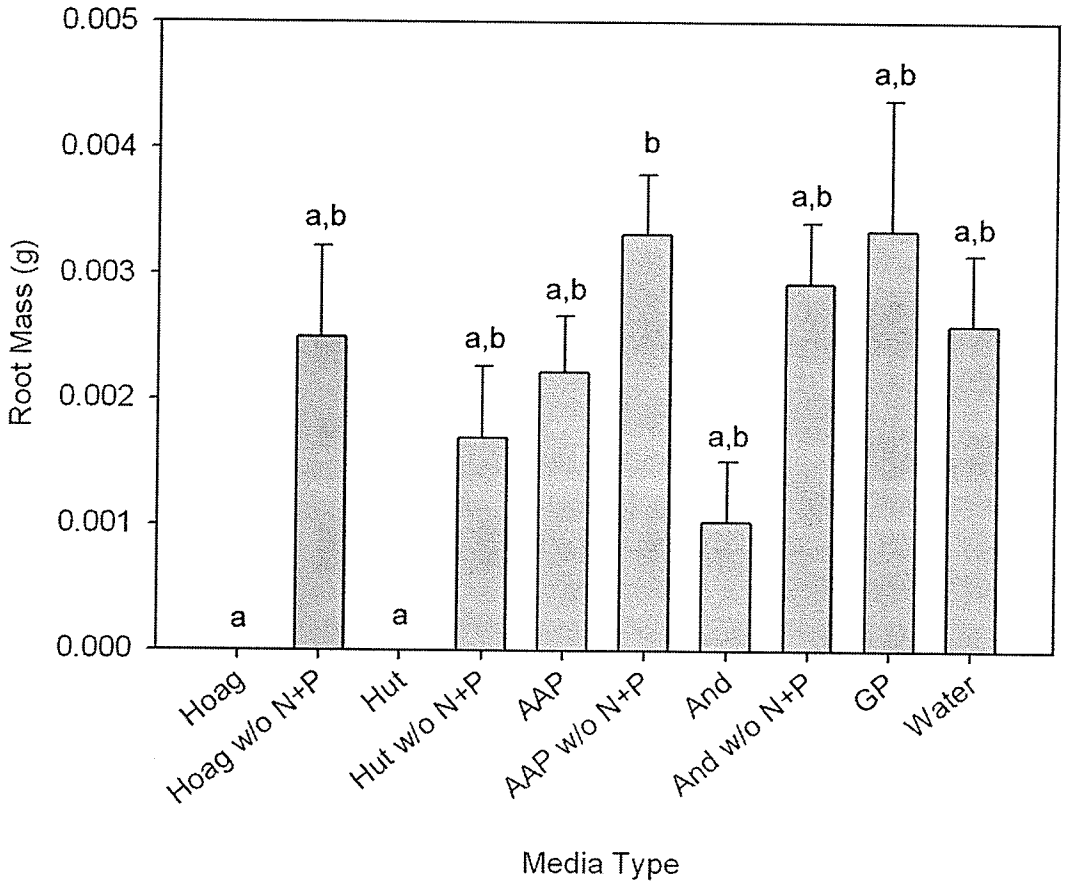
Relative growth rate (RGR) based on dry mass of *Elodea canadensis* shoots grown over a 14-day period in various culture media with root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

5.12 Appendix 12 - Root number of *Elodea canadensis* shoots grown over a 14-day period in various culture media with root substrate



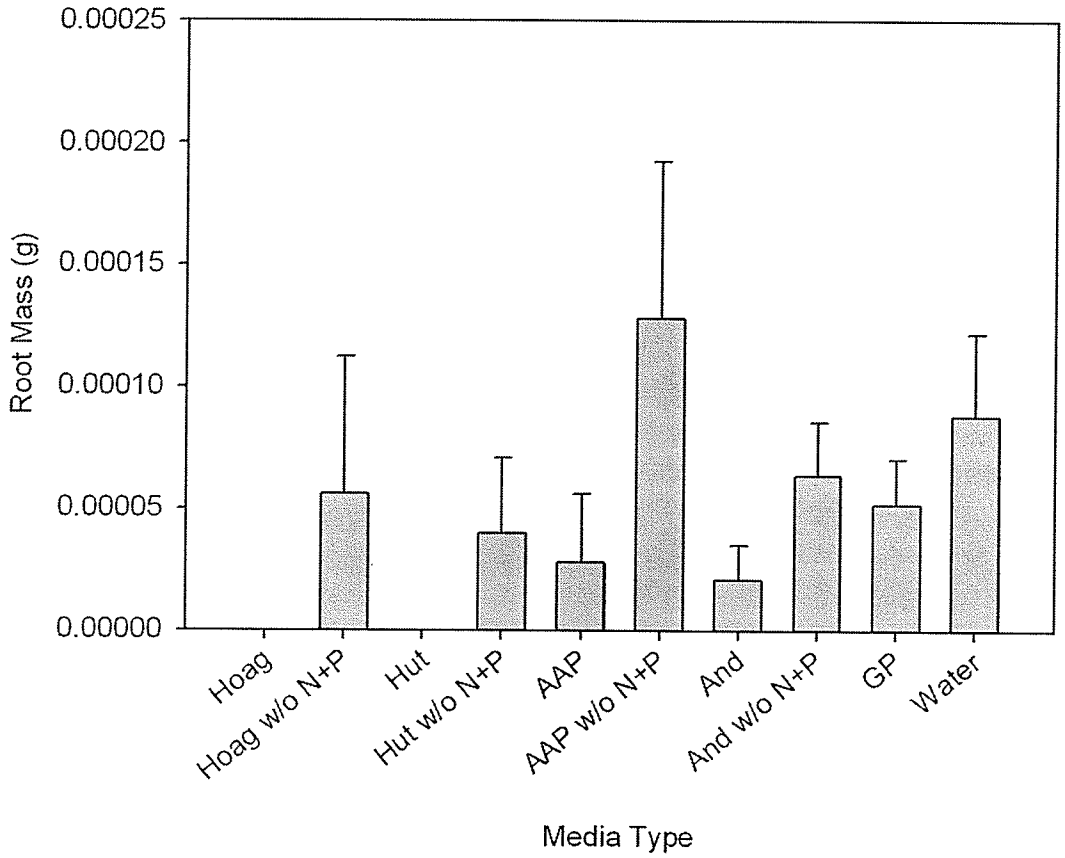
Root number of *Elodea canadensis* shoots grown over a 14-day period in various culture media with root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

5.13 Appendix 13 - Wet root mass of *Elodea canadensis* shoots grown over a 14-day period in various culture media with root substrate



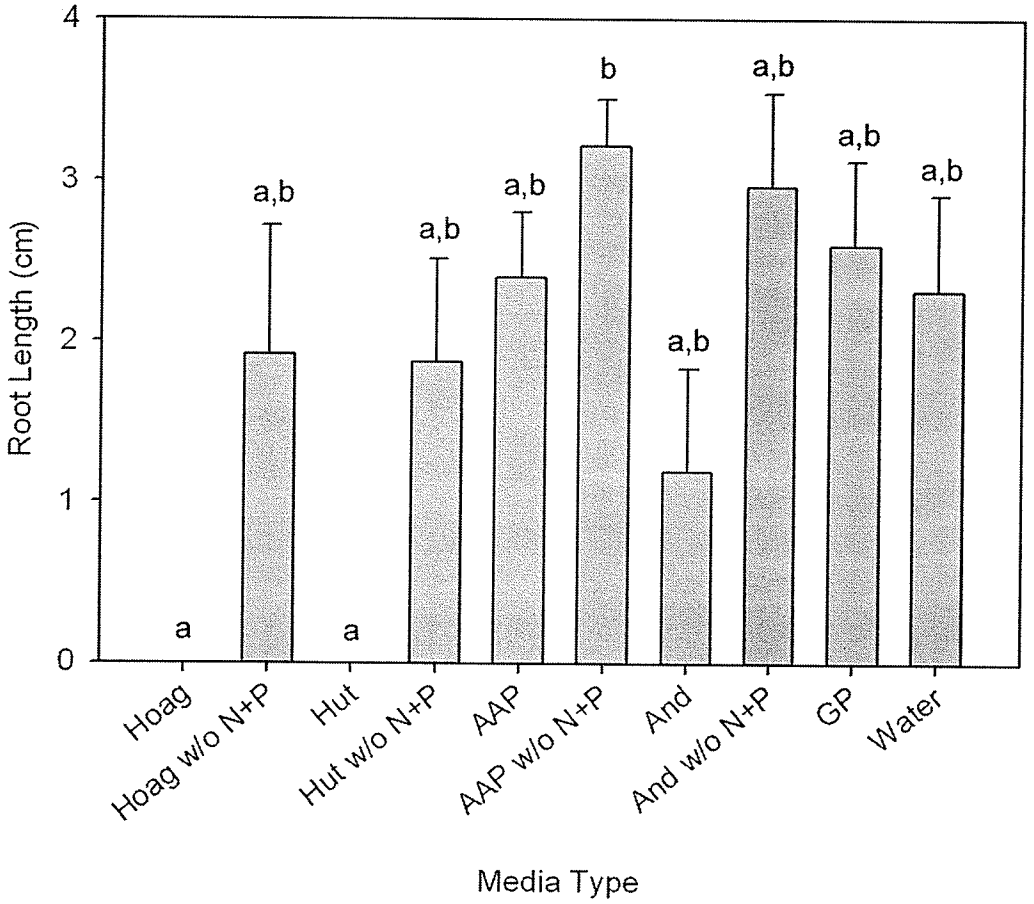
Wet root mass of *Elodea canadensis* shoots grown over a 14-day period in various culture media with root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

5.14 Appendix 14 - Dry root mass of *Elodea canadensis* shoots grown over a 14-day period in various culture media with root substrate



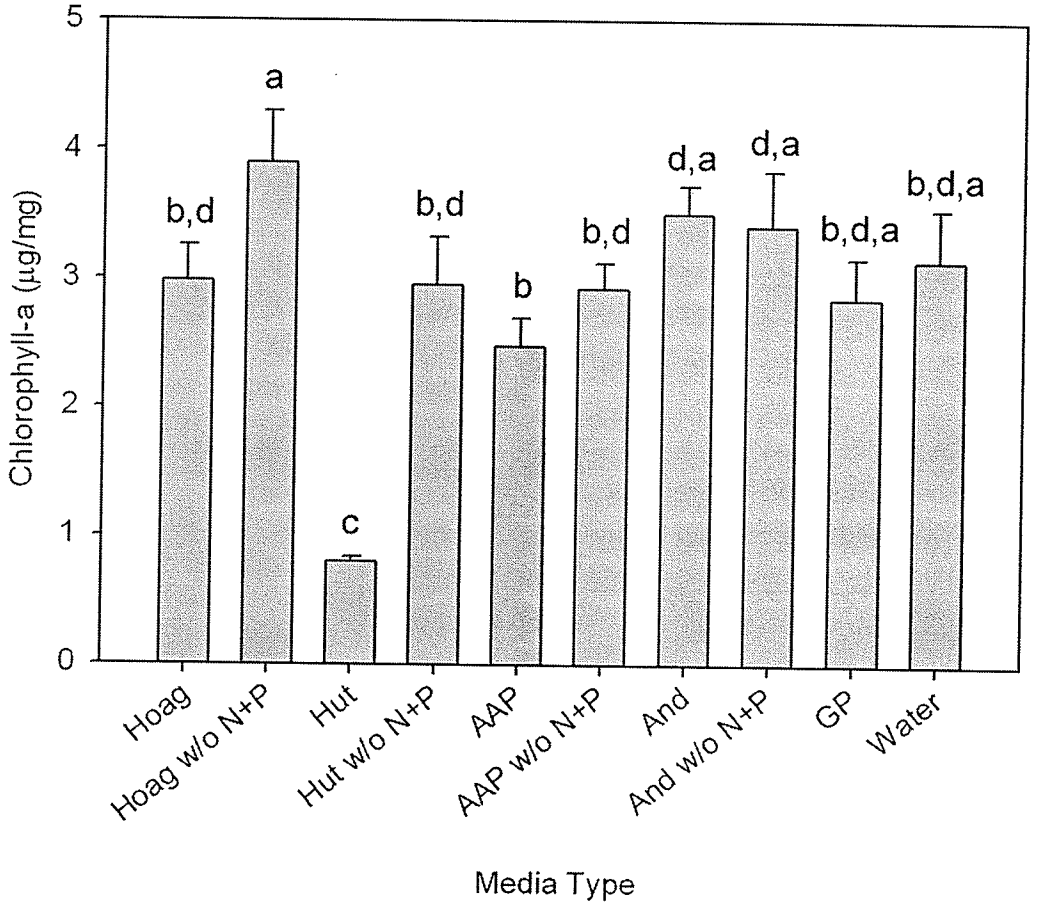
Dry root mass of *Elodea canadensis* shoots grown over a 14-day period in various culture media with root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

5.15 Appendix 15 - Root length of *Elodea canadensis* shoots grown over a 14-day period in various culture media with root substrate



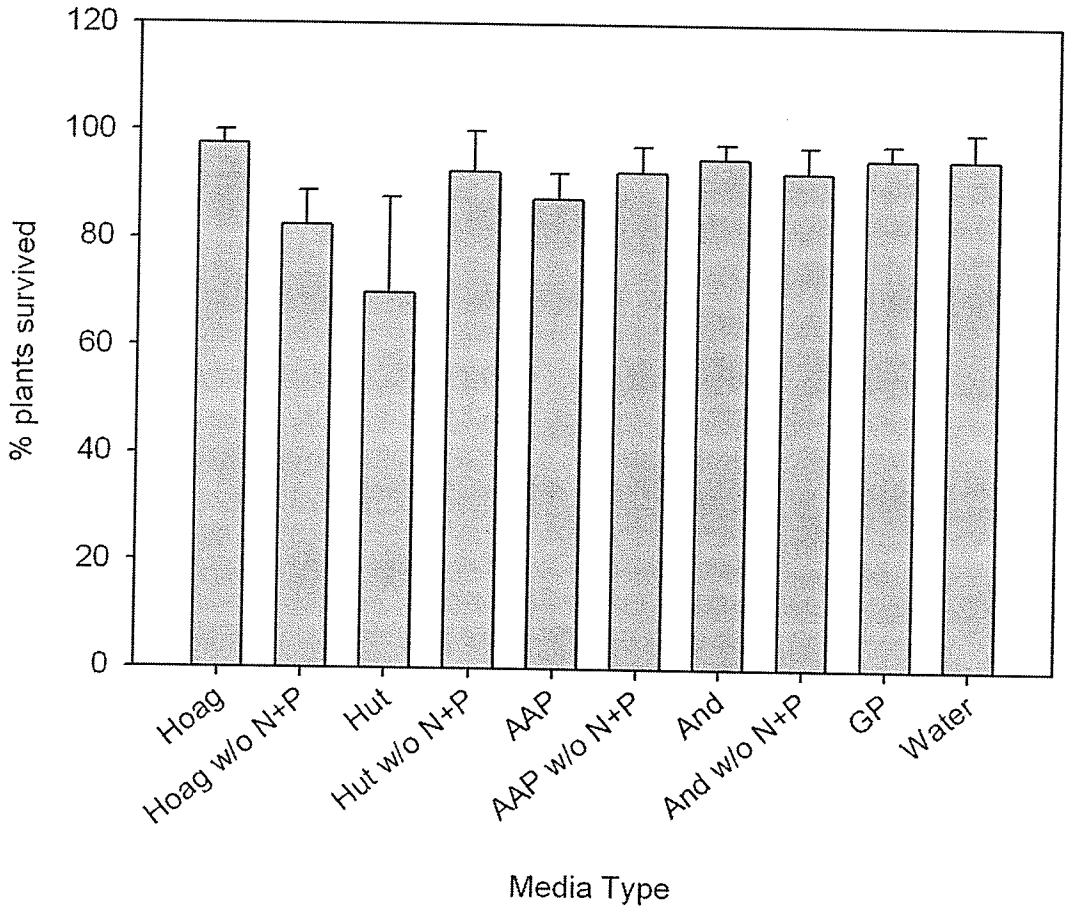
Root length of *Elodea canadensis* shoots grown over a 14-day period in various culture media with root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

5.16 Appendix 16 - Chlorophyll-a content of *Elodea canadensis* shoots grown over a 14-day period in various culture media with root substrate



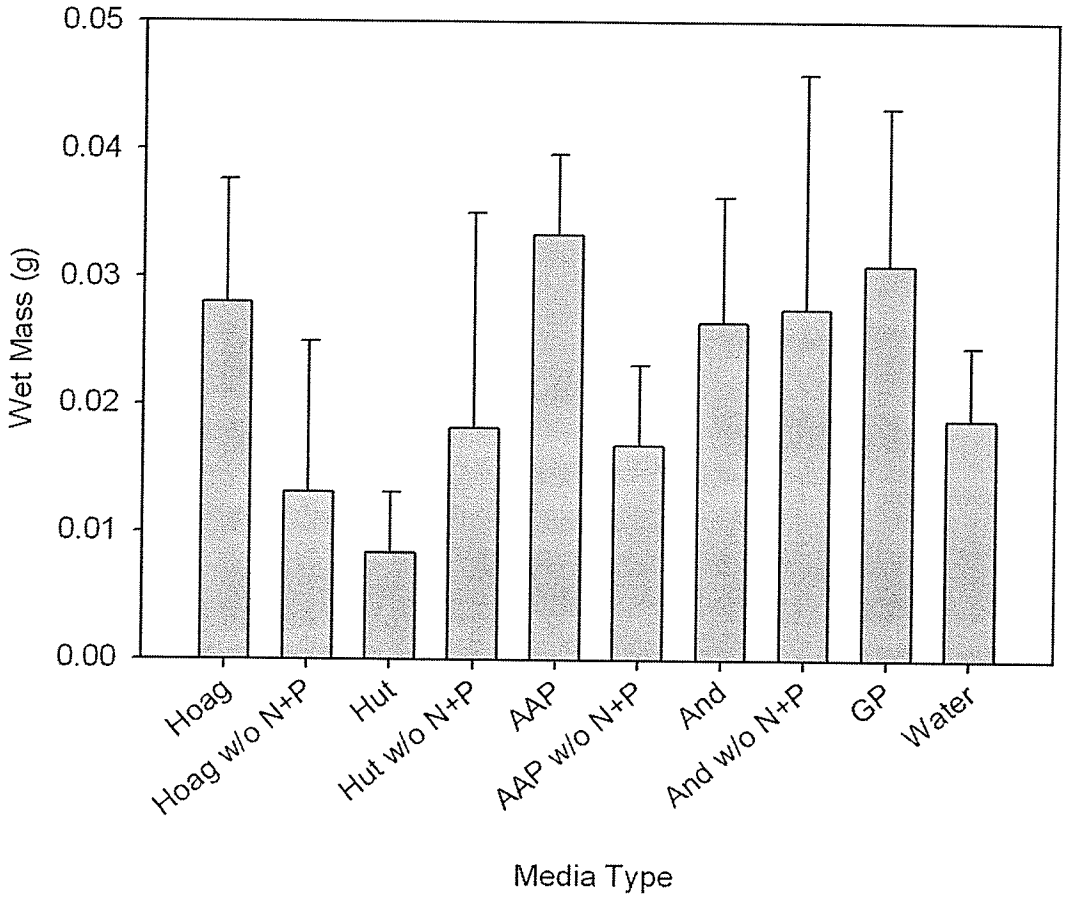
Chlorophyll-a content of *Elodea canadensis* shoots grown over a 14-day period in various culture media with root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

5.17 Appendix 17 - Percent survival of total planted *Elodea canadensis* shoots grown over a 14-day period in various culture media with root substrate



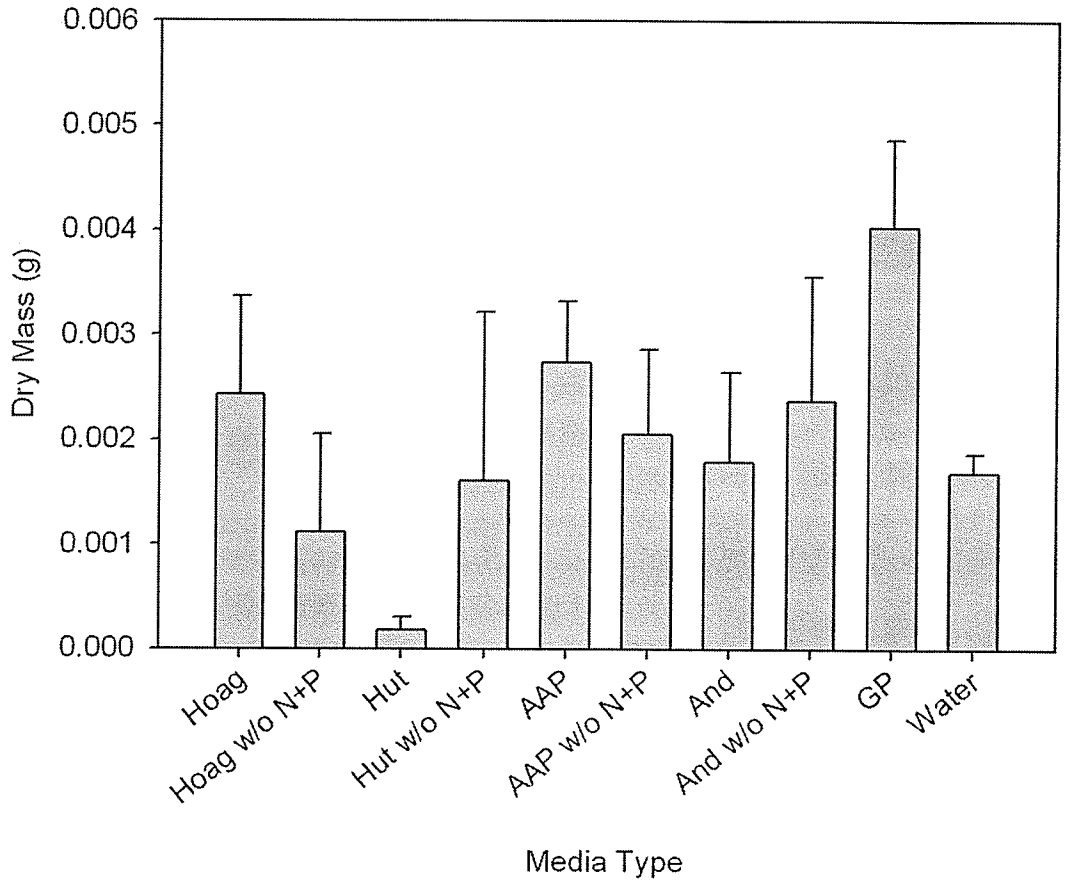
Percent survival of total planted *Elodea canadensis* shoots grown over a 14-day period in various culture media with root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

5.18 Appendix 18 - Wet mass of *Elodea canadensis* shoots grown over a 14-day period in various culture media without root substrate



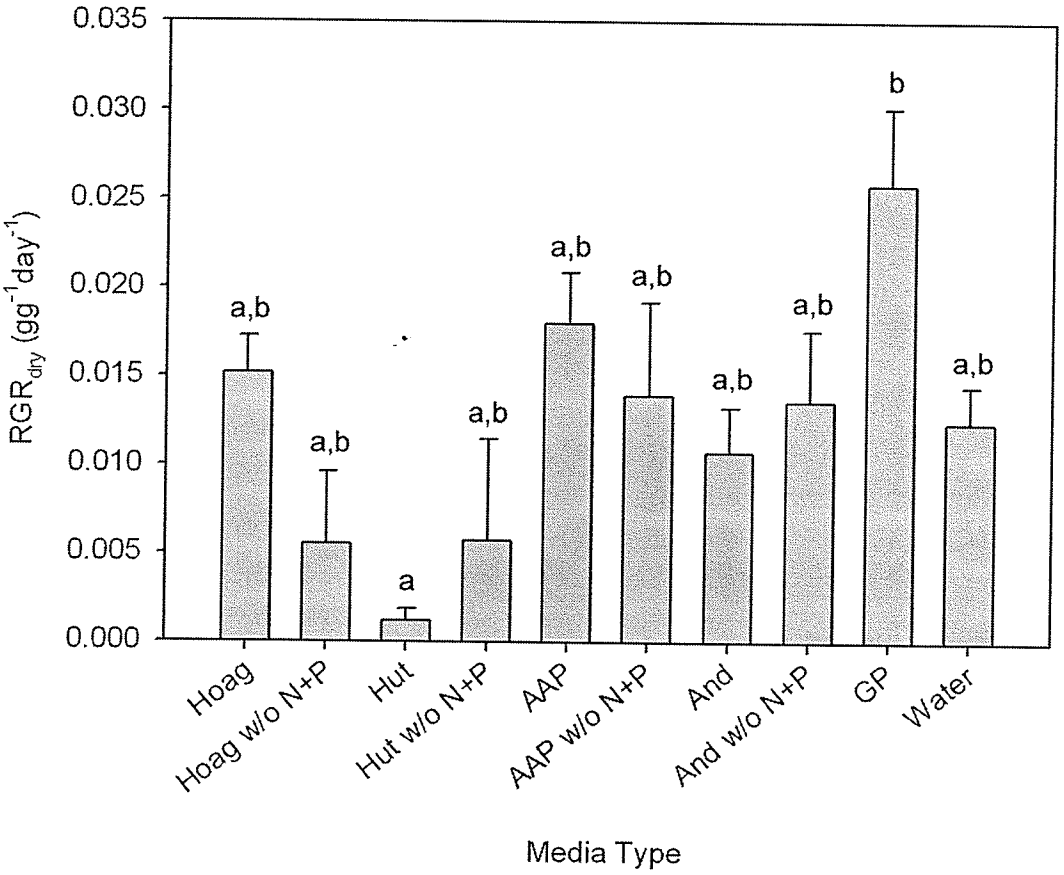
Wet mass of *Elodea canadensis* shoots grown over a 14-day period in various culture media without root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

5.19 Appendix 19 - Dry mass of *Elodea canadensis* shoots grown over a 14-day period in various culture media without root substrate



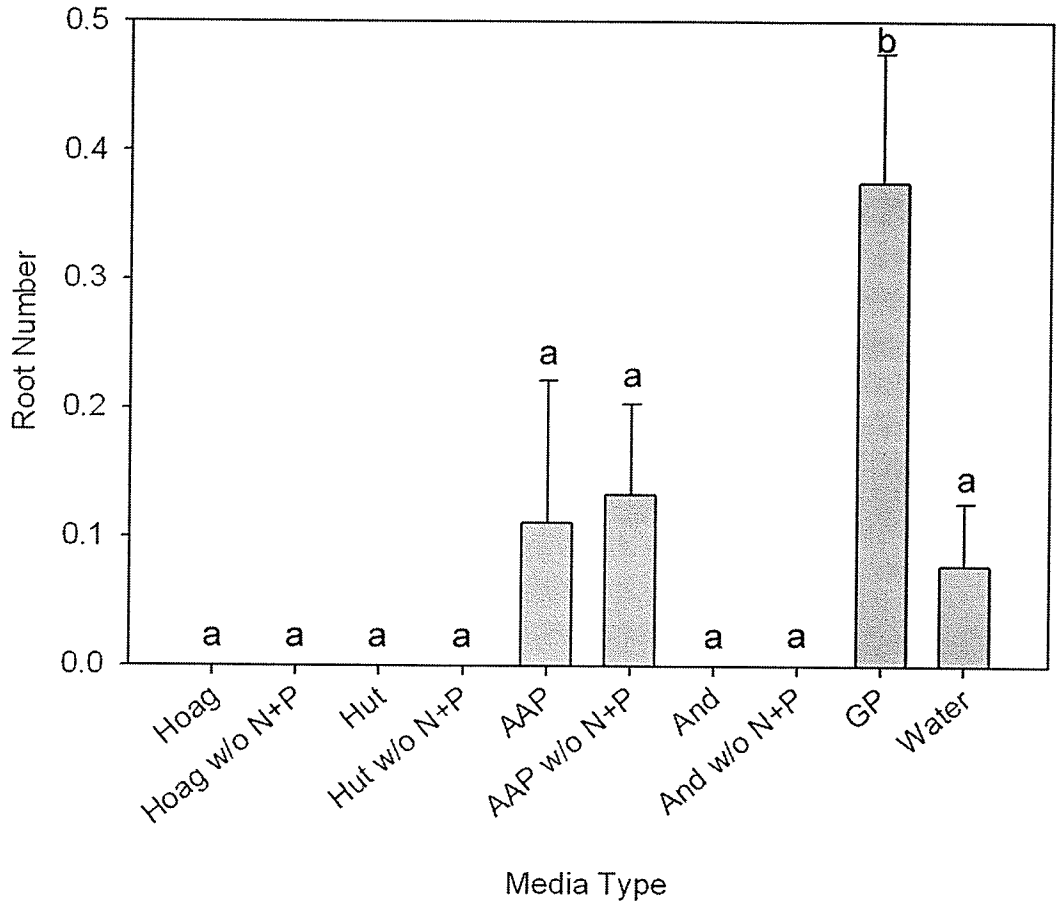
Dry mass of *Elodea canadensis* shoots grown over a 14-day period in various culture media without root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

5.20 Appendix 20 - Relative growth rate (RGR) based on dry mass of *Elodea canadensis* shoots grown over a 14-day period in various culture media without root substrate



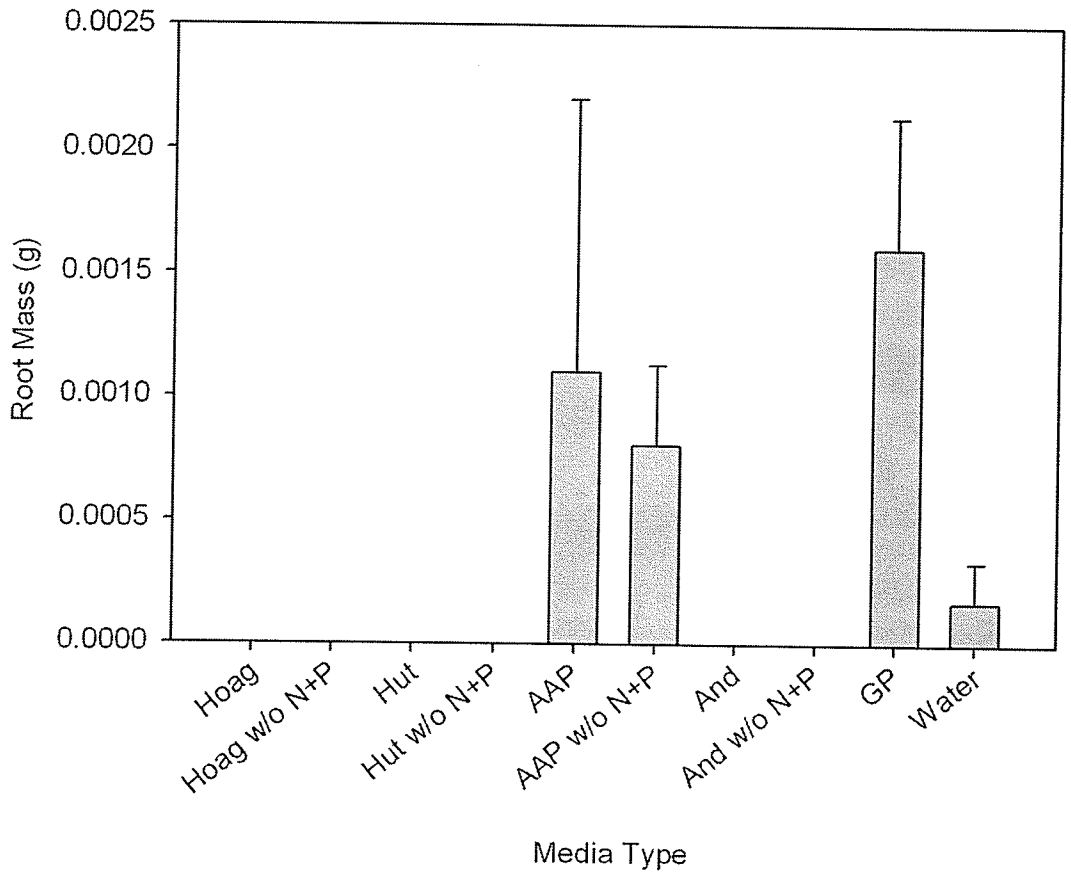
Relative growth rate (RGR) based on dry mass of *Elodea canadensis* shoots grown over a 14-day period in various culture media without root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

5.21 Appendix 21 - Root number of *Elodea canadensis* shoots grown over a 14-day period in various culture media without root substrate



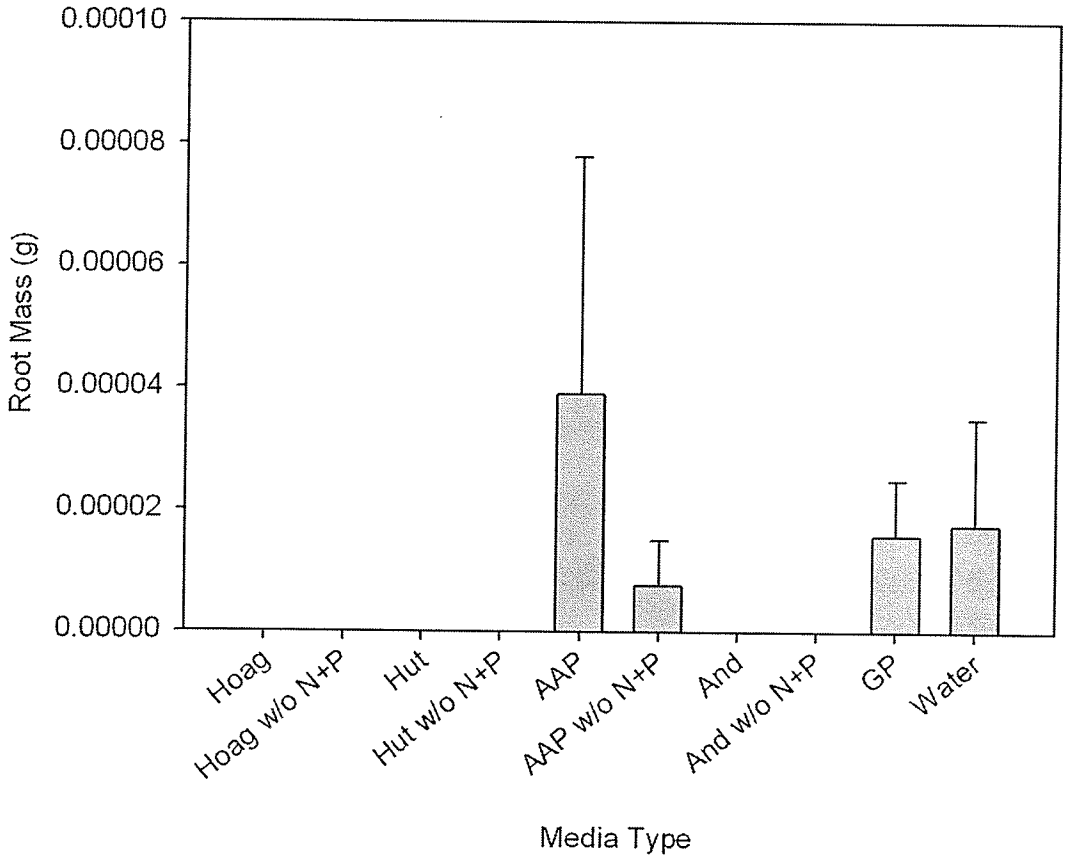
Root number of *Elodea canadensis* shoots grown over a 14-day period in various culture media without root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

5.22 Appendix 22 - Wet root mass of *Elodea canadensis* shoots grown over a 14-day period in various culture media without root substrate



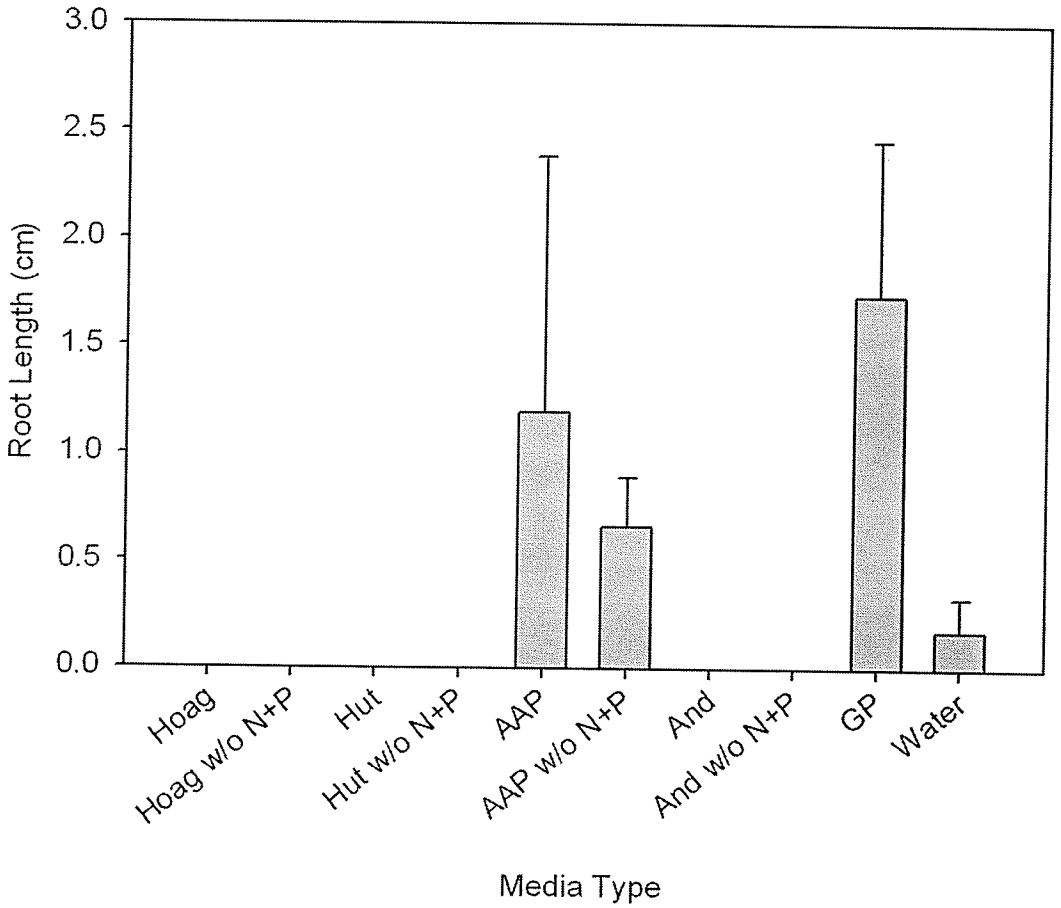
Wet root mass of *Elodea canadensis* shoots grown over a 14-day period in various culture media without root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

5.23 Appendix 23 - Dry root mass of *Elodea canadensis* shoots grown over a 14-day period in various culture media without root substrate



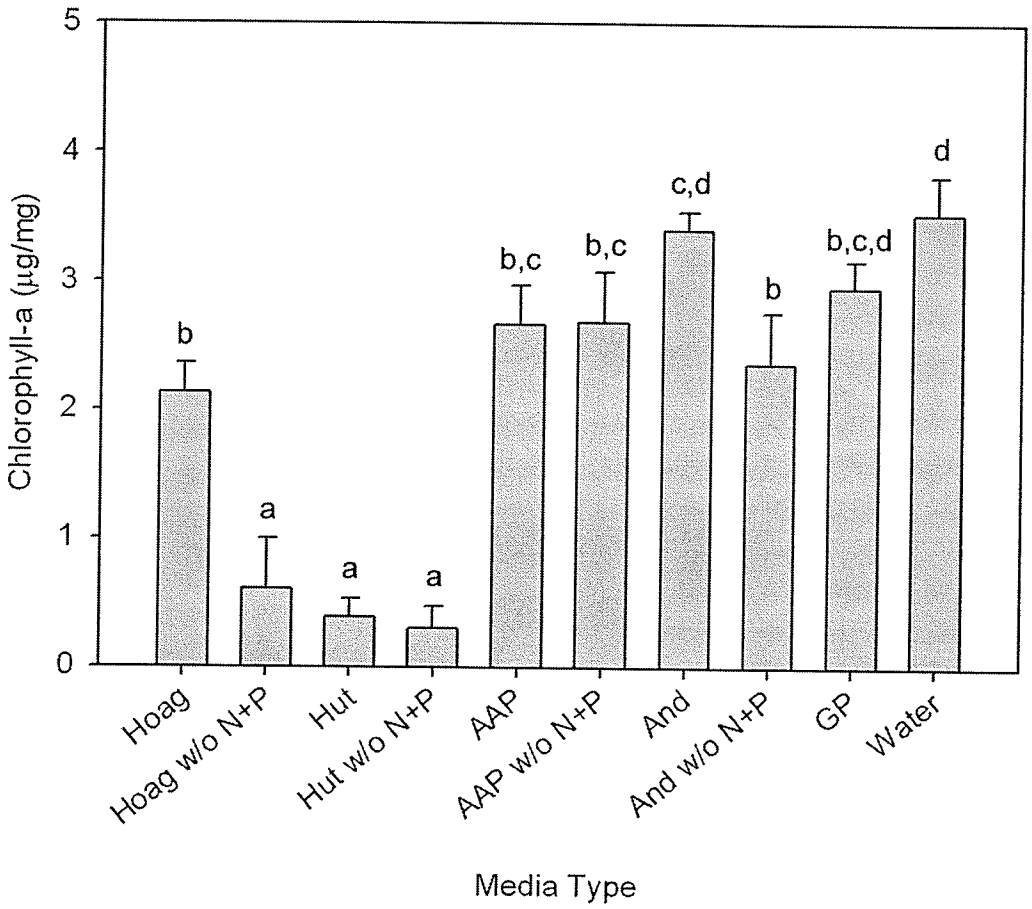
Dry root mass of *Elodea canadensis* shoots grown over a 14-day period in various culture media without root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

5.24 Appendix 24 - Root length of *Elodea canadensis* shoots grown over a 14-day period in various culture media without root substrate



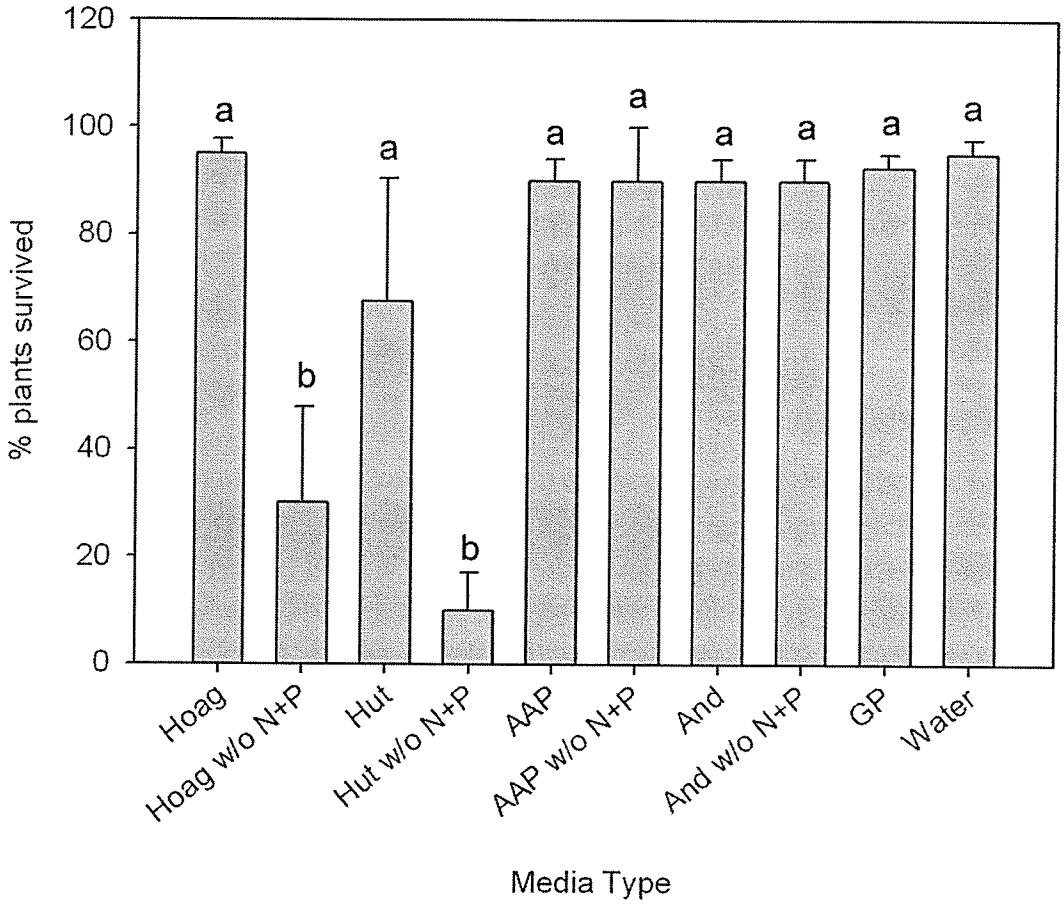
Root length of *Elodea canadensis* shoots grown over a 14-day period in various culture media without root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

5.25 Appendix 25 - Chlorophyll-a content of *Elodea canadensis* shoots grown over a 14-day period in various culture media without root substrate



Chlorophyll-a content of *Elodea canadensis* shoots grown over a 14-day period in various culture media without root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.

5.26 Appendix 26 - Percent survival of total *Elodea canadensis* shoots grown over a 14-day period in various culture media without root substrate



Percent survival of total *Elodea canadensis* shoots grown over a 14-day period in various culture media without root substrate. Bars represent standard error (n = 4). Pairs of media types showing uncommon letters above each bar indicate a statistically significant difference using the Fisher Least Significant Difference method ($p < 0.05$) and the Tukey's test method if ANOVA assumptions were not met. No letters present above bars indicate no significant differences.