Effects of agrochemicals on riparian and aquatic primary producers in an agricultural watershed

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Abstract

In agricultural watersheds, streams are intimately connected with croplands and may be inadvertently exposed to agrochemicals such as fertilizers and herbicides. Riparian plants and aquatic primary producers (aquatic plants, phytoplankton and periphyton) may be particularly affected by agrochemicals due to their taxonomic similarity to the intended targets (crop and weed species). The overall objective of this thesis was to assess the effects of fertilizers and the herbicide atrazine on riparian plants and aquatic primary producers. Effects were assessed across varying scales of observation ranging from empirical field studies at the watershed scale to *in-situ* experimental manipulations in two temperate streams to a laboratory concentration-response experiment.

Twenty-four stream/river sites located across the South Nation River watershed, Canada ranged in surrounding agricultural land use (6.7-97.4 % annual crops) and in-stream concentrations of reactive phosphate (4-102 μg/L) and nitrate (3-5404 μg/L). A gradient of atrazine contamination spanning two orders of magnitude (56 d time-weighted-average concentrations of 4-412 ng/L) was observed using polar organic chemical integrative samplers (POCIS). A total of 285 riparian and aquatic plant species were identified with species richness ranging from 43-107 species per site. Atrazine and the percentage of surrounding annual crops had no statistically significant effects on community structure. In contrast, an increase in the percentage of non-native species, a decrease in submerged macrophytes and a decrease in overall floristic quality was observed along a gradient of increasing nitrate. Similarly, periphyton biomass increased with increasing nitrate across the watershed and was associated with the Chlorophyta. In contrast, no clear response was observed in periphyton exposed to nutrient enrichment and atrazine contamination in *in-situ*

periphytometer experiments in two streams. Greenhouse concentration-response experiments provided evidence that the sensitivity of duckweed (*Lemna minor*) to atrazine was lower in populations previously exposed to the herbicide. However, the overall range in biomass 25% inhibition concentrations was small (19-40 μg/L atrazine). A clear gradient in agrochemical contamination was observed at the watershed scale and this research provided evidence of negative effects on riparian and aquatic primary producers. Effects of nutrients, specifically nitrate, superseded observable effects of the herbicide atrazine.

Résumé

Dans les bassins versants dominés par l'agriculture, les ruisseaux sont étroitement reliés aux champs cultivés et peuvent ainsi être exposés aux produits agrochimiques tels que les fertilisants et les herbicides. Les plantes de milieux riverains et les producteurs primaires (les plantes aquatiques, le phytoplancton et le périphyton) peuvent être particulièrement affectés par les produits agrochimiques à cause de leur similarité taxonomique aux plantes de cultures et mauvaises herbes visées. L'objectif global de cette thèse était de mesurer les effets des produits agrochimiques sur les plantes riveraines et autres producteurs primaires. Les effets ont été étudiés à plusieurs échelles d'observation, lors d'études empiriques sur le terrain au niveau des bassins versants, lors d'expériences de manipulations effectuées *in-situ* sur deux ruisseaux ainsi que lors d'expériences de concentration-réponse en laboratoire.

Les 24 ruisseaux/rivières affluents sélectionnés dans le bassin versant de la rivière South Nation, Canada, avaient une superficie des terres agricoles allant de 6,7% à 97,4% en cultures annuelles avec des concentrations de nitrate de l'eau de surface s'étalant de 3 à 5404 µg/L et de phosphate de 4 à 102 µg/L. Un gradient de contamination d'atrazine couvrant deux ordres de grandeur (56 jours de mesures des concentrations moyennes pondérées dans le temps de 4-412 ng/L) a été observé au moyen d'échantillonneurs intégratifs de substances chimiques polaires organiques (POCIS). Un total de 285 plantes riveraines et aquatiques a été identifié et la richesse spécifique se situait entre 43 à 107 par site. Atrazine et le pourcentage de culture annuelle n'avaient aucun effet direct sur la structure des communautés. Cependant, une augmentation du pourcentage des espèces non indigènes, une diminution des macrophytes submergés et une réduction de la qualité floristique globale ont été observées en relation avec un gradient croissant de nitrate. De la même façon, la

biomasse du périphyton a augmenté avec un accroissement du nitrate et était associé avec les Chlorophytes. Toutefois, aucune réponse mesurable n'a été observée chez le périphyton exposé à l'enrichissement de nutriments et la contamination d'atrazine lors des expériences *in-situ* de périphytomètre effectuées dans deux ruisseaux. Les expériences de concentration-réponse effectuées en serres ont démontré que la sensitivité de la lentille d'eau (*Lemna minor*) à l'atrazine était plus basse chez les populations précédemment exposées à l'herbicide. Cependant, les concentrations d'inhibition de la biomasse (25%) entre les populations étaient rapprochées, allant de 19 à 40 μg/L. Un gradient évident de contamination des produits agrochimiques a été observé au niveau du bassin versant et cette recherche a démontré des effets negatifs sur les producteurs primaires riverains et aquatiques. Les effets des éléments nutritifs, particulièrement le nitrate, ont supplanté les effets observés de l'herbicide atrazine.

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Abbreviations

a.i.	active ingredient
ANOVA	analysis of variance
ATR	atrazine
ATR-D5	deuterated atrazine
CC	coefficient of conservation
CCA	canonical correspondence analysis
CI	confidence intervals
CPCC	Canadian Phycological Culture Centre
CV	coefficient of variation
DIA-D5	deuterated desisopropyl atrazine
ELISA	enzyme-linked immunosorbent assay
ESI^+	positive electrospray ionization
FQI	floristic quality index
Fv/Fm	variable fluorescence/ maximum fluorescence
GC–MS	gas chromatography-mass spectrometry
GLM	general linear model
HLB	hydrophilic-lipophilic balanced
HPLC	high-performance liquid chromatography
IC_{25}	twenty-five percent inhibition concentration
k_e	elimination rate constant
K_{OC}	soil organic carbon/water partition coefficient
K_{OW}	octanol/water partition coefficient
LC–MS/MS	liquid chromatography-tandem mass spectrometry
LED	light-emitting diode
LOD	limit of detection
LOQ	limit of quantitation
MDL	method detection limit
MRM	multiple reaction monitoring
OECD	Organisation for Economic Co-operation and Development

PCC	Pearson's correlation coefficient
PES	polyethersulfone
PET	polyethylene terephthalate
POCIS	polar organic chemical integrative sampler
PRC	performance reference compound
PTFE	polytetrafluoroethylene
PVC	polyvinyl chloride
PVDF	polyvinylidene fluoride
RMS	root mean square
R_s	sampling rate
RSD	relative standard deviation
RSE	relative standard error
SPE	solid phase extraction
S_s	Sørensen coefficient
TWA	time-weighted-average
US EPA	United States Environmental Protection Agency
UV/VIS	ultraviolet/visible

Forms of nitrogen and phosphorus

DIN	dissolved inorganic nitrogen
DON+PON	dissolved and particulate organic nitrogen
N	nitrogen
Na_2HPO_4-$7H_2O$	disodium hydrogen phosphate heptahydrate
$NaNO_3$	sodium nitrate
$NH_3 + NH_4^+$	ammonia + ammonium
NO_2^-	nitrite
NO_3^-	nitrate
P	phosphorus
RP	reactive phosphate
TKN	total Kjeldahl nitrogen
TN	total nitrogen
TP	total phosphorus

Chapter 1: General Introduction

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1.1 Agrochemical pollution

Agrochemicals, including fertilizers and herbicides, are significant contributors of non-point source pollution to surface waters. In agricultural watersheds, water bodies such as ditches, wetlands, streams and rivers are intimately associated with croplands and may be inadvertently exposed to agrochemicals. Nutrients, primarily nitrogen and phosphorus from synthetic fertilizers and manure, enter water bodies via run-off from fields and leaching of nutrients to surface and ground waters (Beaulac and Reckhow, 1982; Haith and Shoemaker, 1987; Carpenter et al., 1998; Ekholm et al., 2000, Dubrovsky et al., 2010). Inadvertent herbicide contamination may occur through similar pathways (Pantone et al., 1992; Waite et al., 1992; Smith et al., 1993; McMahon et al., 1994) as well as through dry deposition and spray drift (Grover et al., 1988; Asman et al., 2003).

Usage of herbicides and commercial fertilizers are closely related and applied on a similar area of cropland in Canada (26,699,392 and 24,917,875 ha of land respectively in 2010) (Statistics Canada, 2011). Manure, including composted, solid and liquid forms, is also a source of nutrient pollution in surface waters and was applied on 2,868,010 ha of cropland in Canada (2010) (Statistics Canada, 2011). Transport of agrochemicals from cropland to surface waters is facilitated by irrigation systems and by surface and subsurface drainage systems such as ditches and tile drains (Blann et al., 2009; Dubrovsky et al., 2010). Drainage systems are most common in regions with clay-rich soils (Dubrovsky et al., 2010) and drain over 80% of watersheds in some North American agricultural regions (Blann et al., 2009). As a consequence, elevated concentrations of both nitrogen and phosphorus were found in 90% of agricultural streams located across the United States (Dubrovsky et al., 2010). In a survey of 186 agriculture, urban, and mixed-use streams, pesticides or their

metabolites were detected in 100% of all streams at least once (Gilliom et al., 2006). These chemicals were detected during 97% of the sampling events throughout the year in streams draining watersheds with agricultural land use (Gilliom et al., 2006). Of the 75 pesticides and 8 metabolites measured, the most frequently detected pesticide in both agricultural streams and ground water was the herbicide atrazine (Fig. 1.1) (Gilliom et al., 2006). Atrazine was also the most commonly detected pesticide in agricultural streams in concentrations >100 ng/L (Gilliom et al., 2006). The atrazine metabolite deethylatrazine was the second and third most commonly detected pesticide/metabolite in agricultural ground and stream water respectively (Gilliom et al., 2006).

1.2 Atrazine

Atrazine (6-chloro-*N*-ethyl-*N*′-1-methylethyl-1,3,5-triazine-2,4-diamine) is a triazine, a class of chemicals characterized by a six-membered aromatic ring with the general molecular formula $C_3H_3N_3$ (Fig. 1.1). Atrazine is a selective systemic inhibitor of photosystem II electron transport and is absorbed mainly through roots but also through leaves (Tomlin, 2000). In tolerant plants such as corn, atrazine is metabolized to hydroxyatrazine (Fig. 1.1) followed by further degradation (Tomlin, 2000). Atrazine may be applied pre-plant incorporated, pre-emergence or post-emergence for selective control of broad-leaved weeds and annual grasses (United Agri Products Canada Inc., 2007).

Atrazine is the most heavily used herbicide in the United States, where an estimated 34.7 million kg are applied each year to approximately 75% of the total corn acreage (US EPA, 2012a). In Canada, atrazine is the second most commonly used pesticide in Ontario after the herbicide glyphosate with 448,071 kg active ingredient applied annually to corn

crops (McGee et al., 2010). In contrast, atrazine has not been registered with the European Commission since 2003 (European Commission, 2003). However, despite its limited use in the last decade, atrazine still remains 1 of 33 priority substances representing a significant risk to the aquatic environment in Europe (European Commission, 2008).

Atrazine is commonly detected in North American surface and ground waters due to its widespread usage as well as its mobility and persistence (Solomon et al., 1996; Gilliom et al., 2006). Atrazine has a low vapour pressure (2.89×10^{-7} mm Hg at 25°C) and Henry's law constant (2.48×10^{-9} atm m^3 mol^{-1}) (Ciba-Geigy Corporation, 1994). Volatilization from soil and surface waters is expected to be negligible (Solomon et al., 1996) but may occur under warm conditions (reviewed in Takacs et al., 2002). In contrast, atrazine is highly mobile in soil due to its low soil organic carbon/water partition coefficient (log K_{OC} 1.40 to 2.19) (Ciba-Geigy Corporation, 1994). It is moderately soluble in water (33 mg/L at 22°C) with a log octanol/water partition coefficient (K_{OW}) of 2.5 (Tomlin, 2000). Atrazine is broken down in the environment primarily through two types of chemical degradation: 1) hydrolysis of the Cl-C bond leading to the formation of hydroxyatrazine and 2) N-dealkylation resulting in the metabolites deethylatrazine, desisopropylatrazine and diaminochlorotriazine (Fig. 1.1) (reviewed in Huber, 1993). Degradation may also occur to a lesser extent through microbial splitting of the triazine ring (reviewed in Huber, 1993). Atrazine is persistent in both soil and water but with highly variable half-lives. For example, the half-life of atrazine in soil ranges from 20 to 385 d or longer (reviewed in Takacs et al., 2002) and atrazine has been detected 22 years after its application (Jablonowski et al., 2009). Tomlin (2000) reported an average half-life of 55 d for atrazine in natural waters. However, various studies have reported half-lives ranging from 1.73 to 742 d with degradation

decreasing with increasing pH and increasing with the addition of fulvic or humic acids (reviewed in Solomon et al., 1996).

A risk assessment for atrazine concluded that inhibitory effects on the most sensitive groups of organisms, phytoplankton, periphyton and macrophytes, were likely to be followed by rapid recovery and that atrazine was not likely to pose a significant risk to the aquatic environment at environmentally relevant concentrations (typically <5 µg/L) (Solomon et al., 1996). However, other studies have shown effects of atrazine on phytoplankton photosynthesis (DeNoyelles et al., 1982), primary production and community structure (Pannard et al., 2009) at concentrations <5 µg/L. Atrazine has been shown to cause reductions in fish egg production due largely to decreased spawning events at concentrations as low as 0.5 µg/L (Tillitt et al., 2010). Additional research has provided evidence that atrazine feminizes male frogs (Hayes et al., 2003) and alters their gonadal differentiation and metamorphosis (Langlois et al., 2010) at concentrations as low as 0.1 µg/L and 1.8 µg/L atrazine respectively. Of particular concern is that the potential for atrazine to demasculinize and feminize male gonads is consistent across vertebrate classes (Hayes et al., 2011).

1.3 Effects of herbicides on riparian and aquatic primary producers

Riparian plants and aquatic primary producers, including aquatic plants (macrophytes), phytoplankton and periphyton, may be particularly at risk of adverse effects of herbicides such as atrazine and nutrients from fertilizers. Primary producers are taxonomically more similar to the intended targets of herbicides (weed species) and fertilizers (crop species) than are other types of organisms. Numerous short-term laboratory

studies have documented the sensitivity of plants and algae to herbicides. For example, the Pesticide Action Network (PAN) pesticide database lists atrazine toxicity data for 494 aquatic plant and 1431 phytoplankton records (Kegley et al., 2011). However, the majority of the data are focused on experiments with a few common laboratory test species and the link between short-term laboratory tests and complex field conditions is often unclear.

Relatively few studies have examined the effects of herbicides or other pesticides on riparian and aquatic plant community structure, particularly *in situ*. Stansfield et al. (1989) suggested that organochlorine pesticides contributed to a shift from aquatic plant dominance to phytoplankton dominance in shallow lakes indirectly through toxic effects on algae-grazing Cladocera. Most documented effects of herbicides on aquatic plant communities come from studies examining effects of direct herbicide application to lakes to control invasive plant species. These studies have had conflicting results ranging from reports of no significant effects on native macrophytes (Jones et al., 2012), to reductions in submerged macrophytes (Parsons et al., 2009) and declines or increases in macrophytes depending on the species present (Wagner et al., 2007).

A more consistent pattern of herbicidal effects on algae has emerged from the literature. Exposure to herbicides appears to favour diatom-dominated periphyton communities. Short-term toxicity tests demonstrated that green algae were severely affected by exposure to the herbicides metribuzin, hexazinone, isoproturon and pendimethalin and did not recover, whereas diatoms and cyanobacteria recovered from herbicide exposure (Gustavson et al., 2003). Similarly, Guasch et al. (1998) and Dorigo et al. (2004) suggested that exposure to the herbicide atrazine shifted algal communities to dominance in diatom species that were less sensitive to atrazine and organic pollution compared to green algae.

1.4 Effects of nutrients on riparian and aquatic primary producers

Eutrophication, in other words the increased primary production resulting from nutrient enrichment, has been studied extensively. The concept was first introduced almost a century ago (Naumann, 1919) and received widespread attention in the 1970's when Schindler (1974) demonstrated that increased phosphorus loading from anthropogenic sources resulted in dramatic increases in the biomass and primary productivity of lakes (also reviewed in Schindler, 2006). Eutrophication is now considered to be a widespread problem in surface waters and has been linked to a range of effects including toxic algal blooms, loss of oxygen, fish kills, reduced biodiversity and loss of habitat-forming aquatic plant beds and coral reefs (Carpenter et al., 1998). In addition to increases in primary productivity, nutrient enrichment of streams has been shown to change algal community structure. For example, Chételat et al. (1999) found a shift in dominance of green algae from *Spirogyra, Oedogonium* and *Coleochaete* at low total phosphorus concentrations to *Cladophora* above 20 µg/L total phosphorus. Dominance of the filamentous green alga *Cladophora* has also been associated with high ammonium concentrations (Dodds, 1991).

Eutrophication can also have severe effects on macrophytes and has been linked to the decline of macrophyte diversity, particularly of submerged macrophytes, observed over the last century (Sand-Jensen et al., 2000; Riis and Sand-Jensen, 2001; Körner, 2002; Egertson et al., 2004; Hilt et al., 2013; Steffen et al., 2013). Enrichment of a limiting nutrient stimulates phytoplankton and epiphyte growth, increasing turbidity and resulting in a shift in dominant macrophyte forms from submerged to floating-leaved to emergent macrophytes. Eventually, a complete loss of macrophytes may occur resulting in phytoplankton dominance as light becomes limiting for all macrophyte forms (Phillips et al.,

1978; Irvine et al., 1989; Scheffer et al., 1993; reviewed in Hilton et al., 2006). While this mechanism has been well established for shallow lakes, eutrophication of rivers is often more complex and there remains some uncertainty over which nutrient, if any, is limiting in river systems (Hilton, et al., 2006).

1.5 Interactions between agrochemicals

Both nutrient enrichment and herbicide contamination pose a potential risk to primary producers in river systems and are associated with the production of cash crops. Despite the close relationship between the application of fertilizers and herbicides, the study of the effects of these agrochemicals on primary producers has generally remained separate. Few studies have examined the interaction between the two stressors and to my knowledge no studies have explicitly examined effects of the interaction on macrophyte communities in the field. Waiser and Robarts (1997) found that the thiocarbamate herbicide triallate stimulated bacterial production only when nitrogen and phosphorus were added and that although the herbicide also stimulated algal growth at low doses, nutrient addition did not affect the response. Guasch et al. (2007) also found that phosphate had no effect on the toxicity of atrazine to periphyton in communities with previous atrazine exposure. However, Barreiro and Pratt (1994) found that recovery from diquat exposure was enhanced by the addition of phosphate and nitrate to natural periphyton communities. In the field, both nutrients and herbicides have been found to alter periphyton communities but to date no study has been able to separate the effects (Guasch et al., 1998; Cuffney et al., 2000). Clearly, the interactions between agrochemicals and primary producers are complex and are

further complicated by recent evidence that individual species within a community have unique response to atrazine, nutrients and mixtures (Murdock and Wetzel, 2012).

1.6 Rationale for thesis

1.6.1 Overview of thesis rationale and structure

The overall objective of this thesis was to assess effects of agrochemicals, specifically fertilizers and atrazine, on riparian and aquatic primary producers in river systems. Agrochemicals have the potential to cause adverse effects on riparian and aquatic primary producers and impair the health and functioning of river systems. Despite the existing literature on agrochemical effects on primary producers, little is known regarding their relative effects and the interactions between nutrients and herbicides on natural communities in the field. In addition, assessment of nutrient limitation and effects of nutrient enrichment in river systems is often difficult.

In mid-sized streams and rivers (Strahler order 4-6), aquatic macrophytes and periphyton are the dominant primary producers (Vannote et al., 1980) and were the focus of this thesis. In Europe, aquatic macrophytes are used as indicators of water quality to assess the ecological status of rivers (European Union, 2000), but typically only vegetation occurring within stream channels is identified (Dawson, 2000). In this thesis, riparian vegetation located along stream banks was also studied because it may be exposed to agrochemicals and has an important role in improving water quality (Osborne and Kovacic, 1993; reviewed in Dosskey et al., 2010). Throughout the thesis, assessment of herbicide contamination focused on atrazine because of its widespread use and concern regarding its environmental impact. Atrazine served as a good model contaminant because of the wealth

of research dedicated to understanding its effects and distribution and because of its likelihood to be detected within an agricultural watershed.

The overall goal of ecotoxicology is to determine effects of chemicals on populations and communities and ultimately on the health and functioning of ecosystems. Achieving this goal is challenging due to issues of scale. Ecological phenomena vary across a range of spatial, temporal and organizational scales and mechanisms underlying patterns may operate on scales different from those in which a pattern was observed (Levin, 1992). As a result, experiments involving a single species and contaminant, although easy to interpret, lack realism. In contrast, field studies are more realistic but are often difficult to interpret due to complex interactions between multiple stressors and ecological drivers.

This thesis examined effects of agrochemicals on primary producers at several scales of observation ranging from empirical studies at the watershed scale (Chapters 3, 4) following characterization of atrazine contamination (Chapter 2), *in-situ* experimental manipulations in two temperate streams (Chapter 4) and laboratory concentration-response experiments (Chapter 5). Chapter 1 consists of the General Introduction and Chapter 6 the Conclusions. Chapters 2, 3, 4, and 5 were written as stand-alone manuscripts intended for publication. Although some repetition was inevitable, an effort was made to reduce repetition between chapters, while ensuring each chapter contained sufficient detail to be read independently of other chapters. Chapters were written in the style required by the journals where the manuscripts have been published or will be submitted.

1.6.2 Assessment of herbicide (atrazine) contamination across a watershed

A key step in assessing effects of agrochemicals on riparian and aquatic primary producers involved the accurate measurement of in-stream herbicide concentrations. Herbicides concentrations may be highly variable, occurring in pulses following rain events. Grab samples may over- or under-estimate actual concentrations and because herbicides are likely to occur in trace concentrations several orders of magnitude lower than nutrients, patterns of contamination may not be obvious. The polar organic chemical integrative sampler (POCIS) was developed to integrate trace concentrations of hydrophilic compounds (Alvarez, et al., 2004) and has the potential to provide time-weighted-average herbicide concentrations that are more reflective of contamination than grab samples. POCIS is an adsorption based sampler containing Oasis hydrophilic-lipophilic balanced (HLB) sorbent (Alvarez et al., 2004), a reversed-phase solid phase extraction (SPE) sorbent capable of retaining acidic, basic and neutral compounds with a range of polarities. To date, few studies have used POCIS to estimate contaminant concentrations at the watershed scale.

Laboratory-derived sampling rates estimate the amount of water cleared by POCIS each day and are used to calculate time-weighted-average concentrations of contaminants. Sampling rates are affected by environmental variables such as temperature, water turbulence, biofouling and pH (Harman et al., 2012). With other types of passive samplers, a performance reference compound (PRC) may be added to passive samplers and its dissipation over time used to correct sampling rates (Booij et al., 1998, 2002; Huckins et al., 2002). However, there is currently no consensus on suitable PRCs for POCIS or even if the PRC approach is appropriate (Harman et al., 2012). The main objective of Chapter 2 was to estimate time-weighted average concentrations of atrazine across an agricultural watershed

using POCIS to estimate chronic atrazine contamination following expected field applications. It was hypothesized that atrazine concentrations would be positively correlated with the intensity of surrounding agricultural land use. A secondary objective was to evaluate effects of environmental variables on spatial and temporal differences in POCIS sampling rates using a PRC approach. This study was the first to relate variability in *in situ* sampling rates with actual environmental conditions. The hypothesis tested was that variability in sampling rates, estimated using a novel PRC approach for POCIS, would vary between field sites and time periods due to differences in environmental variables. Sampling rates were predicted to increase with increasing temperature and stream velocity as has been demonstrated in previous laboratory studies (reviewed Harman et al., 2012).

1.6.3 Empirical field studies

Ecological communities retain information about events in their history at a number of different levels of organization and this information may not always be measurable at a given point in time (Matthews et al., 1996; Landis et al., 1996; Landis et al., 1997). Communities may retain the imprint or "memory" of a stressor long after it occurred (Harding et al., 1998; Dorigo et al., 2004). For example, Harding et al. (1998) found that land use data from the 1950s was better able to predict fish and invertebrate diversity compared to land use data from the 1990s. Dorigo et al. (2004) provided evidence that changes in algal community structure and decreased herbicide (atrazine and isoproturon) sensitivity persisted even when the herbicides were not present. Empirical studies examining natural communities may be the only way to examine the cumulative effects of historic and present day stressors under realistic conditions.

In Chapter 3, the main objective was to assess effects of agrochemicals on riparian and aquatic plant community structure by identifying and comparing vegetation at field sites located across a watershed and along a gradient of agrochemical contamination. The general hypothesis was that surrounding agricultural land use, nutrient enrichment and atrazine contamination have negative effects on plant communities. Agricultural land use and agrochemicals were predicted to lead to an increase in the percentage of non-native species and decreases in species richness, the number and relative frequency of submerged species and overall floristic quality.

In the empirical section of Chapter 4, periphyton communities, located at the same field sites as the vegetation study, were colonized on artificial substrates. Periphyton communities were characterized using a chemotaxonomic approach to classify periphyton into the broad taxonomic groups of Bacillariophyta (diatoms), Chlorophyta (green algae), Chrysophyta (chrysophytes), Cryptophyta (cryptophytes), Cyanophyta (cyanobacteria), Dinophyta (dinoflagellates), Euglenophyta (euglenoids) and Rhodophyta (red algae). The main objective was to assess effects of agrochemicals on periphyton community structure and biomass with the general hypothesis was that agrochemicals were likely to alter community structure and increase periphyton biomass. Sites highly enriched with nutrients were predicted to be dominated by the Chlorophyta and sites highly contaminated by atrazine were predicated to be dominated by the Bacillariophyta. *A priori* it was unclear whether environmental concentrations of atrazine would be high enough to result in observable changes in community structure. However, the interaction between nutrients and atrazine was examined further in *in-situ* experimental manipulations.

1.6.4 *In-situ* experimental manipulations

Field studies are valuable due to their realism but high variability between sites, as well as complex interactions between environmental, chemical and biological factors, can make interpretation of results difficult. One of the problems associated with assessing the effects of herbicides, nutrients and their interaction on primary producer communities is that it may be difficult to determine causal relationships. However, if a variable of interest can be manipulated *in-situ* (e.g. nutrient and herbicide concentrations), it should be possible to quantitatively determine whether nutrients and/or herbicides affect primary production. Nutrients have been manipulated *in-situ* in a number of studies designed to assess nutrient limitation by colonizing natural periphyton communities on nutrient-diffusing substrates or in artificial flow-through channels. For example, Keck and Lepori (2012) analyzed data from 382 studies that used nutrient-diffusing substrates, flow-through systems or periphytometers to assess effects of nutrient-enrichment on periphyton in stream and rivers. In the experimental section of Chapter 4, periphytometers, consisting of a nutrient and herbicide diffusing reservoir and a substrate for periphyton colonization, were used to expose natural periphyton communities to nutrients and atrazine *in-situ* in two temperate streams. The main objective was to assess effects of additions of nutrients (nitrogen and phosphorus) and atrazine on natural periphyton communities. Nutrients were expected to increase biomass and result in a Chlorophyta-dominated community if nutrients were previously limiting. Atrazine was predicted to decrease biomass and result in a Bacillariophyta-dominated community. Effects of atrazine were predicted to supersede those of nutrients in streams where nutrients were not limiting.

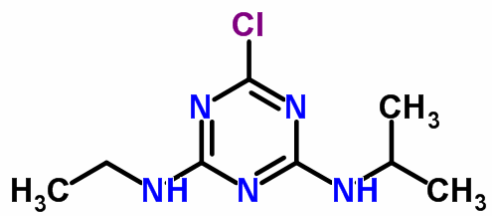
1.6.5 Laboratory concentration-response study

Field studies examining the composition of macrophyte communities do not provide direct information regarding the sensitivity of individual species to herbicides. *In-situ* experimental manipulations provide more direct evidence of herbicidal effects but are limited by the time and effort required to set-up experiments at broad spatial or temporal scales. Concentration-response experiments allow for increased replication and manipulation, albeit under less realistic conditions than studies at broader scales of observation. The pollution induced community tolerance concept predicts that a toxic agent will exert selection pressure towards a global increase in tolerance to that particular toxic agent (Blanck et al., 1988). By comparing the sensitivities of populations of primary producers with differing past exposures to atrazine, it may be possible to determine subtle effects of atrazine that would not be apparent from field studies.

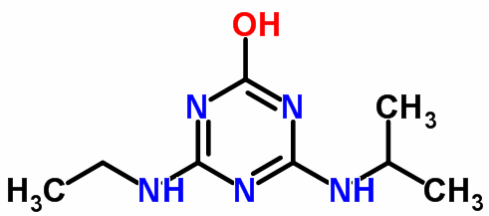
Duckweed species (*Lemna* spp.) are small free-floating aquatic macrophytes commonly used in toxicity testing (reviewed in Wang, 1990) and required in regulatory phytotoxicity testing for the registration of pesticides in several jurisdictions (OECD, 2002; US EPA, 2012b). Data from toxicity tests with *Lemna* spp. are critical because they are used to predict the risk a given chemical poses to all macrophytes in the aquatic environment. It is currently unknown whether the sensitivity of *Lemna* spp. populations to atrazine is modified following exposure as a result of species-level acclimation. If substantial differences between populations exist, extrapolation between laboratory testing and actual field conditions and between different environmental conditions may not be possible. In Chapter 5, duckweed (*Lemna minor*) populations were collected from field sites with differing surrounding land use. The sensitivities of the field populations to atrazine were

compared to determine whether sensitivity was affected by prior exposure to atrazine and other agricultural stressors. The sensitivities of the field populations to atrazine were also compared to that of a commercially available culture to determine the suitability of laboratory cultures in ecotoxicity testing. The hypothesis tested was that exposure of duckweed populations to atrazine in the field alters their sensitivity to atrazine. Sensitivity to atrazine was expected to decrease between populations with increasing past exposure to atrazine in the field.

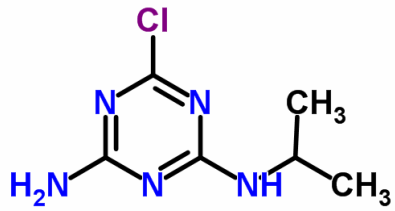
A) Atrazine



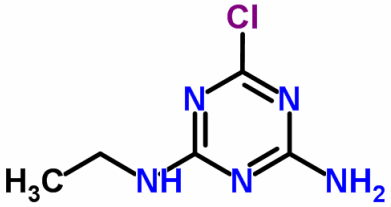
B) Hydroxyatrazine



C) Deethylatrazine



D) Desisopropylatrazine



E) Diaminochlorotriazine

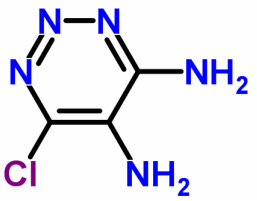


Fig. 1.1 Chemical structure of atrazine and its major metabolites

Chapter 2:

Atrazine contamination at the watershed scale and environmental factors affecting sampling rates of the polar organic chemical integrative sampler (POCIS)

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2.1 Abstract

Polar organic chemical integrative samplers (POCIS) were used to estimate atrazine contamination at 24 stream/river sites located across a watershed with land use ranging from 6.7 to 97.4% annual crops and surface water nitrate concentrations ranging from 3 to 5404 μg/L. A gradient of atrazine contamination spanning two orders of magnitude was observed over two POCIS deployments of 28 d and was positively correlated with measures of agricultural intensity. The metabolite desisopropyl atrazine was used as a performance reference compound in field calibration studies. Sampling rates were similar between field sites but differed seasonally. Temperature had a significant effect on sampling rates while other environmental variables, including water velocity, appeared to have no effect on sampling rates. A performance reference compound approach showed potential in evaluating spatial and temporal differences in field sampling rates and as a tool for further understanding processes governing uptake of polar compounds by POCIS.

Keywords: polar organic chemical integrative sampler (POCIS); performance reference compound; passive sampling rate; herbicide; atrazine

2.2 Highlights

- Atrazine was measured across an agricultural watershed with passive sampling (POCIS).
- 56 d time weighted average concentrations were >100 ng/L at 14 of 24 sites.
- Desisopropyl atrazine was used as a performance reference compound.
- Field corrected sampling rates were similar between sites but differed seasonally.
- Sampling rates appeared to be affected by temperature but not water velocity.

2.3 Introduction

Agricultural herbicides are a significant contributor of non-point source pollution to surface waters through run-off and leaching from agricultural fields (Pantone et al., 1992; Waite et al., 1992; Smith et al., 1993; McMahon et al., 1994) as well as dry deposition and spray drift (Grover et al., 1988; Asman et al., 2003). The triazine herbicide atrazine (ATR) (6-chloro-*N*-ethyl-*N*′-1-methylethyl-1,3,5-triazine-2,4-diamine) is commonly detected in North American surface and ground waters due to its widespread usage, primarily on corn crops, as well as its mobility and persistence (Solomon et al., 1996; Gilliom et al., 2006). Atrazine is the most heavily used herbicide in the United States (US EPA, 2012a) and the second most commonly used pesticide on corn crops in Ontario, Canada (McGee et al., 2010). In contrast, ATR has not been registered with the European Commission since 2003 (European Commission, 2003) but still remains 1 of 33 priority substances posing a significant risk to the European aquatic environment (European Commission, 2008).

A risk assessment for ATR in surface waters concluded that inhibitory effects on the most sensitive organisms, phytoplankton and macrophytes, were likely followed by rapid recovery and ATR was unlikely to pose a significant risk at environmentally relevant concentrations (typically <5 μg/L) (Solomon et al., 1996). However, other studies found effects of ATR on phytoplankton photosynthesis (DeNoyelles et al., 1982), primary production and community structure (Pannard et al., 2009) at concentrations <5 μg/L. Atrazine has been shown to cause reductions in fish egg production due largely to decreased spawning events at concentrations as low as 0.5 μg/L (Tillitt et al., 2010). Additional research provided evidence that ATR feminizes male frogs (Hayes et al., 2003) and alters gonadal differentiation and metamorphosis (Langlois et al., 2010) at concentrations as low as

0.1 µg/L and 1.8 µg/L respectively. Of particular concern is the potential for ATR to demasculinize and feminize male gonads across vertebrate classes (Hayes et al., 2011). Furthermore, ATR is persistent in soil and has for example been detected 22 years following application (Jablonowski et al., 2009).

Assessment of the occurrence of ATR and other herbicides in surface waters, as well as their risk to aquatic organisms, is challenging because herbicide concentrations are often highly variable. Monitoring programs traditionally used point-in-time estimates, such as grab samples, that provide a snapshot of overall contamination. However, pulses in concentration are not integrated, resulting in an over- or underestimation of actual concentrations and a lack of understanding of actual exposures to biota. For example, Rabiet et al. (2010) found that grab sampling largely underestimated herbicide concentrations and fluxes, whereas Petersen et al. (2012) observed that grab sampling failed to account for the variability in the occurrence, duration and concentration of herbicide pulses following rain events. This issue is not unique to herbicides and a number of passive sampling technologies have been developed to provide time-weighted-average (TWA) concentrations of contaminants (reviewed in Vrana et al., 2005; Stuer-Lauridsen et al., 2005). The polar organic chemical integrative sampler (POCIS) was developed to integrate trace concentrations of hydrophilic compounds (log K_{OW} <4) such as pesticides, pharmaceuticals, personal care products and industrial chemicals (Alvarez et al., 2004) and has been used to detect over 300 compounds (Harman et al., 2012).

Sampling rates (R_s) estimate the water volume cleared of chemical per unit time by POCIS and are typically derived from laboratory calibrations. However, experiments have also shown that R_s are affected by factors such temperature, water flow rates, biofouling and

pH (reviewed in Harman et al., 2012). Therefore, under field conditions R_s are expected to vary from those established under laboratory conditions. This issue has been resolved for absorption based passive sampling of hydrophobic compounds (e.g. semi permeable membrane devices) by the addition of performance reference compounds (PRCs) to passive samplers. When both PRCs and target analytes follow isotropic exchange, dissipation of PRCs is equivalent to uptake of target analytes and can be used to correct analyte concentrations for *in situ* R_s (Booij et al., 1998, 2002; Huckins et al., 2002). In contrast, POCIS is an adsorption based sampler that tends to act as an infinite sink for analytes (Alvarez et al., 2004). However, Mazzella et al. (2007) provided evidence of isotropic exchange in POCIS for deuterated desisopropyl atrazine (DIA-D5), a high fugacity metabolite of ATR. Subsequently, Mazzella et al. (2010) used DIA-D5 as a PRC and successfully narrowed the differences in herbicide concentrations obtained with POCIS from those obtained with automatic samplers. Despite this success, it is unclear whether factors affecting the rate of desorption of poorly sorbed PRCs are equivalent to those affecting adsorption of strongly sorbed target analytes, resulting in a gap in knowledge as to whether PRCs can accurately correct R_s of target analytes (Harman et al., 2011). Currently, there is no consensus on suitable PRCs for broad ranges of target analytes or even if the PRC approach is suitable for POCIS (Harman et al., 2012).

In the present study, I used POCIS to determine ATR contamination throughout an agricultural watershed in Eastern Ontario, Canada (Fig. 2.1). Atrazine concentrations obtained with POCIS were compared with those obtained from grab samples and correlated with measures of agricultural intensity. The results represent a comprehensive study using POCIS at the watershed scale, across a gradient of physico-chemical and hydrological

conditions. Despite its recent popularity, POCIS remains poorly characterized in terms of modeling uptake rates and environmental factors (Harman et al., 2012). A PRC approach using DIA-D5 was used to examine factors affecting R_s under complex field conditions at four field sites during two time periods.

2.4 Materials and Methods

2.4.1 Chemicals and materials

Atrazine was purchased from ChemService Inc. (West Chester, USA), while deuterated atrazine (ATR-D5) and deuterated desisopropyl atrazine (DIA-D5) were from CDN Isotopes Inc. (Point-Claire, Canada). The measured chemical purity of each lot was 98.9%, >99% and 98.8% for ATR, ATR-D5 and DIA-D5 respectively. Stock solutions of each standard were prepared gravimetrically at 1 mg/mL in methanol, sonicated and stored in darkness at -30°C. HPLC grade methanol and water were purchased from Sigma-Aldrich Canada (Oakville, Canada). LCMS grade acetonitrile, methanol and water, as well as ACS reagent grade acetone, dimethyl sulfoxide, petroleum ether and sulphuric acid, were from Fisher Scientific (Ottawa, Canada). Oasis hydrophilic-lipophilic balanced (HLB) cartridges (6 mL, 500 mg) were purchased from Waters (Mississauga, Canada). Empty 3 mL polypropylene solid phase extraction (SPE) tubes and polyethylene frits (20 μm pore size) were from Sigma-Aldrich Canada. Oasis HLB bulk sorbent, polyethersulfone (PES) membranes and POCIS hardware were from Environmental Sampling Technologies Inc. (St. Joseph, USA).

2.4.2 Study area and measures of agricultural intensity

Atrazine contamination in the South Nation River watershed, Canada was assessed between 18 May and 22 July 2010. The South Nation River watershed comprises 3915 km^2 in Eastern Ontario, Canada (Fig. 2.1) and has a historical (1915-2011) average annual discharge of 44.3 m^3/s at its mouth (Environment Canada, 2013). The headwaters commence near the St. Lawrence River (44°40'41"N, 75°41'58"W) and the 177 km long river flows north-easterly across a flat landscape until its confluence with the Ottawa River (45°34'24"N, 75°06'00"W). The watershed is predominately agricultural with crops of corn (*Zea mays* L.) and soybean (*Glycine max* L. (Merr.)) planted in tile-drained fields. Usage of ATR is typical of agricultural watersheds in Ontario and peak concentrations are expected following pre-plant incorporated, pre-emergent and post-emergent use on corn crops (late April through to July). Atrazine was previously detected in the watershed from weekly continuous flow surface water samples (mid-April-late October 1991-1992) (Fischer et al., 1995) and more recently from integrated grab samples (June 2008) (Dalton et al., 2013).

Twenty-four sites located throughout the South Nation River watershed were selected for study (Fig. 2.1). Sites were paired along a given tributary with sites surrounded by low levels of agriculture located upstream of sites surrounded by high levels of agriculture. Paired sites are subsequently referred to as low and high agriculture sites respectively. Sites were selected using land use data to identify areas of low and high agriculture (Statistics Canada, 2006), using Google Earth v.4.2.0198.2451 (Google Inc., Mountain View, USA) to verify physical aspects and through field reconnaissance of potential sites. The average distance between paired sites was 9.0 ± 8.5 km (ranging from 1.5 to 33.7 km). Two pairs of sites were located along different tributaries due to a lack of

accessible and suitable sites. Pair 1 sites were 80.4 km apart and pair 6 sites were 10.7 km apart (Fig. 2.1). All sites were matched as closely as possible in terms of visible features such as steam width, bank slope and canopy cover. The 12 pairs were not hydrologically connected except: 1) low agriculture site 1 was upstream of pair 4 sites along the main branch of the South Nation River and 2) high agriculture site 1 and pair 10 sites were upstream of pair 11 sites along the Scotch River (Fig. 2.1).

Agricultural intensity was calculated as the percentage of annual cropland in a 500 m radius surrounding each site (ArcMap v.10, ESRI, Canada Ltd, Toronto, Canada) using data provided by Agriculture and Agri-Food Canada (2008). Elevated nitrate concentrations indicate agricultural contamination from synthetic fertilizers and manure (Dubrovsky et al., 2010) and were used as an additional measure of agricultural intensity. Water samples (300 mL) were collected in polyethylene terephthalate bottles during May, June and July 2010, corresponding to POCIS deployment periods at each site. Integrated, mid-channel samples were taken using a pole sampler to collect water upstream of the canoe/wading location to prevent disturbance and contamination of water and sediments. Nitrate was analyzed at the Robert O. Pickard Environmental Centre Laboratory (Ottawa, Canada) following established methods of the Ontario Ministry of the Environment (2007a). The method detection limit for nitrate was 4 μg/L.

2.4.3 Passive sampling with POCIS

POCIS contained 200 mg of Oasis HLB sorbent (poly(divinylbenzene-co-N-vinylpyrrolidone)) enclosed between two PES membranes and held together with compression between two stainless steel washers (Alvarez et al., 2004). POCIS had a

standardized total sampling surface area of 41 cm^2. POCIS were assembled in the lab and transported to and from the field in methanol rinsed aluminum foil. High density polyethylene shields were designed to be easily assembled, durable, inexpensive and easy to clean (Fig. 2.2). At each site, three replicate POCIS were secured within a shield and deployed mid-stream at a maximum depth of 40 cm below the water's surface, with the deployment depth reduced at shallow sites (Fig. 2.2). POCIS were deployed for two consecutive 28 d exposure periods between: 1) 18 May and 24 June 2010 and 2) 15 June and 22 July 2010, with the deployments slightly staggered temporally to access field sites spread across the watershed.

Recovery of POCIS sorbent was modified from that described by Mazzella et al. (2010). POCIS were gently cleaned with distilled water and frozen at -30°C. Each POCIS was disassembled and the sorbent transferred through a glass funnel into a 3 mL SPE cartridge with a Visiprep SPE Manifold (Sigma-Aldrich). The sorbent was rinsed into the cartridge with 40 mL HPLC grade water and packed with a polypropylene frit. The cartridges were washed with 15 mL of 5% HPLC grade methanol, dried for 20 min under vacuum and frozen at -30°C for storage until elution. Cartridges were brought to room temperature prior to elution and analytes eluted with 5 mL methanol into 15 mL silanized (Surfacil, Fisher Scientific) glass centrifuge tubes. Extracts were evaporated to 0.5 mL at 30°C (CentriVap Centrifugal Concentrator, Labconco, Kansas City, USA), filtered through 0.2 μm PTFE filters (Fisher Scientific), brought to a final volume of 1 mL and spiked with 250 ng/mL AT-D5 prior to analysis.

2.4.4 *In situ* field calibration with deuterated desisopropyl atrazine

Calibration studies, referring here to *in situ* correction of field R_s with a PRC, were conducted at four field sites representing a range of physico-chemical characteristics between 16 September and 14 October 2010 and between 12 July and 9 August 2011. DIA-D5 was used as a PRC and its desorption from POCIS sorbent used to calculate field corrected R_s. Each POCIS was spiked with 5000 ng DIA-D5. For each POCIS, Oasis HLB sorbent (200 mg) was placed on a PES membrane and 100 μL of 50 000 ng/mL DIA-D5 (dissolved in methanol) was added evenly throughout the sorbent by pipette. The methanol was allowed to evaporate before the second PES membrane was placed on top of the sorbent and the membranes secured with stainless steel washers. Six replicate Day 0 POCIS were prepared for both experimental periods to quantify initial DIA-D5 concentrations and account for any losses in recovery. For each experimental period and site, 12 POCIS were deployed on Day 0 and three POCIS removed every 7 d. DIA-D5 was recovered from POCIS as described above.

Environmental variables were measured weekly throughout the calibration experiments (Days 0, 7, 14, 21 and 28). Temperature, pH and conductivity were measured with a HydroLab 4a Sonde (Hach Hydromet, Loveland, USA). Surface water velocity was estimated by measuring the time for an orange wiffle golf ball to travel 1 m. Duplicate mid-channel, integrated water samples (1 L) were taken in polypropylene bottles for turbidity and chlorophyll *a* analysis. All bottles were rinsed 3× with stream/river water at each site and the samples collected with a pole sampler. Planktonic chlorophyll *a* was a proxy of biofouling potential. Turbidity reflects factors affecting water clarity, such as phytoplankton, microbes, suspended sediments and dissolved organic carbon, and was a

proxy of overall membrane fouling potential. Turbidity was measured with a turbidity meter (LaMotte, Chestertown, USA). Water samples (500 mL) were filtered through 1.5 µm Whatman glass fiber filters (type 934-AH, Whatman, Mississauga, Canada), algal pigments extracted from filters (Burnison, 1980) and chlorophyll *a* calculated using a trichromatic equation (Jeffrey and Humphrey, 1975). For each environmental variable, weekly data were averaged separately for each field site and deployment period (fall 2010 or summer 2011).

2.4.5 Solid phase extraction

Amber borosilicate bottles (1 L) were soaked in phosphate-free soap for 24 hrs, rinsed 3× with distilled water, soaked for 72 hrs in 0.5% sulphuric acid, rinsed 3× with distilled water, rinsed 2× with acetone and 2× with petroleum ether. Solvents were evaporated in a fumehood for 1 hr and the glassware subsequently oven baked for 1 hr at 125°C. Water samples (1 L) were collected in pre-cleaned amber borosilicate bottles at each field site at the beginning (Day 0), middle (Day 28) and end (Day 56) of the 56 d POCIS deployment at each site between 18 May and 22 July 2010 and every 7 d during the calibration experiments (Days 0, 7, 14, 21 and 28). Mid-channel, integrated water samples were collected using a pole sampler as described above. Duplicate samples were taken for approximately 10% of the samples and matrix-blank samples (HPLC grade water) used to determine analyte recovery. Samples were filtered through 0.7 µm glass fiber filters (GF/F 47 mm diameter, Whatman) and spiked with 1000 ng/L ATR-D5. Samples for the calibration studies were also spiked with 5000 ng/L DIA-D5. Oasis HLB cartridges (6 mL, 500 mg) were conditioned with 15 mL methanol and equilibrated with 15 mL water. Samples were passed through the cartridges at 4 mL/min, washed with 15 mL 5% methanol,

dried under vacuum for 20 min and frozen at -30°C. Cartridges were brought to room temperature and analytes eluted with 5 mL methanol. Extracts were evaporated to 0.5 mL, filtered through 0.2 μm PTFE syringe filters (13 mm diameter, Fisher Scientific) and brought to a final volume of 1 mL.

2.4.6 LC-MS/MS analysis and validation

LC-MS/MS analyses were performed on a high performance liquid chromatograph hyphenated with a tandem mass spectrometer (3200 QTRAP, AB Sciex, Concord, Canada) at the Laboratory for the Analysis of Natural and Synthetic Environmental Toxins (LANSET) (University of Ottawa, Ottawa, Canada). An Agilent 1200 series HPLC was used to separate analytes using a Zorbax SB-C8 narrow-bore guard column (2.1 mm×12.5 mm, average particle size 5 μm, Agilent Technologies) connected with a Zorbax SB-C18 rapid resolution HT column (2.1 mm×50 mm, average particle size 1.8 μm, pressure limit 600 bar, Agilent Technologies) at a column thermostat temperature of 45°C, flow rate of 300 μL/min, mobile phase of A: water and B: acetonitrile and 1 μL injection volume. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode with turbo ion spray in positive electrospray ionization. Quantitation and confirmation were based on the following MRM transitions: 217.96>176.10 and 217.96>68.10; 223.11>181.20 and 223.11>69.10; and 179.02>69.20 and 179.02>105.10, for ATR, ATR-D5 and DIA-D5 respectively.

All samples, standards and blanks were injected in triplicate. A system blank (0 μL injection) and solvent blanks (acetonitrile, water and methanol) were run before the injection of the lowest concentration standard. A methanol blank was run approximately every six

samples to evaluate and minimize carryover. Standard curves were updated and replaced every 12 h of analysis. External calibration was used for quantitation. Seven point (5-1250 ng/mL) and eight point (2-250 ng/mL) calibration curves were constructed for SPE and POCIS samples respectively. POCIS samples were diluted by a factor of 5 or more in methanol prior to analysis. Quantitation was performed using Analyst 1.4.2 (Applied Biosystems, Foster City, USA).

Calibration models were assessed by evaluating regression model fit (R^2). The percent error at each concentration level was calculated by re-fitting data back to the model (US EPA, 2003):

$$\%Difference = \frac{Cc - Cn}{Cn} \times 100 \quad (1)$$

where, Cc is the calculated standard concentration and Cn is the nominal standard concentration.

The overall model fit was subsequently assessed by calculating its relative standard error (RSE) (US EPA, 2012c):

$$RSE = 100 \times \sqrt{\frac{\sum_{i=1}^{n} \left[\frac{Cc - Cn}{Cn}\right]^2}{[n - p]}} \quad (2)$$

where, n is the number of calibration points and p is the number of terms in the equation (average = 1, linear = 2, quadratic = 3, cubic = 4)

Calibration curves were fit with 1/x weighted quadratic models to emphasize precision at the lower end of the calibration range (US EPA, 2003, 2012c). The relative standard deviation (RSD) between triplicate injections was evaluated for both standards and samples. Concentrations were confirmed by evaluating percent differences between

quantitation and confirmation transition values. Instrument limits of detection (LODs) and limits of quantitation (LOQs) were calculated as 3× and 10× signal to noise respectively (where noise is 6× background signal standard deviation), averaged from triplicate injections of the lowest concentration standard for each calibration curve.

2.4.7 Statistics and modelling

Sampling rates were calculated according to the theory and models developed by Huckins et al. (2002, 2006) and Alvarez et al. (2004, 2007) using nomenclature outlined in Mazzella et al. (2010). TWA concentrations (C_w) (ng/L) of ATR at 24 field sites over 56 d were estimated by:

$$C_w = \frac{m}{R_{scal} \times t} \quad (3)$$

where m is the mass of ATR accumulated in each sampler (ng), R_{scal} is 0.239 L/d, a laboratory calibrated R_s for ATR (Mazzella et al., 2007) and t is the deployment time (d). A 56 d TWA concentration was calculated by summing m from two consecutive 28 d deployments.

For each calibration study site and time period, an *in situ* elimination rate constant ($k_{ePRCinsitu}$) (d^{-1}) for DIA-D5 was estimated:

$$C_{PRC(t)} = C_{PRC(0)} \times e^{-k_{ePRCinsitu} \times t} \quad (4)$$

where $C_{PRC(t)}$ and $C_{PRC(0)}$ are concentrations of DIA-D5 (ng) at time (t) and time (0) respectively.

Concentration data were ln-transformed to linearize the relationship and the slope k_e calculated using linear regression:

$$\ln C_{PRC(t)} = -k_{ePRCinsitu} \times t + \ln C_{PRC(0)} \quad (5)$$

Corrected sampling rates (R_{scorr}) (L/d) for ATR were calculated as:

$$Rscorr = Rscal \times \left(\frac{k\text{ePRCinsitu}}{k\text{ePRCcal}} \right) \quad (6)$$

where $k_{ePRCcal}$ is 0.057 d^{-1}, an elimination rate constant for DIA-D5 determined by Mazzella et al. (2010) in a laboratory calibration experiment.

Statistical analyses were performed using SPSS v.21 (IBM Corp., Armonk, USA). Three paired t-tests were conducted to compare: 1) differences in POCIS ATR concentrations between 12 paired sites (24 sites total), 2) differences in SPE ATR concentrations between 12 paired sites (24 sites total) and 3) differences in ATR concentrations estimated with POCIS and SPE at 24 sites. Differences in ATR between time periods were assessed by calculating the percentage of total ATR at each period to normalize for differences in absolute ATR concentrations between sites and conducting a one-way analysis of variance (ANOVA). For each site and time period, k_e was calculated using linear regression as described above. Pearson's correlations quantified the relationship between ATR concentrations obtained from POCIS and SPE, ATR concentrations and measures of agricultural intensity, and between k_e values and environmental variables. Stepwise linear regression was used to further examine effects of environmental variables on k_e. Differences in k_e between field sites and time periods were modelled using a general linear model with percentage DIA-D5 as the dependent variable, field site and experimental period (fall 2010 or summer 2011) as fixed factors and day since deployment as a covariate. Model assumptions for all tests (normality and heterogeneity of variance) were assessed using Shapiro-Wilk's and Levene's tests respectively. Data were transformed if necessary to meet these assumptions.

2.5 Results and Discussion

2.5.1 Method Validation

Analytical calibration curves met criteria of R^2 values >0.99, percent differences between nominal and calculated concentrations <20% and RSE <20% (EPA, 2003, 2012c). The average R^2 was 0.9980, the average percent difference between nominal and calculated concentrations was 2.9% and the average RSE was 4.6%. Average instrument LODs were 0.83, 0.87 and 0.25 pg on column and average instrument LOQs were 2.76, 2.89 and 0.82 pg on column for ATR, ATR-D5 and DIA-D5 respectively. Overall, RSD between triplicate injections were <15% (average 4.1%). Percent differences between quantitation and confirmation transitions were <20% for ATR and ATR-D5 (average 5.6%) but occasionally >20% for DIA-D5 (average 8.0%). Chromatographic interferences were observed in transition 179.02>105.10 and 179.02>69.20 was subsequently used for quantitation. No carry-over was observed in solvent blanks.

Recoveries of blank spikes (fortified HPLC water samples) were 100.5 ± 12.6% (n=7) for ATR-D5 and 92.8 ± 8.7% (n=4) for DIA-D5 and fell within the acceptable recovery range (70-130%) outlined by US EPA (2003). Recoveries of ATR-D5 and DIA-D5 from field-collected SPE samples were 89.3 ± 14.9% (n=130) and 58.9 ± 7.0% (n=46) respectively. The average difference in ATR concentrations between duplicate SPE samples was 10.6 ± 4.7% (n=11). The average difference in DIA-D5 between duplicate SPE samples was similar but more variable (10.1 ± 11.1%; n=10). Average recovery of ATR-D5 from POCIS samples was 56.7 ± 13.1% (n=239), illustrating that matrix effects were much higher in POCIS samples compared to SPE samples. Matrix effects refer to the effects of all

components of a sample, except the analyte of interest, on an analytical method and are a common challenge in LC-MS/MS analysis (Smeraglia et al., 2002).

2.5.2 Atrazine contamination in the South Nation River watershed

Accumulation of ATR in POCIS at 24 sites over a 56 d period ranged from 59 to 5510 ng/POCIS, spanning two orders of magnitude and demonstrating a clear gradient of ATR contamination across the watershed (Fig. 2.3). A gradient was also observed within tributaries and on average, high agriculture sites had higher concentrations of ATR (2393 ± 1707 ng/POCIS) compared to low agriculture sites (1311 ± 1349 ng/POCIS) (Fig. 2.3; $t=-4.9$; $df=33$; $p<0.001$). Significantly more ATR accumulated in POCIS in the first deployment period (average of 56.2% ATR for 18 May - 24 June 2010) compared to the second deployment period (average of 43.8% ATR for 15 June - 22 July 2010) (Fig. 2.3; $F=10.0$; $df=1,138$; $p=0.002$; $R^2=0.067$).

Atrazine concentrations obtained from SPE-concentrated grab samples followed similar trends to POCIS samples (Fig. 2.4). High agriculture sites had higher average concentrations of ATR (97 ± 62 ng/L) compared to low agriculture sites (58 ± 58 ng/L) (Fig. 2.4; $t=-4.0$; $df=11$; $p=0.002$). A gradient of ATR contamination across the watershed was observed with average ATR concentrations ranging from 6 to 256 ng/L. Atrazine concentrations were higher in June compared to May or July (Fig. 2.4; $F=34.2$; $df=2,69$; $p<0.001$; $R^2=0.498$), indicating POCIS deployment periods bracketed an appropriate timeframe to measure ATR.

ATR concentrations, integrated over a period of 56 d with POCIS, were strongly correlated with ATR concentrations averaged from SPE-concentrated water samples

collected at the beginning, middle and end of the POCIS deployment period (Fig. 2.5). POCIS ATR concentrations were significantly higher than SPE ATR concentrations and ranged from 4 to 412 ng/L (Fig. 2.5; t=3.8; df=23; p=0.001). The point-in-time estimates (SPE ATR) likely underestimated ATR contamination compared to the time-weighted-average estimates (POCIS ATR) because point-in-time estimates do not integrate pulses in concentrations that occur following rain events. ATR concentrations did not exceed Canadian water quality guidelines for the protection of aquatic life (1.8 μg/L) (Canadian Council of Ministers of the Environment, 1999). However, over half of the field sites (14/24) had 56 d average ATR concentrations >100 ng/L. Pulses in ATR concentrations may be almost 30× higher than post-pulse concentrations (Knight et al., 2013) suggesting potential for pulses above the guideline value.

The gradient in atrazine contamination across the watershed was associated with surrounding land use, specifically with measures of agricultural intensity. Atrazine concentrations were positively correlated with both the percentage of annual crops surrounding field sites and nitrate concentrations (Fig. 2.6). Annual crops in the South Nation River watershed often rotate annually between corn and soy crops and ATR is used on corn crops in Canada. Corn crops are typically treated with nitrogen-based fertilizers and in-stream nitrate concentrations >240 μg/L are indicative of anthropogenic nitrate contamination (Dubrovsky et al., 2010). Both percentage of surrounding annual crops and nitrate concentrations may be useful to identify areas of potential ATR contamination. However, a few sites had unexpectedly high ATR concentrations (Fig. 2.3, 2.6). The discrepancies may be due to localized inputs of ATR or from groundwater which can also be a significant source of ATR during baseflow (Squillace et al., 1993; Fischer et al., 1995).

2.5.3 *In situ* field calibration with deuterated desisopropyl atrazine

Desorption of DIA-D5 from spiked POCIS over 28 d was monitored at four sites in fall 2010 and again in summer 2011 (Fig. 2.7). Recoveries of ATR-D5 and DIA-D5 from SPE samples taken weekly at each field site during the two time periods were 99.3 ± 6.3 % and 58.9 ± 7.0 % respectively (n=46), demonstrating that while extraction efficiency was high, there was substantial signal suppression of DIA-D5 due to matrix effects. Average recovery of DIA-D5 from Day 0 POCIS samples was 86.9 ± 10.7% (n=12). Recoveries for Day 0 POCIS samples were further adjusted for estimated site specific matrix effects based on recovery of DIA-D5 from SPE samples. Direct assessment of matrix effects for DIA-D5 in POCIS samples was not possible as measured concentrations in field samples reflect both matrix effects and desorption of DIA-D5 over time. Desorption of ln(DIA-D5) was modelled using linear regression to calculate *in situ* rate elimination constants (k_e) from the slope of the regression line (Table 2.1). Mazzella et al. (2010) calibrated DIA-D5 desorption in a French stream and obtained a k_e of 0.022 /d, comparable to the values observed in the fall experiment but lower than those observed in the summer experiment of this study (Table 2.1).

A general linear model was used to assess effects of field site, experimental period (fall 2010 or summer 2011) and the number of days following deployment on the percentage of DIA-D5 remaining at sampler retrieval as a function of Day 0 concentrations. Significant effects of day ($F=192.0$; $p<0.001$), experimental period ($F=21.6$; $p<0.001$) and an interaction between day and experimental period ($F=32.1$; $p<0.001$) were observed. Desorption of DIA-D5 over time was greater and faster in summer 2011 compared to fall 2010 (Fig. 2.7). Desorption of DIA-D5 from POCIS did not differ significantly between field sites (Fig. 2.7;

F=1.4; p=0.247). The corresponding k_e and R_{scorr} values illustrated that desorption of DIA-D5 and uptake of ATR was higher in summer 2011 compared to fall 2010 (Table 2.1). A recent review found R_s for ATR were similar between six studies, averaging 0.25 ± 0.03 L/d (Harman et al., 2012). The average field corrected R_s obtained in the present study was 0.23 ± 0.12 L/d (Table 2.1), suggesting that laboratory derived R_s and *in situ* PRC corrected R_s were comparable. However, the larger standard deviation observed in the present study compared to the six calibration studies and the differences observed between deployment periods (Table 2.1), highlighted that factors affecting R_s under field conditions warranted further investigation.

2.5.4 Effect of environmental variables on POCIS sampling rates

POCIS remains poorly characterized in terms of modeling uptake rates and environmental factors (Harman et al., 2012). Only three studies have published *in situ* R_s (Zhang, et al., 2008; Mazzella et al., 2010; Jacquet, et al., 2012) and none have related variability in *in situ* R_s with environmental parameters (reviewed in Morin et al., 2012). I examined the effect of environmental variables on R_s under field conditions. Gradients in a number of environmental variables were observed between field sites and experimental periods (Table 2.2). However, only temperature was significantly correlated with DIA-D5 k_e values, with desorption of DIA-D5 increasing with increasing temperature (Table 2.2). Desorption of DIA-D5 increased by an average of 2.7 ± 0.3 fold between the cooler fall and warmer summer experimental periods (Table 2.1). Previous studies found R_s to increase by <2 fold over a similar temperature range (reviewed in Harman et al. 2012).

Maximum analyte uptake occurs when the rate-limiting barrier to solute transport is the external aqueous boundary (i.e. the thin layer of water between the POCIS membrane

and surrounding water) (Huckins et al., 2002). POCIS was under boundary layer control in previous laboratory studies (Alvarez et al., 2004; Mazzella et al., 2010). The observed increase in R_s with increasing temperature was in agreement with theoretical models that predict analyte diffusion across the aqueous boundary to be directly proportional to temperature (Alvarez et al., 2004 and references therein). Under boundary layer control, increases in flow velocity are expected to reduce the thickness of the boundary layer and increase R_s (Huckins et al., 2002; Alvarez et al., 2004). Previous studies found increases in R_s from <2 to 9 fold in turbulent conditions, with most studies comparing static versus stirred conditions and flow rates ranging from 2.6 to 37 cm/s for the studies that did measure flow rates (Harman et al., 2012). In the present study, R_s did not increase with increasing stream velocity, despite a range in velocity from 0.6 to 59 cm/s (Table 2.2). Harman et al. (2012) noted that measured flow rates may poorly represent actual flow rates at the sampler surface. Despite the limitation in accurately measuring flow rates at the sampler surface, the present study found that R_s did not appear to be affected by flow rates across a range of surface velocities measured in actual field deployment conditions.

Under turbulent conditions, the aqueous boundary layer may thin to the point that the rate-limiting barrier to solute transport becomes the PES membrane rather than the boundary layer and further increases in turbulence do not increase R_s (Alvarez et al., 2004). In-stream turbidity, planktonic chlorophyll *a* and conductivity were measured as proxies of concentrations of suspended particles, biofouling potential and dissolved inorganic ions respectively. While no direct effect of these environmental factors was observed (Table 2.2), they may have been present in sufficient concentrations at the four sites to impede solute

transport across the PES membrane and result in membrane control at stream velocities lower than would be predicted by laboratory studies.

Stepwise linear regression confirmed that of the environmental variables measured, only temperature had a significant effect on k_e values ($F=79$; $df=2,37$; $p<0.001$). Both temperature and velocity are expected to have positive effects on k_e values and in this study a weak negative correlation between temperature and velocity may have confounded detection of subtle effects of velocity on k_e (Pearson's correlation coefficient (PCC)= -0.712; p=0.047), with temperature overriding effects of velocity.

2.5.5 Field calibration and the performance reference compound approach for POCIS

Harman et al. (2011) state that one of the biggest challenges in quantitative use of POCIS is the lack of a method to correct for factors known to affect R_s. There is currently no consensus on whether the PRC approach is suitable for POCIS (Harman et al., 2012), given that POCIS tends to act as an infinite sink during the integrative uptake phase (Alvarez et al., 2004) but may also exhibit two-way isotropic exchange for some compounds (Mazzella et al., 2007). For a PRC to be effective, it must follow first order kinetics with equal uptake, release and resistance to mass transfer across boundaries in both directions (Alvarez et al., 2007). Data shown in the present study demonstrated that similarly to Mazzella et al. (2010), loss of DIA-D5 followed pseudo first order kinetics (Fig. 2.7). One further challenge with the PRC approach for POCIS is that PRCs must be poorly sorbed to be useful and are therefore likely to elute early along with interfering compounds that complicate LC-MS/MS analysis. Signal suppressing matrix effects were observed in this study, whereas Mazzella et al. (2010) observed enhancing matrix effects.

Further work is needed to better understand the displacement of PRCs by compounds with a greater affinity for POCIS sorbent (Harman et al., 2011), the effects of interactions between PRCs, target analytes and PES membranes (Vermeirssen et al., 2012) and whether factors controlling the release of DIA-D5 and those controlling uptake of target analytes are equivalent (Harman et al., 2012). Despite these challenges, the use of PRCs such as DIA-D5 has potential for improving quantitative use of POCIS that warrants further investigation. Desorption of DIA-D5 demonstrated that R_s between four field sites appeared to differ temporally but not spatially (Fig. 2.7; Table 2.1) and was useful in identifying potential factors affecting field R_s (Table 2.2). However, further understanding of the mechanisms governing PRC desorption and target analyte uptake is necessary before the PRC approach can accurately correct R_s for a broad suite of target analytes.

2.6 Conclusions

A gradient of atrazine (ATR) contamination across the South Nation River watershed in Eastern Ontario was observed. While time-weighted-average concentrations did not exceed Canadian water quality guidelines, the detection of elevated concentrations at a number of sites is cause for concern. POCIS was an effective tool to assess ATR contamination at the watershed level and ATR concentrations were positively correlated with measures of agricultural intensity. Field calibration studies using a performance reference compound (PRC) demonstrated that sampling rates (R_s) were similar between four field sites but differed seasonally. Temperature appeared to be the only significant environmental factor affecting R_s and future work could be directed to develop temperature corrected R_s. While further work is needed to validate a PRC approach for POCIS, the

inclusion of a PRC can provide valuable information on environmental factors with potential to affect R_s and function as an alternative and complement to *in situ* uptake calibration studies.

2.7 Acknowledgements

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Table 2.1 Deuterated desisopropyl atrazine (DIA-D5) *in situ* elimination rate constants ($k_{ePRCinsitu}$) and corrected atrazine (ATR) sampling rates (R_{scorr}) (± standard error (SE)) determined during fall 2010 and summer 2011 calibration studies in four tributaries of the South Nation River watershed, Canada

Site[a]	Deployment period	$k_{ePRCinsitu}$ ± SE (d^{-1})	Regression statistics	R_{scorr} ± SE (L/d)[b]
Little Castor (8 ●)	16 Sep - 14 Oct 2010	0.030 ± 0.004	F=69; df=1,17; p<0.001; R^2=0.812	0.124 ± 0.015
Middle Castor (6 ■)	16 Sep - 14 Oct 2010	0.023 ± 0.003	F=47; df=1,17; p<0.001; R^2=0.746	0.094 ± 0.014
North Branch (5 ■)	16 Sep - 14 Oct 2010	0.034 ± 0.004	F=94; df=1,16; p<0.001; R^2=0.862	0.143 ± 0.015
South Castor (7 ■)	16 Sep - 14 Oct 2010	0.031 ± 0.005	F=39; df=1,17; p<0.001; R^2=0.708	0.131 ± 0.021
Little Castor (8 ●)	12 Jul - 9 Aug 2011	0.083 ± 0.007	F=164; df=1,17; p<0.001; R^2=0.911	0.349 ± 0.027
Middle Castor (6 ■)	12 Jul - 9 Aug 2011	0.063 ± 0.005	F=137; df=1,17; p<0.001; R^2=0.895	0.264 ± 0.023
North Branch (5 ■)	12 Jul - 9 Aug 2011	0.080 ± 0.006	F=169; df=1,17; p<0.001; R^2=0.914	0.334 ± 0.026
South Castor (7 ■)	12 Jul - 9 Aug 2011	0.093 ± 0.008	F=125; df=1,17; p<0.001; R^2=0.886	0.391 ± 0.035

[a]Numbers and symbols following site names correspond to Fig. 2.1.

[b]R_{scorr} values were calculated using published $k_{ePRCcal}$ (0.057 d^{-1}) and R_{scal} (0.239 L/d) values (Mazzella et al., 2010).

Table 2.2 Environmental variables measured weekly at four field sites during fall 2010 (n=40) and summer 2011 (n=40) deployment of polar organic chemical integrative samplers (POCIS) and their correlation with *in situ* elimination rate constants ($k_{ePRCinsitu}$) (n=8). Averages are shown with minimum and maximum values in brackets. Significant correlations (p<0.001) are indicated in **bold**.

Variable	Fall 2010 (16 Sep - 14 Oct)	Summer 2011 (12 Jul - 9 Aug)	Pearson correlation coefficient (p)[a]
Temperature (°C)	12.73 (9.37-14.97)	23.73 (21.30-26.44)	**0.952 (<0.001)**
Velocity (cm/s)	21.0 (3.8-59.0)	4.5 (0.6-18.2)	-0.582 (0.130)
Turbidity (NTU)	13.7 (2.7-47.0)	8.5 (3.1-23.8)	-0.486 (0.222)
pH	8.08 (7.70-8.66)	8.12 (7.78-8.45)	0.283 (0.498)
Planktonic chlorophyll *a* (µg/L)	3.6 (1.2-11.9)	4.5 (1.7-16.4)	0.245 (0.558)
Conductivity (µS/cm)	634.5 (391.9-825.4)	632.8 (465.1-894.2)	-0.214 (0.612)

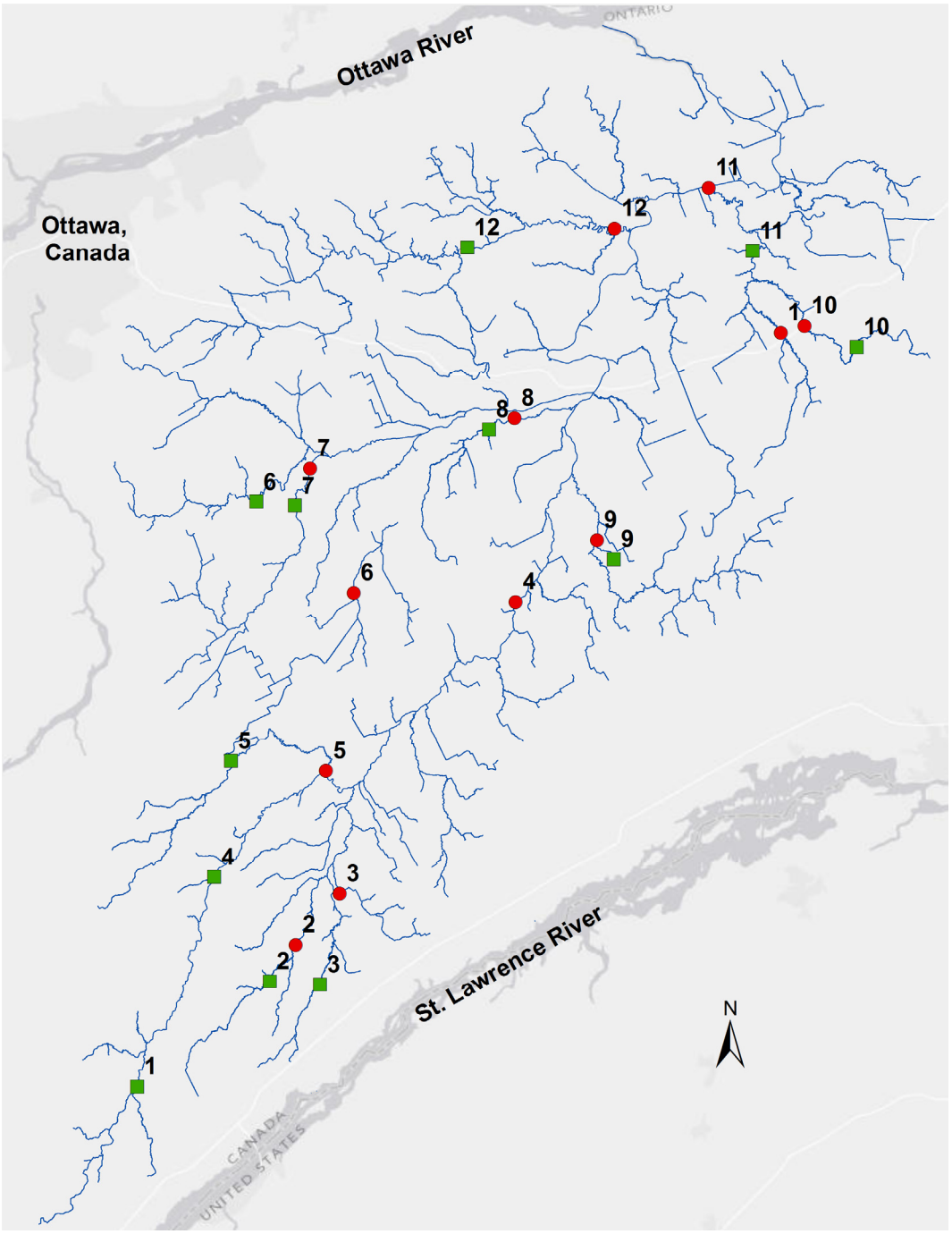

Fig. 2.1 Twelve paired field sites (total of 24) in the South Nation River watershed (3915 km^2), Canada. Sites were surrounded by low ■ or high ● levels of agriculture

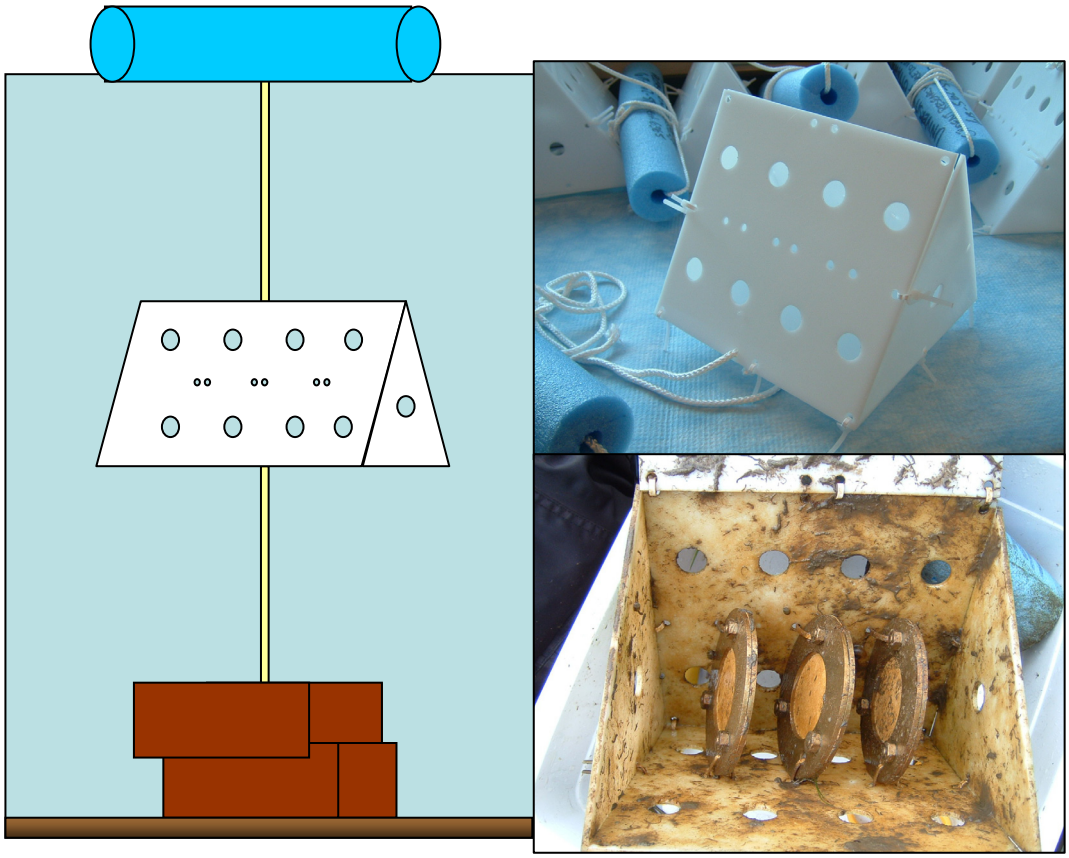

Fig. 2.2 Schematic view of triplicate polar organic chemical integrative samplers (POCIS) contained within a protective high density polyethylene shield (with holes to allow water exchange), supported with a float and secured with bricks

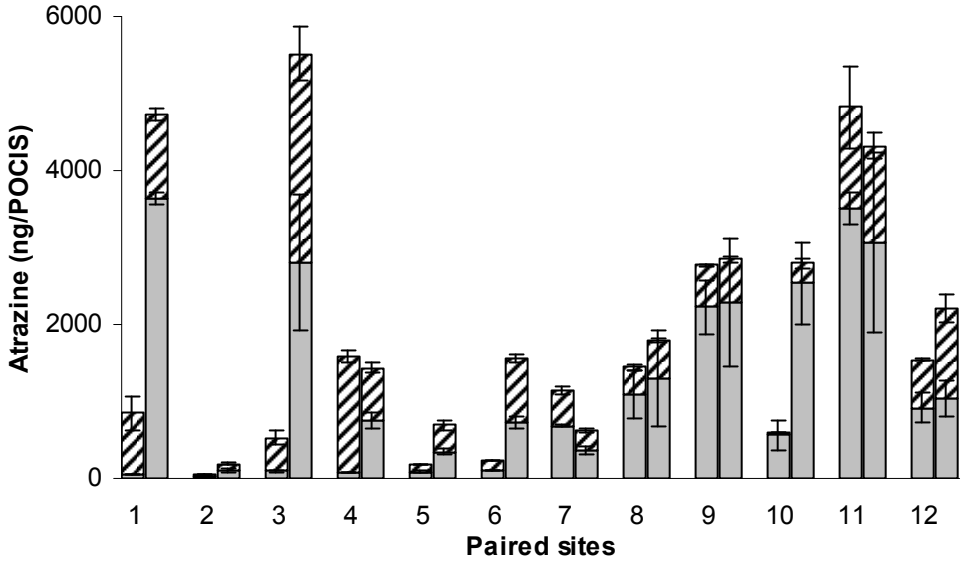

Fig. 2.3 Atrazine (ng) (± standard deviation) per polar organic chemical integrative sampler (POCIS) deployed over a 56 d period at 12 paired sites (total of 24) located throughout the South Nation River watershed. POCIS were deployed for 28 d between ▭ 18 May-24 June and ▨ 15 June-22 July 2010. Sites were paired along tributaries. Low agriculture sites (left column) were located upstream of high agriculture sites (right column). Site numbers correspond to Fig. 2.1

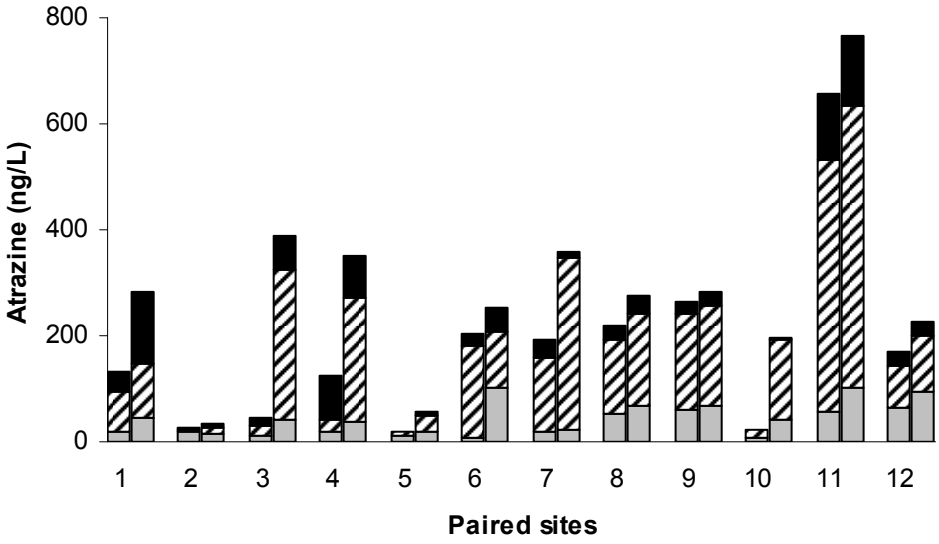

Fig. 2.4 Concentration of atrazine (ng/L) in grab samples (1 L) concentrated with solid phase extraction (SPE) and taken at 12 paired sites (total of 24) located throughout the South Nation River watershed. Samples were collected between ☐ 18-27 May, ▨ 15-24 June and ■ 13-22 July 2010. Sites were paired along tributaries. Low agriculture sites (left column) were located upstream of high agriculture sites (right column). Site numbers correspond to Fig. 2.1

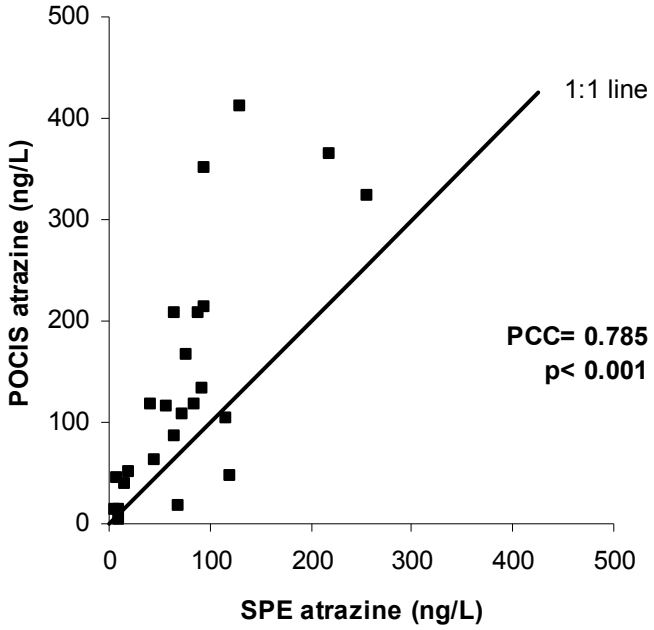

Fig. 2.5 Correlation between atrazine concentrations (ng/L) obtained from polar organic chemical integrative samplers (POCIS) and grab samples (1 L) concentrated with solid phase extraction (SPE). Time-weighted-average atrazine concentrations are shown for POCIS deployed for 56 d (two consecutive deployments of 28 d). Average SPE atrazine concentrations are shown for water samples taken on day 0, 28 and 56 of POCIS deployment. Pearson's correlation coefficient (PCC) and p value are shown

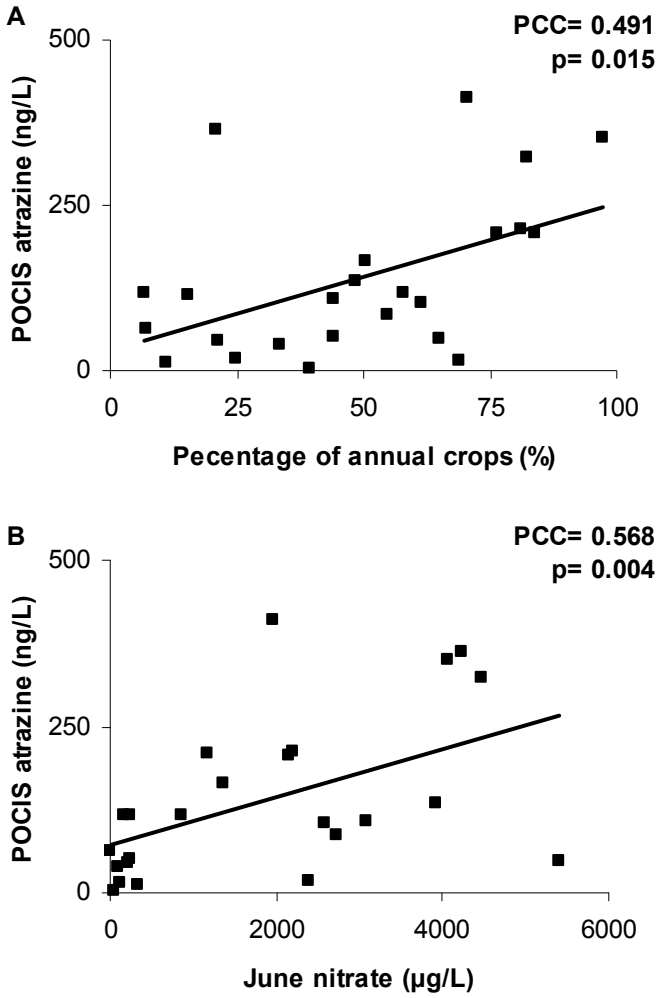

Fig. 2.6 Correlations between atrazine and A) the percentage of annual crops in a 500 m radius surrounding each site and B) June in-stream nitrate concentrations. Pearson's correlation coefficients (PCCs) and p values are shown. The line of best fit was illustrated using linear regression

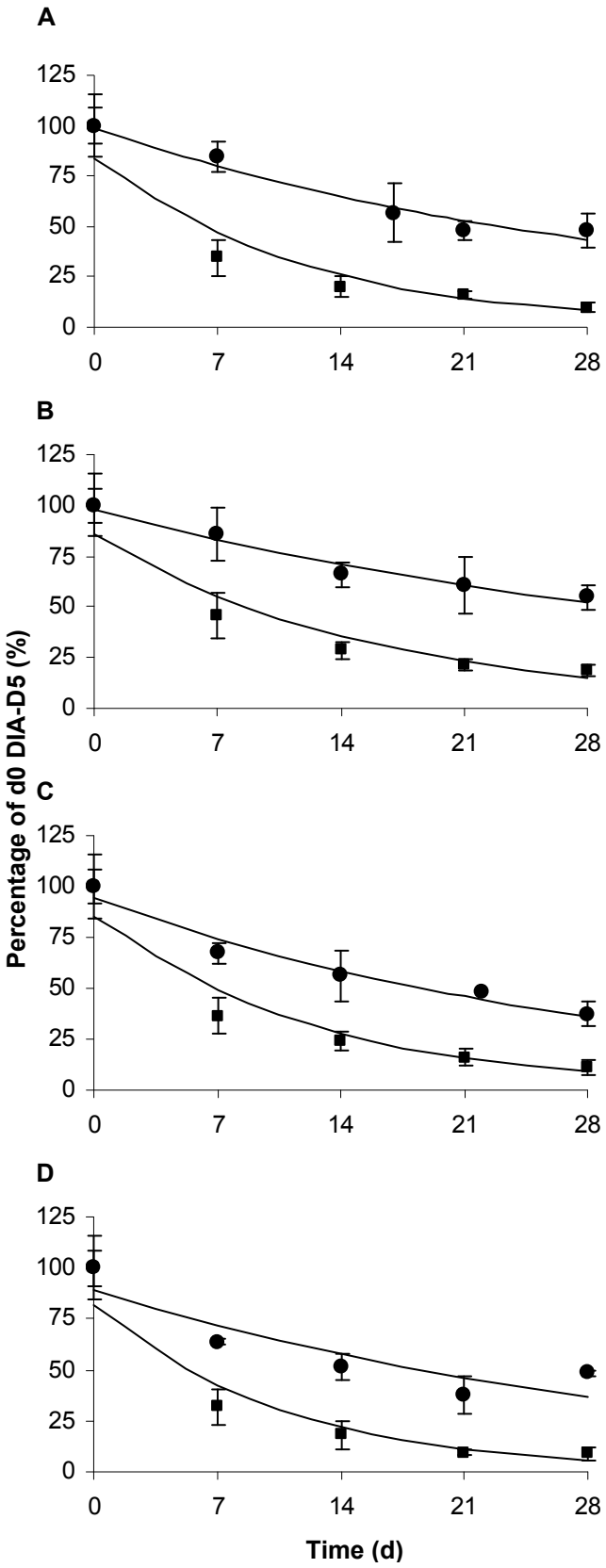

Fig. 2.7 Desorption of deuterated desisopropyl atrazine (DIA-D5) from polar organic chemical integrative samplers (POCIS) deployed in A) Little Castor R, B) Middle Castor R, C) North Branch South Nation R, D) South Castor R during ● 16 Sep-14 Oct 2010 and ■ 12 Jul-9 Aug 2011. Averages ± standard deviation and modelled response (solid lines) are shown

Chapter 3:
Nitrate overrides atrazine effects on riparian and aquatic plant community structure in an agricultural watershed

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3.1 Summary

1. In agricultural watersheds, streams are intimately connected with croplands and are likely to be exposed to agrochemicals such as fertilizers and herbicides. Plants, including riparian and aquatic species may be specifically affected by agrochemicals because they are more taxonomically similar to the intended targets, crop and weed species, than other types of organisms.

2. In shallow lakes, nutrient enrichment contributes to a shift from clear water conditions dominated by submerged species to floating-leaved species to emergent species and to the eventual loss of macrophytes (aquatic plants) characterized by turbid, phytoplankton-dominated conditions. This process may also occur in streams and rivers but it is often unclear which, if any, nutrient is limiting in these systems. Effects of herbicides on riparian and aquatic plant community structure are poorly characterized.

3. The riparian and aquatic plant community structure was assessed at 12 paired stream/ river sites (24 in total), across a watershed with a gradient of agricultural land use. Effects of agricultural impact were evaluated by examining species richness, percentage of non-native species, the number and relative frequency of submerged macrophytes and the overall floristic quality at each site.

4. A gradient in agricultural impact was observed in field sites located across the watershed in terms of differences in the percentage of surrounding annual crops and concentrations of nitrogen, phosphorus and the herbicide atrazine. Nitrate and atrazine concentrations were highly correlated. However, only in-stream nitrate had a significant effect on riparian and aquatic plant community structure.

5. In total, 285 riparian and aquatic plants were identified. Nitrate had no effect on species richness. Along a gradient of increasing nitrate, an increase in the percentage of non-native species, a decrease in the number of submerged macrophytes and a decrease in the overall floristic quality of field sites was observed.

6. Species positively associated with nitrate generally had low or negative coefficients of conservation, whereas species negatively associated with nitrate had higher coefficients of conservation. The floristic quality assessment system provided more information and was more sensitive to effects of agriculture than measures of species richness and percentage of non-native species.

7. Overall, there was evidence that nitrate enrichment across an eastern Ontario (Canada) watershed reduced the quality of the riparian and aquatic plant community, overriding any observable effects of atrazine.

Running head: Community structure changes along an agrochemical gradient

Keywords: riparian; macrophyte; nitrate; atrazine; agriculture impact

3.2 Introduction

Agrochemicals, including fertilizers and herbicides, are a significant contributor of non-point source pollution to surface waters. In agricultural watersheds, water bodies such as ditches, wetlands, streams and rivers are intimately associated with croplands and may be inadvertently exposed to agrochemicals. Nutrients, primarily nitrogen and phosphorus from synthetic fertilizers and manure, enter water bodies via run-off from fields and leaching of nutrients to surface and ground waters (Beaulac and Reckhow, 1982; Haith and Shoemaker, 1987; Carpenter et al., 1998; Ekholm et al., 2000, Dubrovsky et al., 2010). Off-target herbicide contamination may occur through similar pathways (Pantone et al., 1992; Waite et al., 1992; Smith et al., 1993; McMahon et al., 1994) as well as through dry deposition and spray drift (Grover et al., 1988; Asman et al., 2003). Transport of agrochemicals is facilitated by surface and subsurface drainage systems, primarily ditches and tile drains, which drain over 80% of catchment basins in some North American agricultural regions (Blann et al., 2009). As a consequence, comprehensive surveys in the United States have found elevated nutrient concentrations and the presence of pesticides in 90% and 97% of agricultural streams respectively (Dubrovsky et al., 2010; Gilliom et al., 2006). The triazine herbicide atrazine (6-chloro-*N*-ethyl-*N*′-1-methylethyl-1,3,5-triazine-2,4-diamine) is commonly detected in North American surface and ground waters due to its widespread usage on corn crops and is of particular concern due to its toxicity, mobility and persistence (Solomon et al., 1996; Gilliom et al., 2006).

Plants, including macrophytes (aquatic plants) and riparian species, may be especially affected by agrochemicals because they are more taxonomically similar to the intended targets, crop and weed species, than other types of organisms. Numerous studies

have documented the sensitivity of plant test species such as *Lemna minor* L. to pesticides (e.g. Kegley et al., 2011). However, the effects of pesticides on the structure of plant communities in actual field conditions are poorly understood and rarely examined. Stansfield et al. (1989) suggested that organochlorine pesticides contributed to a shift from macrophyte dominance to phytoplankton dominance in shallow lakes through toxic effects on algae-grazing Cladocera. Most documented effects of herbicides on macrophyte communities come from studies examining effects of direct herbicide application to lakes to control invasive species. These studies have had conflicting results ranging from reports of no significant effects on native macrophytes (Jones et al., 2012), reductions in submerged macrophytes (Parsons et al., 2009) and declines or increases in macrophytes depending on the species present (Wagner et al., 2007). The effects of current applications of herbicides on native riparian and aquatic plant communities in agricultural landscapes are largely unknown. However, short-lived, grassy and non-native plant species are more common in habitats such as woodlots and hedgerows adjacent to intensively farmed fields (Boutin and Jobin, 1998).

Unlike herbicides, effects of nutrients on plant communities have been better characterized by far. Eutrophication (increased primary production resulting from nutrient enrichment) is a significant factor in the decline of macrophyte diversity, particularly of submerged species over the last century and has been reported in lakes and streams in Europe (Sand-Jensen et al., 2000; Riis and Sand-Jensen, 2001; Körner, 2002; Hilt et al., 2013; Steffen et al., 2013) and North America (Egertson et al., 2004). A mechanism for the loss of macrophytes has been established: enrichment of a limiting nutrient stimulates phytoplankton and epiphyte growth, increasing turbidity and resulting in a shift in dominant

macrophyte forms from submerged to floating-leaved to emergent species and eventually resulting in phytoplankton dominance as light becomes limiting for all macrophyte forms (Phillips et al., 1978; Irvine et al., 1989; Scheffer et al., 1993; reviewed in Hilton et al., 2006).

Aquatic macrophytes are good indicators of water quality due to their sensitivity to eutrophication and are now used as biological quality elements to assess the ecological status of rivers in Europe as part of the Water Framework Directive (European Union, 2000). Although riparian bank vegetation has an important role in improving water quality (Osborne and Kovacic, 1993; reviewed in Dosskey et al., 2010), typically only species within river channels are identified (Dawson, 2002). A number of metrics have been developed to detect eutrophication using macrophytes in Europe (Haury et al., 2006; Holmes et al., 1999). However, the sensitivity of particular macrophyte species to eutrophication is not well characterized in many regions outside of Europe, including Canada. The issue is complicated in streams and rivers where it is often unclear which, if any nutrients, are limiting (Hilton et al., 2006).

While specific eutrophication metrics have not been developed in North America, a general methodology for evaluating and assessing natural areas using floristic composition has been developed for the Chicago region (Wilhelm and Ladd, 1988; Wilhelm and Masters, 1995), Michigan (Herman et al., 1997), Ohio (Andreas et al., 2004) and southern Ontario (Oldham et al., 1995). The general theory is that native plant species within a region vary in their tolerance to disturbance and display a quantifiable degree of fidelity to specific habitats (Oldham et al., 1995). Non-native species can be evaluated by rating their impact on natural areas. For southern Ontario, coefficient of conservation (CC) values were determined for

1615 native and 712 non-native plant species with all species, including riparian and aquatic species, assigned a rank from -3 to 10 (Oldham et al., 1995). Non-native species range from -3 to -1 with lower scores representing problematic species. Native species were assigned a rank from 0-10 as follows: species with scores from 0-3 are found in a wide variety of habitats, including disturbed areas, scores of 4-6 represent species that tolerate moderate disturbance, scores of 7-8 are characteristic of species that belong to a community that has undergone minor disturbance and scores of 9-10 represent species with a high degree of fidelity to specific environmental conditions (Oldham et al., 1995). Coefficient of conservation values can be used to calculate the overall floristic quality index (FQI) of the plant community, allowing for a quantitative comparison of different areas.

The objective of the present study was to assess effects of agrochemicals on riparian and aquatic plant community structure by identifying and comparing vegetation at 24 field sites located across a watershed and along a gradient of agrochemical contamination. The general hypothesis was that intense agricultural land use, nutrient enrichment and atrazine contamination have negative effects on plant communities. The floristic quality assessment system for southern Ontario (Oldham et al., 1995) was used to assess the quality of riparian and aquatic plant communities. Agricultural land use and agrochemicals were predicted to lead to an increase in the percentage of non-native species and decreases in species richness, the number and relative frequency of submerged species and overall floristic quality. Canonical correspondence analysis (CCA) was used to further assess changes in plant species composition while normalizing for differences in physico-chemical parameters between field sites.

3.3 Methods

3.3.1 Study area and site selection

The South Nation River watershed covers most of Eastern Ontario, Canada (3915 km^2) (Fig. 2.1) and has a historical (1915-2011) average annual discharge of 44.3 m^3/s at its mouth (Environment Canada, 2013). The headwaters commence near the St. Lawrence River (44°40'41"N, 75°41'58"W) and the river flows north-easterly across a flat, poorly drained landscape for 177 km until its confluence with the Ottawa River (45°34'24"N, 75°06'00"W). The watershed is predominately agricultural with crops of corn (*Zea mays* L.) and soybean (*Glycine max* L. (Merr.)) typically planted in tile-drained fields.

Twenty-four sites located throughout the South Nation River watershed were selected for study in 2007 (Fig. 2.1). Sites were paired along a given tributary with sites surrounded by low levels of agriculture located upstream of sites surrounded by high levels of agriculture. Sites were selected using land use data to identify areas of low and high impact (Statistics Canada, 2006), using Google Earth v.4.2.0198.2451 (Google Inc., Mountain View, USA) to verify physical aspects and through reconnaissance of potential field sites. The average distance between paired sites was 9.0 ± 8.5 km (ranging from 1.5 to 33.7 km). Two pairs of sites were located along different tributaries due to a lack of accessible and suitable sites. Pair 1 sites were 80.4 km apart and pair 6 sites were 10.7 km apart (Fig. 2.1). All sites were matched as closely as possible in terms of visible features such as steam width, bank slope and canopy cover. The 12 pairs were not hydrologically connected except: 1) low agriculture site 1 was upstream of pair 4 sites along the main branch of the South Nation River and 2) high agriculture site 1 and pair 10 sites were

upstream of pair 11 sites along the Scotch River (Fig. 2.1). Each site was defined by a 20 m stream length.

3.3.2 Physical characteristics

Strahler stream order was determined from data provided by the South Nation River Conservation Authority and Ontario Ministry of Natural Resources as part of the Water Resources Information Project (WRIP). Data were produced by the South Nation Conservation Authority under license with the Ontario Ministry of Natural Resources (© Queen's Printer, 2013). Bank slope was measured in triplicate along each bank and ranged from 0 % for flat banks to 100 % for banks cut-away at 90°. Aggregate soil samples were collected from both banks in 2010 to characterize soil structure (percentage sand, silt and clay) using an established hydrometer method (analyzed by A&L Canada Laboratories Inc., London, Canada using Klute, 1986). Stream width during base flow was measured in triplicate along the length of each site.

Stream depth and surface velocity were measured in triplicate during aquatic vegetation surveys in August 2007 and 2010. Surface velocity was estimated by measuring the time for an orange wiffle golf ball to travel 1 m. Maximum depth was measured mid-channel, while average depth was measured mid-channel as well as halfway between the mid-channel and each bank. Data were averaged from all sampling dates.

3.3.3 General water chemistry

Dissolved oxygen, pH, temperature and conductivity were measured with a HydroLab 4a Sonde (Hach Hydromet, Loveland, USA). Measurements were taken once in

2007 (August), three times in 2008 (early June, late June and August) and four times in 2010 (monthly from May to August). Duplicate mid-channel, integrated water samples were taken in 1 L Nalgene polypropylene bottles for turbidity and chlorophyll *a* analysis (during the periods described above except from May to July only in 2010). All bottles were rinsed 3× with stream/river water at each site. Samples were taken using a pole sampler to collect water upstream of each canoe/wading location to avoid disturbance and contamination of water and sediments. Turbidity was measured with a portable turbidity meter (LaMotte, Chestertown, USA).

Water samples (500 mL) were filtered through 1.5 μm Whatman glass fiber filters (type 934-AH, Whatman, Mississauga, Canada) and frozen at -30°C until extraction of algal pigments. Thawed glass fiber filters were heated in 4 mL dimethyl sulfoxide for 10 min at 65°C and pigments extracted with the addition of 90% acetone to a final volume of 15-18 mL (Burnison, 1980). Optical density was measured with a spectrophotometer (Pye Unicam SP8-100 UV-Visible spectrophotometer, Thermo Fisher Scientific Inc., Waltham, USA or Varian Cary100 UV-Visible spectrophotometer, Agilent Technologies, Mississauga, Canada) at 630, 647, 664 and 750 nm and chlorophyll *a* calculated using a trichromatic equation (Jeffrey and Humphrey, 1975). Data were averaged from all sampling dates.

3.3.4 Measures of agricultural impact

Agricultural intensity at each field site was determined in two ways. First, the percentage of annual cropland was calculated in a 500 m radius surrounding each site as well as the percentage of land in perennial crops and pasture. The percentage of undisturbed natural habitat was estimated similarly by calculating the percentage of wetland, forest,

shrub land and exposed land surrounding each site. Exposed land was predominately non-vegetated and non-developed and included bare soil, rock and sediments. The remaining land area consisted of developed land, including suburban development, roads, buildings, parks, farmsteads and golf courses. A 500 m radius allowed for the determination of land use immediately surrounding each field site as well as in the adjacent area. Second, land use upstream of each site was calculated in a 1000 m long, 100 m wide area to compare the influence of upstream land use. All calculations were made from data provided by Agriculture and Agri-Food Canada (2008).

Water quality at field sites was characterized by measuring in-stream nitrogen and phosphorus concentrations, with elevated concentrations representing agricultural contamination from synthetic fertilizers and manure (Dubrovsky et al., 2010). Mid-channel, integrated water samples were collected using a pole sampler in 300 mL PET bottles for nutrient analysis seven times between 2007 and 2010. Duplicate samples were taken once in 2007 (August) and three times in 2008 (early June, late June and August). In 2010, single samples were taken three times (monthly from May to July), with duplicate samples collected for approximately 10% of all samples. Nutrients were analyzed at the Robert O. Pickard Environmental Centre Laboratory of the City of Ottawa (Canada) following established methods of the Ontario Ministry of the Environment (2007a, b). Nitrate (NO_3^-) and nitrite (NO_2^-) were measured with an ion chromatograph system (Dionex® DX100, Thermo Fisher Scientific Inc. Sunnyvale, USA). Reactive phosphate (RP) and ammonia + ammonium ($NH_3 + NH_4^+$) were measured with colorimetric assays at 880 nm and 630 nm respectively using an autoanalyzer (Skalar™ 1070 Autoanalyzer, Skalar, Inc, Brampton, Canada). Total phosphorus (TP) and total Kjeldahl nitrogen (TKN) were converted to RP

and NH_3 respectively following an acid digestion and analyzed as above. Dissolved inorganic nitrogen (DIN) was calculated as the sum of NO_3^-, NO_2^- and $NH_3 + NH_4^+$ and total nitrogen (TN) was calculated as the sum of TKN, $NO_3^-$and NO_2^-. Dissolved and particulate organic nitrogen (DON+PON) were calculated as TKN minus $NH_3 + NH_4^+$. Method detection limits (MDLs) were 2, 3, 5, 20, 20 and 40 μg/L for RP, $NH_3 + NH_4^+$, TP, NO_3^-, TKN and NO_2^- respectively. For each parameter, concentrations for spring/ summer were estimated by averaging concentrations from all sampling dates. June concentrations were estimated by averaging June 2008 (two sampling dates averaged) and June 2010 concentrations. June concentrations were of particular interest because they were expected to reflect peak nutrient concentrations following run-off of fertilizers applied on crops during early spring.

In-stream concentrations of atrazine were measured using three methods: enzyme linked immunosorbent assays (ELISAs), active sampling and passive sampling. In 2008, duplicate water samples (1 L) for atrazine analysis were collected in pre-cleaned amber borosilicate bottles at the beginning and end of June. Twenty mL sub-samples were frozen and stored at -30°C in 40 mL pre-cleaned amber borosilicate vials with PTFE caps until analysis using microtiter plate format ELISAs (Abraxis LLC, Warminster, USA). Atrazine concentrations were quantified with a Spectramax® Plus UV/VIS spectrophotometer with a microplate reader using Softmax® Pro V. 3.1 (Molecular Devices, Sunnyvale, USA). Atrazine in each sample was measured in duplicate wells and on two different days. Atrazine values for each site were averaged from these values for both sampling days. The MDL was 0.050 μg/L.

Active sampling was conducted in June 2010. Water samples (1 L) were collected in pre-cleaned amber borosilicate bottles and concentrated onto Oasis HLB solid phase extraction cartridges (6 mL, 500 mg, Waters, Mississauga, Canada). Duplicate samples were taken for approximately 10% of the samples. Time-weighted-average atrazine concentrations (56 d) were also determined by passive sampling with polar organic chemical integrative samplers (POCIS), deployed in triplicate for two consecutive 28 d periods between 18 May and 22 July 2010. Atrazine concentrations were analyzed with a high performance liquid chromatograph coupled to a tandem mass spectrometer (LC-MS/MS). The limit of quantitation was 2.76 pg on column. Details of active and passive sampling, extraction of analytes and LC-MS/MS analysis are described in Dalton et al., 2014.

3.3.5 Macrophyte survey

Vegetation was surveyed along a 20 m open canopy stream length in four belt transects orientated perpendicular to the shore in 2007 and again in 2010 (Fig. 3.1). All vascular plants were identified in 1 m^2 quadrats spanning both stream banks. Quadrats in the riparian zone were located just above the water's edge on both banks as well as 2 m from the water's edge (Fig. 3.1). Each transect typically had five quadrats spanning the stream channel, with the number of quadrats across the channel adjusted for very narrow or wide sites. Most sites had 20 aquatic quadrats and all sites had 16 riparian quadrats (Fig. 3.1; average 36 quadrats per site). Low order narrow streams had as few as 12 aquatic quadrats and high order wide rivers had as many as 24 aquatic quadrats. Bank vegetation was surveyed twice per year (late spring and late summer) so that the majority of species could be identified while in flower. Aquatic vegetation in the stream channels was surveyed once

per year when biomass was likely to be greatest (July-August) by securing quadrats in the water. Species were identified following Gleason and Cronquist (1991) and Crow and Hellquist (2000) with nomenclature updated according to USDA (2013).

3.3.6 Statistics

Paired t-tests were conducted to compare physical, chemical, land use, nutrient and atrazine characteristics between paired sites surrounded by low and high levels of agriculture. Paired t-tests were also used to compare atrazine concentrations obtained with ELISA and LC-MS/MS (passive and active sampling) between paired sites. The assumption of normality of differences between paired t-tests was evaluated with a Shapiro-Wilk's test. Data were transformed to best meet this assumption. A non-parametric Wilcoxon signed rank test was conducted when transformations did not improve normality.

The total number of species (species richness) was calculated at each site. The native status of each species was assigned according to Oldham et al. (1995) and the percentage of non-native species calculated for each site. For each site, the frequency of each species was calculated as the percentage of quadrats in which a given species was recorded. Frequency was initially calculated separately by year (2007 and 2010) and by location (riparian or in-stream) and subsequently merged. Where species appeared in both the riparian zone and aquatic surveys, the highest frequency was kept and represented the frequency of a species within its preferred habitat. Similarly, frequency between years was compared and the highest frequency retained to avoid bias against rare species missed in one year. Also, frequency may fluctuate between years as well as seasonally and the highest frequency was chosen to estimate the maximum potential frequency of a given species.

Each species was assigneed a CC score ranging from -3 to 10 as listed in Oldham et al., 1995. The overall floristic quality index of each site was calculated by multiplying the average CC for each site by the square root of the number of species at that site (Oldham et al., 1995). Non-native species were included in the calculation of FQI. The presence of each species at low and high agriculture sites was tabulated and the difference between paired sites calculated.

The aquatic macrophyte community was examined further using data from the aquatic surveys and including only species identified as being obligate wetland species according to Oldham et al. (1995). At each site, the number of emergent, floating-leaved and submerged species was identified. The relative frequency of each type of aquatic species was also determined by dividing the sum of frequencies for a given growth form by the total frequency of all aquatic species. Paired t-tests were used to compare sites surrounded by low and high levels of agriculture as described above. The relationship between agrochemical impact (indicators measured in terms of NO_3^-, atrazine and annual crops) on wetland and aquatic plant community characteristics was assessed using linear regression.

The similarity in plant species composition between paired sites was evaluated using the Sørensen coefficient (S_s) (1948):

$$Ss = \frac{2a}{2a+b+c} \times 100 \qquad (1)$$

where a is the number of species common to both sites, b is the number of species in the low impact site and c is the number of species in the high impact site. Both the Sørensen and Jaccard coefficients are popular measures of expressing similarity, with the Sørensen coefficient generally preferred because it gives weight to species common to two sites as

opposed to giving weight to species that only occur in one site (Kent and Coker, 1992). All analyses described above were conducted using SPSS v.21 (IBM Corp., Armonk, USA).

Canonical correspondence analysis (CCA) was used to ordinate species and sites relative to measures of agrochemical impact, after accounting for variation in species composition due to intrinsic physical and chemical differences between field sites. Pearson's correlations were used to correlate measures of agrochemical impact with physical and chemical site characteristics and highly correlated environmental variables were excluded from the CCA. The inclusion of multiple highly correlated variables leads to unreliable constrained ordination models (Lepš and Šmilauer, 2003) and would possibly mask agrochemical effects. Variables were normalized if necessary following an assessment of normality using Shapiro-Wilk's tests. Variables were standardized to a mean of 0 and standard deviation of 1 (z-score transformation). An initial CCA was conducted with environmental variables uncorrelated with agrochemical stress to determine which environmental variables had an influence on species composition. Biplot scaling was used and focused on inter-species differences. Rare species were down-weighted. Stepwise regression and Monte Carlo permutations were used to test the significance of the environmental variables. Significant environmental variables were then used as covariables in a subsequent partial CCA where the ordination was constrained to species variation explained by measures of agrochemical impact. Species with significant positive and negative associations with measures of agrochemical stress were identified with t-value biplots and Van Dobben circles (Ter Braak and Looman, 1994). Multivariate analyses were conducted using CANOCO v.4.5 (Plant Research International, Wageningen, The Netherlands).

3.4 Results

3.4.1 Site characteristics

Physical and chemical characteristics varied across the watershed but were typically similar between paired sites (Table 3.1). Sites were located along streams ranging from a Strahler order of 3 near the headwaters to 6 along the main branch of the South Nation River (Table 3.1). Across the watershed, sites differed in terms of bank slope, stream width, depth and surface velocity. However, paired sites were similar in terms of these physical characteristics, as well chemical characteristics such as temperature, conductivity and suspended chlorophyll *a* (Table 3.1). Paired sites differed in their bank soil structure, with sites surrounded by low levels of agriculture tending to have a higher percentage of sand and lower percentage of silt compared to sites surrounded by high levels of agriculture (Table 3.1). High agriculture sites were more turbid, more alkaline and had a higher concentration of dissolved oxygen compared to low agriculture sites (Table 3.1).

3.4.2 Measures of agricultural impact

Land use

Land use surrounding field sites located across the watershed varied from 6.7-97.4 % annual crops and from 1.1-77.8 % natural habitat (Table 3.2). High agriculture sites had a higher percentage of surrounding annual crop land and lower percentage of natural habitat compared to low agriculture sites (Table 3.2). The percentage of perennial crops and pasture land was similar between paired sites (Table 3.2). The South Nation River watershed is predominately agricultural and was characterized by having a low percentage of urban development (Table 3.2). Trends observed in a 500 m radius surrounding each field site

(Table 3.2) were also observed along stream banks immediately upstream of each field site (1 km long, 100 m wide areas on either side of the streams) (Appendix B, Table B.1). Crop data quantifying the percentage of corn, soybean and cereal crops (wheat, barley, rye and oats) were available for 16 of 24 field sites (Agriculture and Agri-Food Canada, 2008). Within 500 m radius areas surrounding field sites, crops consisted of an average of 55.5% corn, 37.4 soybean and 7.1% cereal crops. Minimum and maximum percentages ranged from 3.8-98.9% corn, 0.2-86.1% soybean and 0-56.1% cereal crops (Agriculture and Agri-Food Canada, 2008).

Nitrogen and phosphorus

Concentrations of RP and TP were not significantly different between paired sites surrounded by varying levels of agriculture (Table 3.3). Subsequent analyses focused primarily on nitrogen and contamination from nitrogen-based fertilizers. Organic nitrogen and NH_3 + NH_4^+ were similar between paired sites and seasonally (Table 3.3). Nitrite concentrations were typically below detection and were not reported. Nitrate was higher in June compared to the spring/summer and was higher at high agriculture sites compared to low agriculture sites during both time periods (Table 3.3). Total nitrogen followed similar trends, driven by changes in NO_3^- (Table 3.3). The ratio of TN:TP was higher at sites surrounded by high levels of agriculture compared to sites surrounded by low levels of agriculture and this trend was stronger when the ratio between bioavailable nitrogen and phosphorus, DIN: RP, was examined (Table 3.3).

Atrazine

In-stream atrazine concentrations measured with ELISAs in 2008 were significantly higher than concentrations measured with LC-MS/MS following both active and passive sampling in 2010 (Fig. 3.2). Atrazine concentrations from grab samples taken in June 2010 were similar to time-weighted-average concentrations estimated from passive sampling during a 56 d time period between mid May and mid July 2010 (Fig. 3.2; t=-0.464; df=23; p=0.647). Time-weighted-average concentrations of atrazine ranged from 4 to 412 ng/L (Fig. 3.3) and these values were used in subsequent statistical analyses. Sites surrounded by high levels of agriculture had significantly higher concentrations of atrazine compared to sites surrounded by low levels of agriculture (Fig. 3.3).

3.4.3 Riparian and aquatic plants

In total, 285 riparian and aquatic plants were identified in 2007 and 2010. The complete list of species is given in Appendix B (Table B.2). Of the 285 species, 39 were unique to the stream channel, 198 were unique to the riparian bank and 48 species were identified in both habitats. Average species richness per site was 77 and ranged from 43-107 species (Fig. 3.4). Species richness was similar between paired sites and across a gradient of NO_3^- contamination (Fig. 3.4 A, D). The percentage of non-native species ranged from 9.0 to 38.8% (average 28.5%) and was similar between paired sites but declined significantly across the watershed as NO_3^- increased (Fig. 3.4 B, E).

Despite similarities in species richness and percentage of non-native species, species composition was dissimilar between paired sites and Sørensen coefficients were below 50% for all paired sites. The overall average Sørensen coefficient was 36.7% with values ranging

from 27.7-40.8% (Appendix B; Table B.3). This dissimilarity between paired sites did not translate into a reduction in floristic quality at paired sites surrounded by high agriculture compared to low agriculture (Fig. 3.4C) and a number of species were common to most sites (Table 3.4). However, species more commonly found at low agriculture sites had higher CC values compared to species more commonly found at high agriculture sites (Table 3.4). At the watershed scale, a reduction in floristic quality was observed along a gradient of NO_3^- (Fig. 3.4F). Floristic quality also decreased as the percentage of surrounding agriculture increased ($F=6.552$; $df=1,23$; $p=0.018$; $R^2=0.229$; Appendix B, Table B.4). This relationship was examined further in a general linear model assessing the effects of NO_3^-, percentage annual crops and their interaction on FQI. The percentage of annual crops had no effect on FQI ($F=1.776$; $p=0.198$) once the effect of NO_3^- was evaluated ($F=12.054$; $p=0.002$) and there was no significant interaction ($F=3.321$; $p=0.083$). No other effect of percentage of annual crops ($F\leq2.963$; $p\geq0.099$) and no effects of atrazine ($F\leq1.044$; $p\geq0.318$) on descriptors of plant community structure were observed (Appendix B, Table B.4).

The effect of NO_3^- was examined further for the aquatic macrophyte community. A total of 67 obligate wetland species were identified in the aquatic macrophyte surveys. Of these species, 36 were emergents, 13 were floating-leaved and 18 were submerged species. The number of aquatic species at each site ranged from 4-38 (average 15). The number of submerged species was similar between paired sites (Fig. 3.5A) but decreased with increasing NO_3^- at the watershed scale (Fig. 3.5C). In contrast, the relative frequency of submerged species was higher at low agriculture sites compared to high agriculture sites (Fig. 3.5B), whereas emergent species displayed the opposite trend (Appendix B; Table B.4). The relative frequency of submerged species was similar across the NO_3^- gradient (Fig.

3.5D). The number and relative frequency of floating-leaved species did not differ between paired sites or across the watershed (Appendix B; Table B.4). The number of emergent species was similar between sites and but declined as NO_3^- increased (Appendix B; Table B.4). Overall, the number of aquatic species was similar between paired sites and declined as NO_3^- increased (Appendix B; Table B.4).

Canonical correspondence analysis was used to further examine the influence of agricultural intensity on community structure, while normalizing for differences in physico-chemical characteristics between field sites. An initial CCA was conducted to determine which environmental variables influenced community structure. These variables were subsequently used as covariables in a partial CCA so that the influence of agricultural intensity could be assessed after accounting for physico-chemical differences between field sites. Only variables uncorrelated with agrochemical impact were considered for inclusion as covariables. Pearson's correlations indicated that bank slope, stream width, average depth, maximum depth, stream velocity, temperature, turbidity and chlorophyll *a* were the only physical and chemical variables not significantly correlated with NO_3^-, atrazine or percentage of annual crops. Temperature was excluded from the analysis because it was not thought to be a driver of community structure at the watershed scale and maximum depth was excluded because average depth explained slightly more variation. Bank slope, stream width, average depth, stream velocity, turbidity and chlorophyll *a* were included in the initial CCA as environmental variables and forward regression followed by Monte Carlo permutations were used to evaluate the significance of the axes and variables (Fig. 3.6). Bank slope and stream width explained a significant amount of variation in species composition (Fig. 3.6) and were used as covariables in subsequent CCAs. Paired sites were

generally ordinated close together (Fig. 3.6). A CCA was conducted to illustrate the relationship between measures of agrochemical impact and correlated environmental variables, with related variables orientated in the same direction (Fig. 3.7). Measures of agricultural intensity were positively associated with a number of environmental factors including turbidity, stream order, conductivity, pH and dissolved oxygen as well as the percentages of silt and clay in bank soil (Fig. 3.7). The percentage of sand in bank soil was positively associated with the percentage of surrounding natural vegetation (Fig. 3.7). When a number of highly correlated variables were included in the analysis, atrazine concentration, the percentage of sand and stream order explained a significant amount of species variation (Fig. 3.7). Highly correlated variables were not included in the final CCA because the inclusion of multiple highly correlated variables masks the effects of agrochemicals and can lead to an unreliable constrained ordination model (Lepš and Šmilauer, 2003).

A partial CCA was conducted using bank slope and stream width as covariables and constrained to species composition variation explained by measures of agricultural intensity (Fig. 3.8). Axis 1 explained 9.2% of the species composition and Axis 2 explained 6.0% (Fig. 3.8). Along Axis 1 and 2, 46.6% and 30.9% of the species-environment relationship was explained (Fig. 3.8). Of the measures of agricultural intensity, only NO_3^- explained a significant amount of species variation and was primarily explained by Axis 1 (Fig. 3.8). It is interesting to note that atrazine was best explained by Axis 2 and is significant at the $p<0.1$ level (Fig. 3.8). Sites with low NO_3^- tended to have higher FQI values compared to high NO_3^- sites (Fig. 3.8). A number of species were positively or negatively associated with NO_3^- (Fig. 3.8; Table 3.5). Species positively associated with NO_3^- had lower CC values compared to species negatively associated with NO_3^- (Table 3.5). The exception was

the high CC emergent species, wild rice (*Zizania aquatica*) which was positively associated with NO_3^-. Other species positively associated with NO_3^- were riparian species, whereas species negatively associated with NO_3^- included a number of growth forms (Table 3.5).

3.5 Discussion

3.5.1 Agricultural impact

Land use

A clear gradient of agricultural impact was observed both between paired sites and across the watershed. The South Nation River watershed has a sparse human population, no major industry and little urban development. Agriculture, particularly the cultivation of annual crops, represented the major anthropogenic disturbance across the watershed. High agriculture, high NO_3^- sites had a higher percentage of surrounding annual crops and lower percentage of natural habitat. Although a gradient of land use was observed, the percentage of surrounding annual crops did not have direct effects on riparian and aquatic plant community structure.

Atrazine

Concentrations of atrazine were higher in 2008 samples measured with ELISA compared to 2010 samples, obtained from both active and passive sampling and measured with LC-MS/MS. While atrazine is the second most commonly used herbicide on corn crops in Ontario, its use in recent years has declined as glyphosate resistant corn crops have increased (McGee, et al., 2010; Environment Canada, 2011). In addition, ELISAs may overestimate atrazine concentrations due to cross-reactivity with atrazine metabolites and

similar pesticides (Abraxis LLC, 2010). Atrazine concentrations obtained with passive sampling were used in further analysis because they represented concentrations integrated over time rather than point-in-time estimates. Time-weighted-average atrazine concentrations (maximum 412 ng/L) were well below the Canadian water quality guidelines for the protection of aquatic life (1.8 μg/L) (Canadian Council of Ministers of the Environment, 1999). At these concentrations, atrazine is not likely to have direct toxic effects on riparian and aquatic plants (Solomon et al., 1996) and in the present study, no direct effects were detected. However, effects of pulses and chronic low concentrations on plant communities are not well established. Furthermore, indirect effects are plausible due to effects on phytoplankton communities at concentrations less than 5 μg/L (DeNoyelles et al., 1982; Pannard et al., 2009). It is notable that atrazine contamination was widespread across the watershed and despite the low concentrations observed, multivariate analysis provided evidence of subtle effects of atrazine on riparian and aquatic plant community structure.

Nitrogen and Phosphorus

Of the measures of agricultural impact, the main driver of change in plant community structure appeared to be NO_3^-. Dubrovsky et al. (2010) estimated background nutrient concentrations from stream sites located across the United States. Based on this assessment, nutrient concentrations were elevated above background concentrations (240 μg/L NO_3^- and 10 μg/L RP) in the South Nation River watershed, suggesting non-point source pollution from synthetic fertilizers and manure at a number of sites. However, both RP and TP were similar between paired sites indicating that annual crops did not lead to a systematic increase

in P at high agriculture sites. Concentrations of RP and TP tended to be lower in June compared to spring/summer concentration. In contrast, NO_3^- and TN concentrations were higher at high agriculture sites and higher in June following expected applications of synthetic nitrogen-based fertilizers. For high agriculture sites, the average NO_3^- concentration was 10× higher than the expected background concentration estimated by Dubrovsky et al. (2010). Elevated NO_3^- concentrations did not translate into changes in NH_3 + NH_4^+ or increases in DON+PON and these concentrations were similar between paired sites and time periods.

A number of similarities were observed between sites at both the tributary and watershed level. Low agriculture, low NO_3^- sites were associated with high percentages of surrounding natural vegetation and sandy soils, whereas high agriculture, high NO_3^- sites were associated with high percentages of annual crops and higher percentages of silt. Sandy soils retain less NO_3^- than loamy soils (Pedersen et al., 2009) and high agriculture sites tended to be located on loamy soils, which are ideal for crop growth. Paired sites were similar in stream order but at the watershed level, higher order sites tended to have higher NO_3^- concentrations because higher order streams drain a larger area. Atrazine was best explained by the secondary CCA axis and explained a significant amount of variation when a number of highly correlated environmental variables were included. However, the trend was no longer significant when highly correlated environmental variables were removed from the analysis. Turbidity increased with stream order and at downstream high agriculture sites as result of downstream transport of algae, particulate organic carbon and sediments. Increased dissolved oxygen and pH at high agriculture, high NO_3^- sites suggested there was increased photosynthesis, producing dissolved oxygen and increasing pH through uptake of

carbon dioxide. However, further research would be needed to verify that observed results were not due to a decrease in microbial respiration at high agriculture sites.

The lack of increased chlorophyll *a* at high agriculture sites, the overall low chlorophyll *a* concentrations in the watershed and the overall high N:P ratios suggest that suspended algae may be limited by phosphorus or environmental factors such as light availability in turbid waters. However, overall high concentrations of both nitrogen and phosphorus and evidence of increased primary production at high agriculture sites suggested that phosphorus and nitrogen were likely not limiting primary production, particularly of riparian and aquatic plants. Westlake (1981) estimated that macrophytes were not nutrient limited in rivers at >30 µg/L phosphate and >1000 µg/L NO_3^-, conditions observed in some but not all field sites. In general, aquatic macrophytes are thought to have lower nutrient requirements than phytoplankton due to their slower growth, ability to conserve nutrients and ability to access sediment nutrient pools (Sand-Jensen and Borum, 1991). McJannet et al. (1995) hypothesized that fast-growing annual wetland plants were able to efficiently exploit nutrients through rapid production of new tissues. In the present study, a possible mechanism to explain observed changes in riparian and aquatic plant community structure is that nutrient enrichment leads to a shift in community structure towards species best able to exploit nutrients.

3.5.2 Changes in riparian and aquatic plant communities

Species Richness

Species richness did not differ between paired sites or along a NO_3^- gradient across the watershed and was not a sensitive indicator of agricultural impact. Bowers and Boutin

(2008) also found that species richness was a not a sensitive indicator of disturbance for riparian plant species. Within a given tributary, species richness was governed by physical and chemical factors. On a broad geographical scale, aquatic macrophytes are influenced by factors such as temperature, precipitation, latitude and altitude (reviewed in Lacoul and Freedman, 2006). On smaller geographical scale, aquatic macrophytes are also influenced by the area of suitable habitat, light availability and substrate type (Lacoul and Freedman, 2006). In the present study, bank slope and stream width explained a significant amount of variation in riparian and aquatic plant community structure, regardless of agricultural impact. Bank slope varied across the watershed from flat banks to banks cut-away at 90°. Steep banks had more terrestrial vegetation and a sharp transition from terrestrial to aquatic vegetation, whereas flat banks had a more gradual transition with a larger area of wetland species. Stream width was also an important factor in structuring plant communities with wider sites having a larger area for colonization. In contrast, Dybkjær et al. (2012) found the number of plant communities increased with stream depth and not width. However, both factors serve as indirect measures of stream size.

The Intermediate Disturbance Hypothesis predicts high species richness at medium levels of disturbance (Connell, 1978). Although the watershed had a gradient of disturbance, the disturbance was not large enough to eliminate species. No site was completely undisturbed, with all sites exposed to some degree of disturbance in the form of seasonal changes in hydrology. However, individual species were replaced by other species as disturbance from agriculture (NO_3^-) increased. Sorenson coefficients indicated that paired sites had more species uncommon than common to each other and evidence of specific changes in community structure are presented below.

Loss of submerged macrophytes

The number of submerged macrophytes was similar within paired tributaries but declined across the watershed as NO_3^- increased. Similarly to overall species richness, the number of macrophytes within a tributary was likely governed by its relative level of disturbance compared to other tributaries and by a number of physical and chemical factors. Within tributaries, while the absolute number of submerged macrophytes did not differ significantly between paired sites, the relative frequency of submerged macrophytes was lower in high agriculture sites. These observations introduce a central issue of pattern and scale in ecology. Ecological phenomena vary on a range of spatial, temporal and organizational scales and mechanisms underlying patterns may operate on scales different from those in which a pattern was observed (Levin, 1992). By examining community structure at both the tributary and watershed scale, a decline in the relative frequency of submerged macrophytes as well as a decline in the number of submerged macrophytes with increasing NO_3^- was observed. These results are in agreement with studies that concluded that nutrient enrichment was a significant factor in the decline submerged macrophyte diversity in European (Sand-Jensen et al., 2000; Riis and Sand-Jensen, 2001; Körner, 2002; Hilt et al., 2013; Steffen et al., 2013) and North American (Egertson et al., 2004) waters. The loss of submerged macrophytes has been attributed to light limitation from eutrophication (Phillips et al., 1978; Irvine et al., 1989; Scheffer et al., 1993; reviewed in Hilton et al., 2006) but could also be related to increased turbidity at high agriculture sites from increases in soil erosion. At the tributary level, fast growing emergent species may have been better able to take advantage of increased nutrients at high agriculture sites.

However at the watershed level, an overall decline in the number of aquatic species was observed with increasing NO_3^-.

Percentage of non-native species

Although the percentage of non-native species tended to be higher at high agriculture sites, the trend was not significant. Dispersal of seeds by wind and dispersal of seeds and vegetative structures through water facilitates the spread of both non-native and native species throughout a tributary. A number of non-native species such as *Bromus inermis*, *Lythrum salicaria* and *Phalaris arundinacea* (*P. arundinacea* is represented by both native and non-native ecotypes that can only be differentiated with molecular techniques) were widespread through the watershed and present in at least 75% of all sites. Both *L. salicaria* and *P. arundinacea* have become dominant in many North American wetlands and are capable of reducing diversity (Schooler et al., 2006). The tributaries differed in their overall level of disturbance and the percentage of non-native species increased with increasing NO_3^-. Non-native species commonly possess increased vigour and reduced herbivory (reviewed in Bossdorf et al. 2005) and are more likely to be short-lived (annuals and biennials) (Sutherland, 2004). Short-lived species are characterized by rapid population growth and short lag-times during succession (Meiners, 2007), with both early and mid sucessional stages being sensitive to invasions by non-native species due to their strength as both colonizers and competitors (Catford et al., 2012). These characteristics make non-native species suited to invade disturbed, nutrient enriched sites.

Changes in community structure and floristic quality

In addition to changes in percentage of non-native species, other significant changes in the riparian and aquatic plant community structure were observed. While the overall floristic quality index was similar between paired sites, there was a significant decline in FQI across the watershed as NO_3^- increased. Species with high CC values are associated with minor disturbance, whereas species with low CC values are associated with disturbed sites (Oldham et al., 1995). Species positively associated with NO_3^- generally had low or negative CC values and were all riparian species. One important exception was the emergent *Zizania aquatica* which was positively associated with nitrate and has a high CC value. However, this species is sown annually in the South Nation River watershed as part of a restoration program (Pat Piitz, South Nation Conservation Authority, personal communication). Similarly to some weedy species, *Z. aquatica* is a fast growing annual and resistant to shading due to its height. Species negatively associated with NO_3^- had higher CC values compared to species positively associated with NO_3^- and were composed of a range of growth forms, included submerged. The present study demonstrated that in lieu of metrics specifically designed to detect eutrophication, such as those developed in Europe (e.g. Haury et al., 2006; Holmes et al., 1999), the floristic quality assessment system can be a useful tool for evaluating agrochemical effects on riparian and aquatic plant communities. The system was more sensitive to agricultural impact than species richness and provided more comprehensive information than percentage of non-native species.

3.6 Conclusions

Among the measures of agricultural impact, only nitrate (NO_3^-) appeared to have a significant effect on riparian and aquatic plant community structure. Although, a number of measures of agricultural impact were correlated, there was no evidence of direct impacts of atrazine or the percentage of surrounding annual crops on plant communities. However, atrazine contamination was widespread throughout the watershed and it is notable that a subtle influence of atrazine on riparian and aquatic plants was observed ($p<0.1$) despite the low concentrations detected. Nitrate had no effect on species richness. However, the percentage of non-native species increased and both the number of submerged macrophytes and overall the floristic quality index (FQI) of field sites decreased along a gradient of increasing NO_3^-. At the tributary level, species more commonly found at low agriculture sites had higher coefficient of conservation (CC) values than those more commonly found at high agriculture sites. At the watershed level, species positively associated with NO_3^- had low or negative CC values whereas species negatively associated with NO_3^- had higher CC values. Overall, this study provided evidence that NO_3^- is a factor in reducing the quality of riparian and aquatic plant communities.

3.7 Acknowledgements

This research was funded by grants to F. R. Pick and C. Boutin from the Natural Sciences and Engineering Research Council of Canada and to C. Boutin from Environment Canada. We thank David Carpenter and Kirk Bowers for field and plant identification assistance, Philippe Thomas for field assistance and analysis of GIS land use data and Elias Collette, Daniel Gregoire, Bettina Henkelman and Andrea White for field assistance.

Table 3.1. Physical and chemical characteristics of 12 paired (24 in total) stream/ river sites in the South Nation River watershed, Canada. Statistics in **bold** are significant at $p \leq 0.05$

Variable	Units	Average ± standard deviation (range in brackets) Low agriculture sites	Average ± standard deviation (range in brackets) High agriculture sites	n	Paired t-test comparing average values (df=11)
*Bank characteristic*s					
Bank slope	%	41.8 ± 25.0 (0.0-100)	52.9 ± 31.8 (1.8-100)	144	t=-1.098; p=0.296
Bank soil structure	% sand	56.6 ± 14.4 (35.3-73.2)	46.5 ± 9.6 (27.2-64.2)	48	**t=2.928; p=0.014**
	% clay	14.3 ± 6.2 (6.0-26.5)	18.3 ± 5.9 (10.8-27.4)		t=-1.922; p=0.081
	% silt	29.1 ± 10.5 (14.6-46.6)	35.3 ± 6.7 (24.6-46.6)		**t=-2.873; p=0.015**
	class	sandy loam	loam		
Physical stream characteristics					
Strahler stream order	n/a	4.5 ± 0.90 (3-6)	4.7 ± 0.89 (4-6)	24	Wilcoxon p=0.157[a]
Stream width	m	14.2 ± 7.6 (4.7-32.6)	16.5 ± 11.2 (5.6-48.5)	72	t=-0.919; p=0.378
Average baseflow depth	cm	62 ± 29 (32-114)	63 ± 47 (10-183)	432	t=-0.075; p=0.942
Maximum baseflow depth	cm	82 ± 41 (40-173)	84 ± 65 (14-248)	144	t=-0.137; p=0.893
Surface baseflow velocity	m/s	0.071 ± 0.057 (0.008-0.179)	0.080 ± 0.052 (0.028-0.166)	144	t=-0.389; p=0.705
Water chemistry					
pH	n/a	7.85 ± 0.25 (7.45-8.11)	8.00 ± 0.15 (7.75-8.34)	192	**t=-2.515; p=0.029**
Dissolved oxygen	mg/L	6.49 ± 1.62 (3.07-8.14)	7.81 ± 1.30 (6.21-10.60)	192	**t=-2.503; p=0.029**
Temperature	°C	20.93 ± 1.13 (19.61-22.71)	21.48 ± 1.20 (19.50-23.31)	192	t=-1.814; p=0.097
Conductivity	µS/cm	514.6 ± 123.2 (359.7-738.0)	520.3 ± 84.8 (373.8-628.2)	192	t=-0.386; p=0.707
Turbidity	NTU	8.7 ± 6.4 (2.2-19.1)	15.0 ± 8.4 (2.4-33.1)	336	**t=-3.574; p=0.004**
Chlorophyll *a*	µg/L	6.5 ± 2.5 (3.5-11.3)	7.4 ± 2.5 (4.3-11.2)	336	t=-0.976; p=0.350

[a]Non-parametric Wilcoxon signed rank test p-value

Table 3.2. Land use in 500 m radius areas surrounding 12 paired (24 in total) stream/ river sites in the South Nation River watershed, Canada. Statistics in **bold** are significant at $p \leq 0.05$

Land use	Low agriculture sites	High agriculture sites	Paired t-test (df=11)
Annual crops (%)	30.2 ± 22.7 (6.7-83.9)	67.0 ± 15.8 (44.3-97.4)	**t=-4.865; p<0.001**
Perennial crops and pasture (%)	19.5 ± 14.3 (1.2-54.2)	17.7 ± 10.7 (1.5-35.2)	t=0.564; p=0.584
Natural habitat (%)[a]	48.7 ± 22.1 (3.5-77.8)	14.0 ± 9.7 (1.1-27.2)	**t=4.405; p=0.001**
Developed land (%)[b]	1.5 ± 1.6 (0.0-3.5)	1.3 ± 1.9 (0.0-4.6)	t=0.362; p=0.724

[a]Composed of forest, wetland, grassland, shrub land, water, rock, soil and sediments

[b]Composed of suburban development, roads, buildings, parks, farmsteads, golf courses

Table 3.3 Concentrations of major nutrient forms and their ratios across field sites. Statistics in **bold** are significant at $p \leq 0.05$

Nutrient Form	Abbreviation	Units	Time period	Low agriculture sites	High agriculture sites	Paired t-test (df=11)
Reactive phosphate	RP	µg/L	June	29 ± 14 (11-49)	32 ± 16 (6-65)	t=-0.966; p=0.355
			Spring/Summer	42 ± 26 (16-99)	48 ± 24 (13-97)	t=-1.141; p=0.278
Total phosphorus	TP	µg/L	June	52 ± 17 (30-82)	55 ± 15 (25-79)	t=-0.684; p=0.508
			Spring/Summer	70 ± 31 (40-131)	77 ± 31 (35-142)	t=-0.794; p=0.444
Ammonia + Ammonium	$NH_3 + NH_4^+$	µg/L	June	48 ± 31 (12-103)	41 ± 19 (10-71)	t=0.712; p=0.491
			Spring/Summer	49 ± 25 (14-93)	47 ± 21 (10-82)	t=0.166; p=0.871
Nitrate	NO_3^-	µg/L	June	1333 ± 1375 (3-3889)	2494 ± 1385 (137-3981)	**Wilcoxon p=0.002** [a]
			Spring/Summer	682 ± 679 (4-1850)	1368 ± 814 (82-2562)	**Wilcoxon p=0.002** [a]
Dissolved + particulate organic nitrogen	DON+PON	µg/L	June	882 ± 160 (577-1110)	809 ± 160 (523-1036)	t=1.467; p=0.171
			Spring/Summer	896 ± 175 (648-1138)	856 ± 182 (609-1153)	t=0.764; p=0.461
Total nitrogen	TN	µg/L	June	2278 ± 1297 (882-4692)	3367 ± 1325 (1064-4701)	**Wilcoxon p=0.005** [a]
			Spring/Summer	1636 ± 590 (941-2690)	2286 ± 758 (1293-3610)	**Wilcoxon p=0.006** [a]
Dissolved inorganic nitrogen: reactive phosphate	DIN: RP	n/a	June	57 ± 63 (1-220)	123 ± 122 (6-450)	**Wilcoxon p=0.010** [a]
			Spring/Summer	31 ± 31 (3-108)	63 ± 52 (4-183)	**Wilcoxon p=0.010** [a]
Total nitrogen: phosphorus	TN: TP	n/a	June	47 ± 25 (21-99)	69 ± 37 (20-135)	**Wilcoxon p=0.023** [a]
			Spring/Summer	32 ± 12 (17-56)	42 ± 20 (15-75)	t=-2.124; p=0.057

[a]Non-parametric Wilcoxon signed rank test p-value

Table 3.4 The ten[a] most common species, based on presence, at 24 sites within the South Nation River watershed. Latin names are followed by coefficients of conservation (CC) and the total number of sites (S) where the species was present. Species most commonly found at low and high agriculture sites are also shown along with CC values and the number of low (L) and high (H) agriculture sites where the species was present indicated following the latin name.

Species common to all sites	Species common to low agriculture sites	High > low agriculture
Acer negundo; CC0; S24	*Carex* sp.; CC7; L7>H2	*Artemisia vulgaris*; CC-1; H7>L3
Bidens frondosa; CC3; S22	*Equisetum fluviatile*; CC7; L7>H3	*Atriplex patula*; CC0; H8>L3
Galium palustre; CC5; S24	*Eutrochium maculatum*; CC3; L11>H4	*Cerastium fontanum*; CC-1; H5>L1
Impatiens capensis; CC4; S24	*Hydrocharis morsus-ranae*; CC-3; L5>H1	*Echinochloa muricata*; CC4; H4>L0
Leersia oryzoides; CC3; S24	*Lycopus americanus*; CC4; L11>H5	*Erysimum cheiranthoides*; CC-1; H12>L7
Lythrum salicaria; CC-3; S23	*Poa compressa*; CC0; L8>H4	*Rubus idaeus*; CC0; H6>L2
Phalaris arundinacea; CC0; S24	*Polygonum sagittatum*; CC5; L6>H2	*Sinapis arvensis*; CC-1; H6>L2
Pilea pumila; CC5; S24	*Potamogeton natans*; CC5; L7>H2	*Sonchus asper*; CC1; H7>L3
Plantago major; CC-1; S22	*Solidago canadensis*; CC1; L6>H1	*Sonchus oleraceus*; CC-1; H7>L3
Sagittaria latifolia; CC4; S22	*Thalictrum pubescens*; CC5; L6>H1	*Trifolium pratense*; CC-2; H5>L0
Urtica dioica; CC2; S22	*Verbena urticifolia*; CC4; L5>H1	
Vicia cracca; CC-1; S22		
Average CC 1.75	Average CC 3.45	Average CC -0.20

[a] Additional species were included when multiple species were present at the same number of sites

Table 3.5 Species positively and negatively associated with nitrate

Species	Acronym	Positive (+) or negative (-) regression coefficients	Coefficient of conservation (CC)	Growth form[a]
Ambrosia trifida	*At*	+	0	R
Arctium minus	*Am*	+	-2	R
Daucus carota	*Dc*	+	-2	R
Zizania aquatica	*Za*	+	9	E
Average +		+	1.3. with *Za*, -1.3 without *Za*	
Boehmeria cylindrica	*Bc*	-	4	R
Campanula aparinoides	*Ca*	-	7	R
Ceratophyllum demersum	*Cd*	-	4	S
Elodea canadensis	*Ec*	-	4	S
Equisetum fluviatile	*Ef*	-	7	E
Ludwigia palustris	*Lp*	-	5	E
Nymphaea odorata	*No*	-	5	F
Onoclea sensibilis	*Os*	-	4	E
Potamogeton epihydrus	*Pe*	-	5	F/S
Schoenoplectus tabernaemontani	*St*	-	5	E
Symphyotrichum lanceolatum	*Sl*	-	3	R
Utricularia macrorhiza	*Um*	-	4	S
Average -		-	4.8	

[a] E- emergent, F- floating-leaved, R- riparian, S- submerged, F/S- both floating-leaved and submerged forms

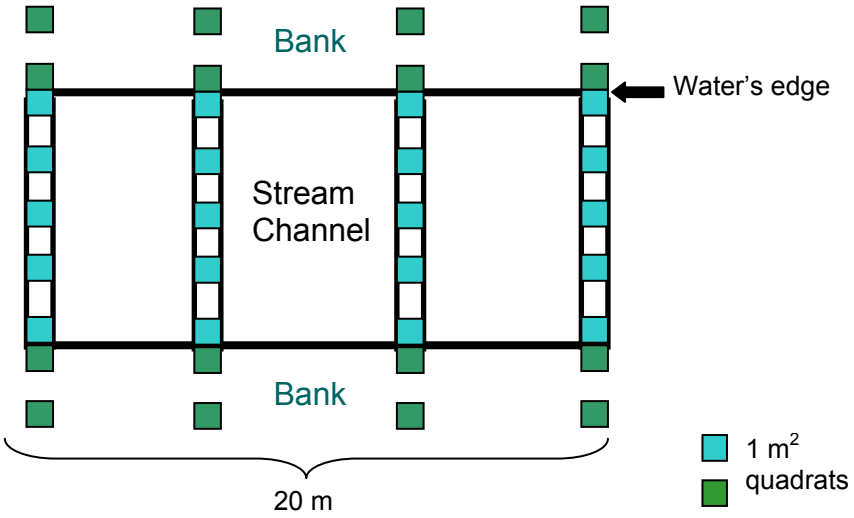

Fig. 3.1 Schematic view of a typical sampling plan used to survey riparian and aquatic plants in the South Nation River watershed, Canada.

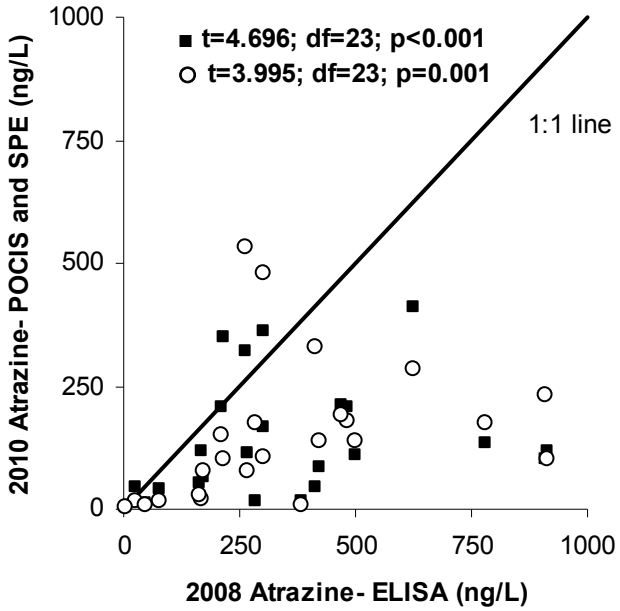

Fig. 3.2 Comparison of atrazine concentrations measured via enzyme linked immunosorbent assays (ELISAs) in June 2008 and by LC-MS/MS analyses of samples obtained with ○ active sampling in June 2010 followed by solid phase extraction (SPE) and ■ passive sampling with polar organic chemical integrative samplers (POCIS) over a 56 d period from mid May to mid July 2010.

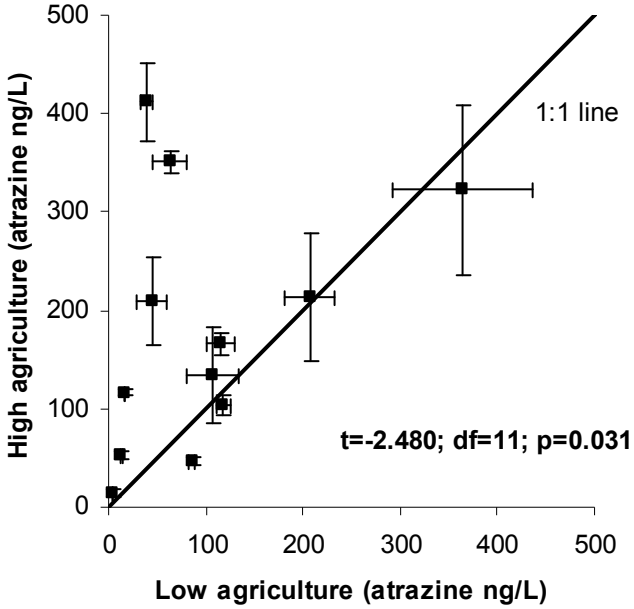

Fig. 3.3 Comparison of 56 d time-weighted-average concentrations of atrazine obtained using passive sampling at 12 paired sites (24 in total) surrounded by low and high agriculture. Averages (n=3) ± standard deviation are shown.

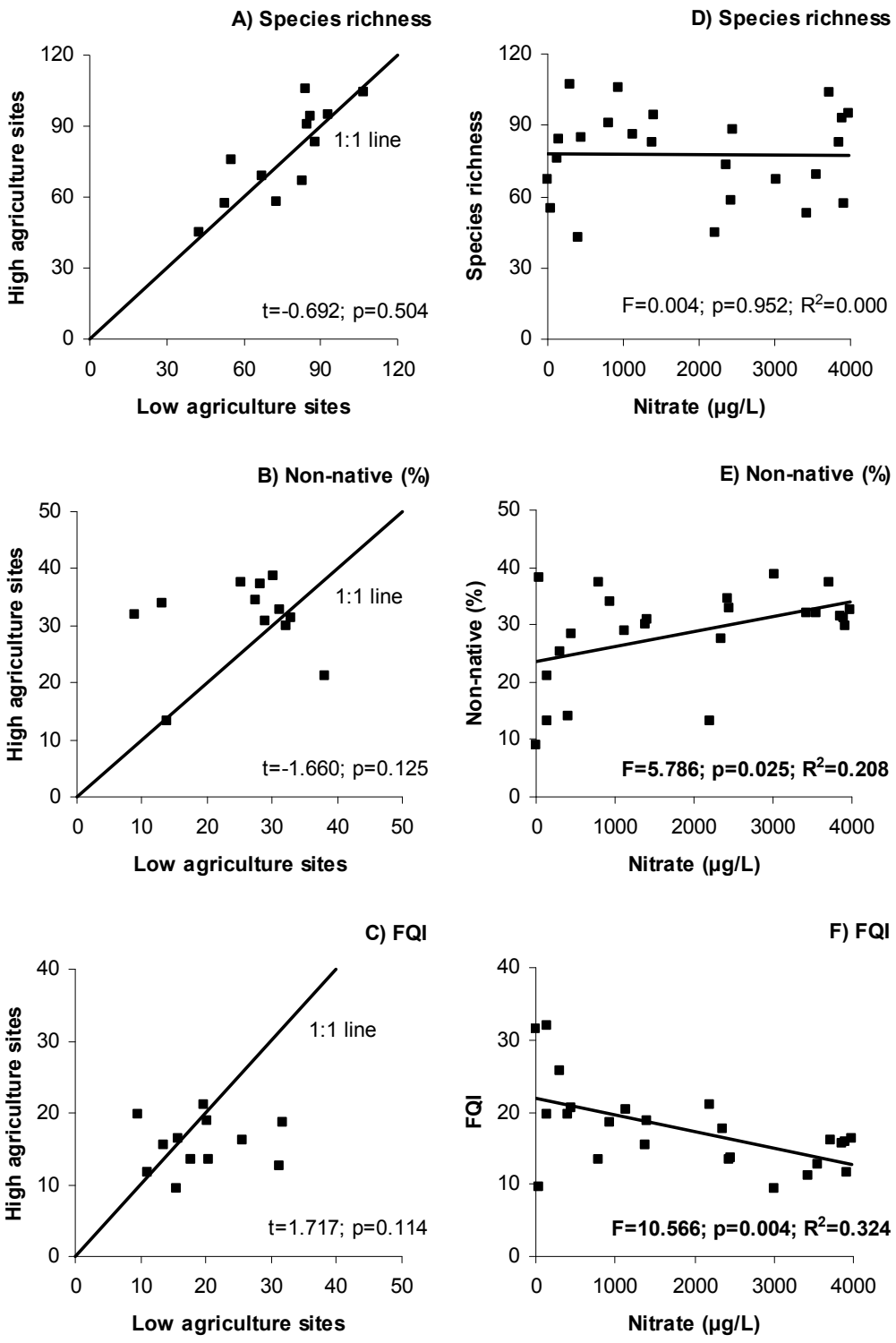

Fig. 3.4 Comparison of plant communities between sites surrounded by A-C) low and high levels of agriculture (paired t-tests, df=11) and D-F) across the watershed along a gradient of nitrate contamination (linear regression, df=23). FQI- floristic quality index.

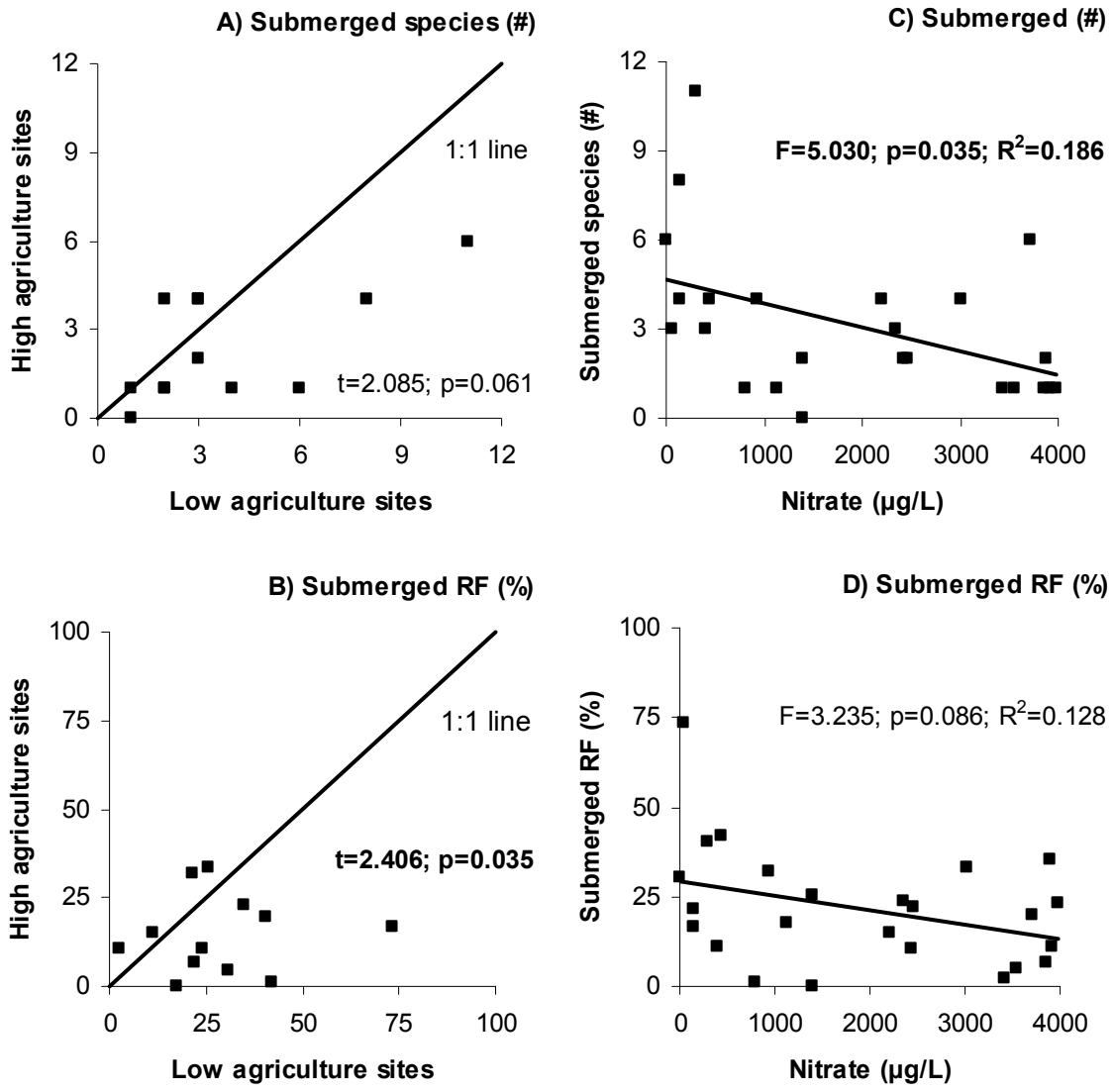

Fig. 3.5 Comparison of submerged species between sites surrounded by A-B) low and high levels of agriculture (paired t-tests, df=11) and C-D) across the watershed along a gradient of nitrate contamination (linear regression, df=23). RF- relative frequency

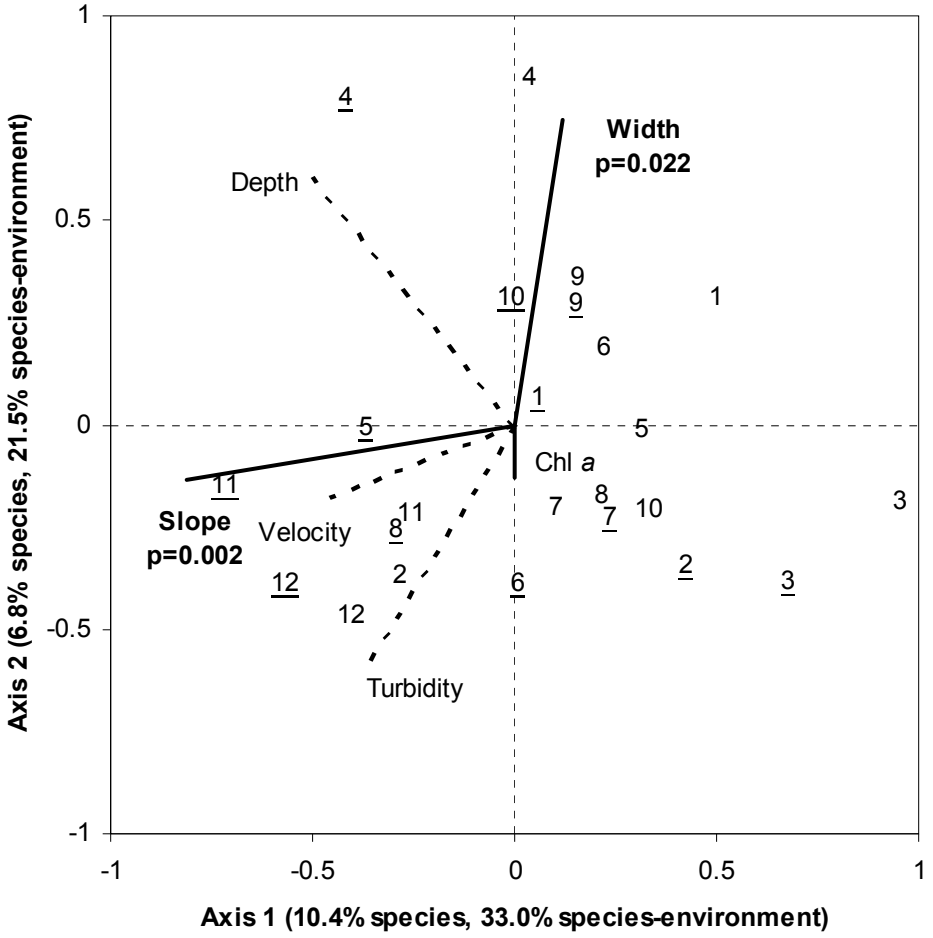

Fig. 3.6 Canonical correspondence analysis (CCA) ordinating field sites by species variation explainable by environmental variables uncorrelated to agrochemicals (bank slope, stream width, average depth, velocity and turbidity). Twelve paired sites (24 in total) are shown with paired sites represented by the same number and high agriculture sites underlined.

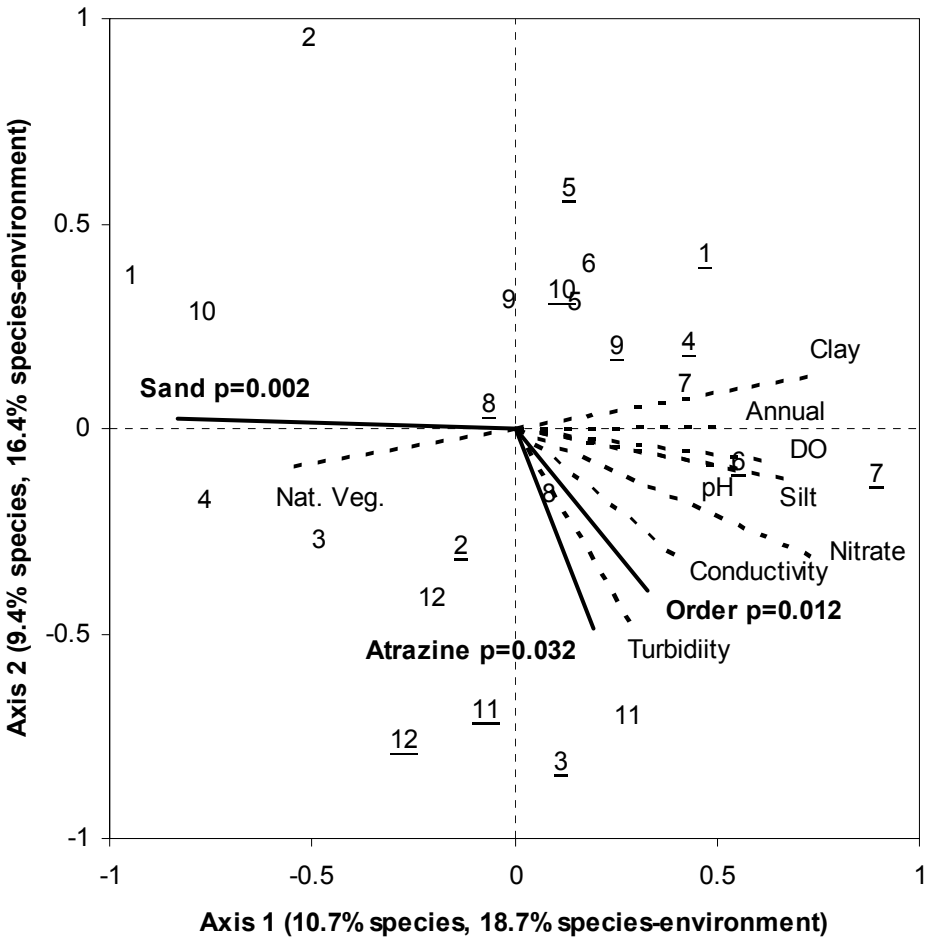

Fig. 3.7 Partial canonical correspondence analysis (partial CCA) illustrating species variation explainable by measures of agrochemical impact and correlated environmental variables after accounting for the covariables, slope and width. Twelve paired sites (24 in total) are shown with pairs represented by the same number and high agriculture sites underlined.

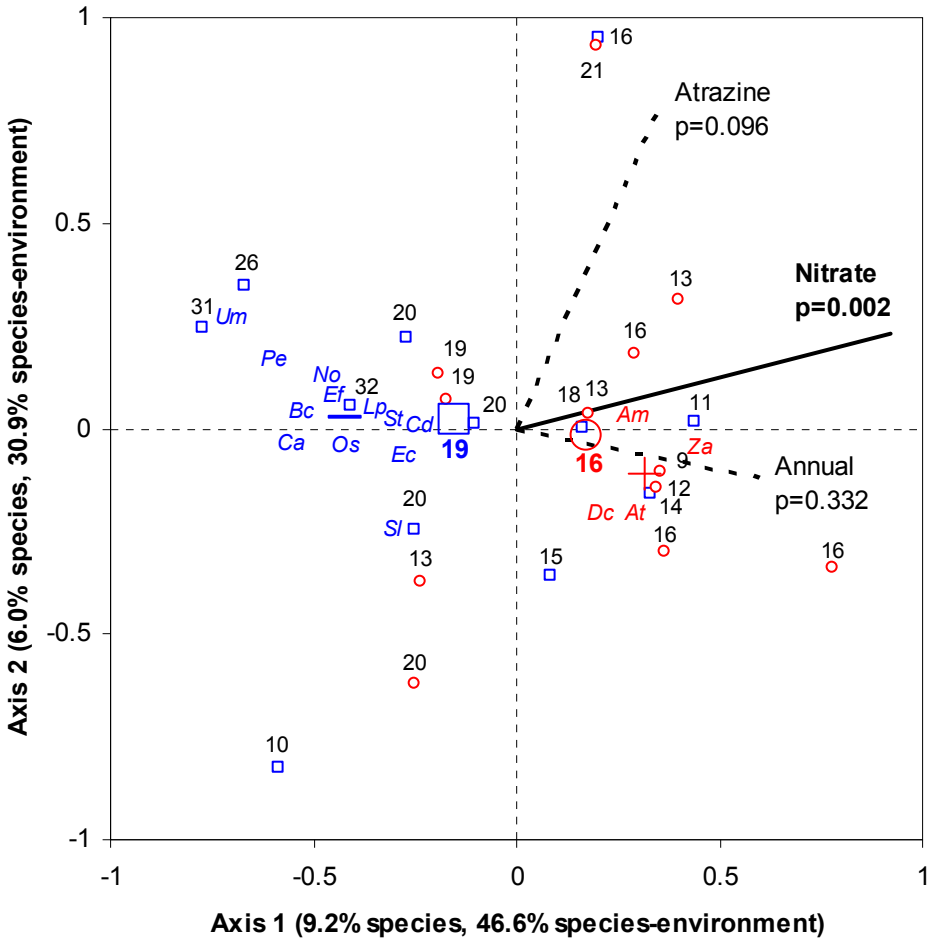

Fig. 3.8 Partial canonical correspondence analysis (partial CCA) ordinating species composition and constrained to variation explained by agrochemical impact (nitrate, atrazine, percentage annual crops) using bank slope and stream width as covariables. Field sites (24 in total) were surrounded by low □ or high ○ levels of agriculture. The floristic quality index (FQI) of each site is located above each site's coordinates (designated as □ or ○). The average coordinates for the 12 low and 12 high agriculture sites were designated by larger symbols (□ or ○), with their corresponding average FQIs in bold. Four species were positively associated with nitrate (*At*, *Am*, *Dc* and *Za*, average coordinates +) and 12 species negatively associated with nitrate (*Bc*, *Ca*, *Cd*, *Ec*, *Ef*, *Lp*, *No*, *Os*, *Pe*, *St*, *Sl*, *Um*, average coordinates –). Species names are given in Table 3.5.

Chapter 4: Periphyton community responses to nutrient and herbicide gradients: experimental and empirical evidence

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4.1 Abstract

The objective of this study was to assess of the effects of nutrient enrichment and exposure to the herbicide atrazine on periphyton communities using experimental and empirical evidence. An HPLC-chemotaxonomic approach (CHEMTAX) was effective in classifying periphyton communities into the dominant taxonomic groups Bacillariophyta, Chlorophyta, Cryptophyta and Euglenophyta. Nutrient (20 mg/L nitrate and 1.25 mg/L phosphate) and atrazine (20 or 200 μg/L) diffusing periphytometers were deployed in two temperate streams for 14 d experimental periods. In 2008, the addition of nutrients, atrazine (20 μg/L) and a combination of both nutrients and atrazine had no effect on periphyton biomass or community structure. Similarly, few effects of nutrients, atrazine (200 μg/L) and a combination of both nutrients and atrazine were observed in 2009 experiments. In 2010, periphyton was colonized on artificial substrates at 24 sites located along a gradient of nutrient and atrazine contamination in an eastern Ontario agricultural watershed. Periphyton biomass increased with increasing nitrate while no direct effects of reactive phosphate or atrazine were observed. Periphyton communities were composed of Bacillariophyta (60.9%), Chlorophyta (28.1%), Cryptophyta (6.9%) and Euglenophyta (4.1%), with the Bacillariophyta associated with high turbidity and the Chlorophyta with high nitrate. Overall, effects of nitrate on periphyton biomass and community structure superseded effects of phosphate and atrazine.

Keywords: periphyton, nutrients, herbicide, environmental gradient, flowing waters

4.2 Introduction

Periphyton is a complex mixture of attached algae, microbes and detritus that plays a significant role in the synthesis of organic carbon in flowing waters. The growth of periphyton is controlled by physical factors such as hydrology, solar radiation and temperature; chemical factors such as nutrient availability; and disturbance factors such as changes in hydrodynamics and invertebrate grazing (Biggs, 1995; Biggs, 1996). As a result, the biomass of periphyton communities is highly variable and epilithic periphyton biomass can span up to six orders of magnitude from 0.01 to 10 0000 mg/m^2 chlorophyll *a* (Morin and Cattaneo, 1992). Despite the complexity of factors affecting periphyton communities, they are useful in ecotoxicological studies because they are sessile, have a high growth rate (Stevenson and Lowe, 1986) and are sensitive to environmental stressors. Algal communities have been used to assess stream condition as early as 1948 (Patrick, 1949) and diatom communities have since been used to evaluate effects of organic pollution (Descy and Coste, 1991), acidification (Steinberg and Putz, 1991) and eutrophication (Sabater et al., 1996).

In agricultural watersheds, primary producer communities, including periphyton, may be altered by exposure to herbicides and nutrients from fertilizers. Previous studies have shown shifts in periphyton communities under nutrient rich, eutrophic conditions. For example, Chételat et al. (1999) found a shift in dominance of green algal taxa from *Spirogyra, Oedogonium* and *Coleochaete* at low total phosphorus (TP) concentrations to *Cladophora* above 20 µg/L TP. Dominance of the filamentous green alga *Cladophora* has also been associated with high ammonium (NH_4^+) (Dodds, 1991). Winter and Duthie (2000) found that patterns of epilithic diatom species distribution were related to total

nitrogen (TN) and TP concentrations and could be used to indicate eutrophic conditions. However, Nelson et al. (2013) found no consistent response to nutrient augmentation when species were organized into the dominant taxonomic groups, Bacillariophyta, Chlorophyta and Cyanophyta.

Effects of pesticides on the Bacillariophyta, Chlorophyta and Cyanophyta have also been examined. A study of 23 commonly used pesticides found that triazine herbicides, such as atrazine, were highly phototoxic and that considerable differences occurred in the sensitivity of algal test species to pesticides (Peterson et al., 1994). Exposure to herbicides appears to favour Bacillariophyta-dominated periphyton communities. Short-term toxicity tests demonstrated that green algae were severely affected by exposure to the herbicides metribuzin, hexazinone, isoproturon and pendimethalin and did not recover, whereas diatoms and cyanobacteria recovered from herbicide exposure (Gustavson et al., 2003). Similarly, Guasch et al. (1998) and Dorigo et al. (2004) suggested that exposure to the herbicide atrazine shifted algal communities to dominance in diatom species that were less sensitive to atrazine and organic pollution compared to green algae. However, it was unclear from these field-based studies whether the observed changes in community structure were attributed solely to atrazine or also to correlated factors such as high nutrient concentrations and turbidity.

Assessment of the effects of herbicides and nutrients on stream periphyton is challenging because exposure to herbicides is difficult to quantify and it is often unclear which nutrients, if any, are limiting in streams. Concentrations of herbicides are highly variable with peak concentrations occurring in pulses following rain events. Passive sampling techniques, such as the polar organic chemical integrative sampler (POCIS)

(Alvarez et al., 2004) may be used to provide time-weighted-average concentrations that better estimate periphyton exposure to herbicides. Nutrient limitation may be assessed by examining the effects of experimental nutrient enrichment using several techniques. Keck and Lepori (2012) analyzed data from 382 studies that used nutrient-diffusing substrates, flow-through systems and periphytometers to assess effects of nutrient-enrichment on periphyton. Periphytometers may be particularly suitable to examine effects of both nutrient enrichment and herbicide contamination. The devices consist of a solution reservoir, a membrane to reduce microbial growth in the solution reservoir and a glass fibre filter that functions as substrate for periphyton colonization (Matlock et al., 1998). Periphytometers may be modified to allow injection of herbicides following a period of growth (Kish, 2006; Fig. 4.1).

Traditionally, algal communities have been enumerated with microscopy but more recently chemotaxonomic methods have been used as an alternative to microscopy. These methods, coupled with improvements in high performance liquid chromatography (HPLC) methods of pigment identification and quantitation, substantially reduce the time required to process samples, as well as the need for the considerable taxonomic expertise required to accurately identify diverse periphyton assemblages. Algal pigments, particularly the carotenoids and xanthophylls, vary among major algal groups with some marker pigments unique to particular groups (reviewed in Jeffrey et al., 2011). CHEMTAX was developed to calculate algal class abundances from concentration ratios of marker pigments to chlorophyll *a* quantified using HPLC (Mackey et al., 1996). CHEMTAX is an iterative approach that uses factor analysis and a steepest descent algorithm to determine the best fit of pigment data to algal classes based on an initial pigment ratio matrix (Mackey et al.,

1996). The program was initially developed for classification of phytoplankton in the Southern Ocean (Mackey et al., 1996) and has been used primarily for classification of marine phytoplankton to date (Higgins et al., 2011 and references therein). Few studies have used CHEMTAX for classification of freshwater periphyton communities. However, Lauridsen et al. (2011) recently demonstrated that the CHEMTAX approach was suitable for quantifying benthic diatom communities in three Danish streams and three lakes.

The objective of the present study was to assess effects of high nutrient concentrations and exposure to the herbicide atrazine on periphyton community structure using experimental and empirical approaches. Atrazine is of particular interest because of its widespread usage on North American corn crops, frequent detection in surface and ground waters and concerns over its toxicity, mobility and persistence (Solomon et al., 1996; Gilliom et al., 2006). In the experimental portion of the study, periphytometers were used to expose natural communities of periphyton to nutrients and atrazine *in-situ* in two streams. Nutrients additions were expected to increase periphyton biomass and result in a Chlorophyta-dominated community if nutrients were previously limiting. Atrazine was predicted to decrease biomass and result in a Bacillariophyta-dominated community. Effects of atrazine were predicted to supersede those of nutrients in streams where nutrients were not limiting. In the empirical portion of the study, artificial substrates were used to colonize periphyton at 24 sites located throughout an agricultural watershed to compare community structure across existing gradients of nutrient and atrazine exposure. Sites highly enriched with nutrients were predicted to be dominated by Chlorophyta and sites highly contaminated by atrazine were predicated to be dominated by Bacillariophyta. *A priori* it was unclear

whether environmental concentrations of atrazine would be high enough to result in observable changes in community structure.

4.3 Materials and Methods

4.3.1 Study area

The South Nation River watershed comprises 3915 km^2 in Eastern Ontario, Canada and has a historical (1915-2011) average annual discharge of 44.3 m^3/s at its mouth (Environment Canada, 2013). The headwaters commence near the St. Lawrence River (44°40'41"N, 75°41'58"W) and the river flows north-easterly across a flat, poorly drained landscape for 177 km until its confluence with the Ottawa River (45°34'24"N, 75°06'00"W). The watershed is predominately agricultural with crops of corn (*Zea mays* L.) and soybean (*Glycine max* L. (Merr.)) typically planted in tile-drained fields.

Twenty-four sites located throughout the South Nation River watershed were selected for study (Fig. 2.1). Sites were paired along a given tributary with sites surrounded by low levels of agriculture located upstream of sites surrounded by high levels of agriculture. Sites were selected using land use data to identify areas of low and high agriculture (Statistics Canada, 2006), using Google Earth v.4.2.0198.2451 (Google Inc., Mountain View, USA) to verify physical aspects and through field reconnaissance of potential sites. The average distance between paired sites was 9.0 ± 8.5 km (ranging from 1.5 to 33.7 km). Two pairs of sites were located along different tributaries due to a lack of accessible and suitable sites. Pair 1 sites were 80.4 km apart and pair 6 sites were 10.7 km apart (Fig. 2.1). All sites were matched as closely as possible in terms of visible features such as steam width, bank slope and canopy cover. The 12 pairs were not hydrologically connected to each other except: 1) low agriculture site 1 was upstream of pair 4 sites along

the main branch of the South Nation River and 2) high agriculture site 1 and pair 10 sites were upstream of pair 11 sites along the Scotch River (Fig. 2.1). Each site was defined by a 20 m stream length.

4.3.2 Periphytometer experiment

Passive-diffusion periphytometers were deployed in the South Castor River (45º12'51.322"N 75º25'23.120"W) in 2008 and in both the South Castor River and North Branch of the South Nation River (45°1'24.550"N 75°29'19.780"W) in 2009 (Table 4.1). Periphytometers were modified from Matlock et al. (1998) and Kish (2006) (Fig. 4.1). Each periphytometer consisted of a 250 mL Nalgene® amber high density polyethylene solution reservoir with a 2.54 cm diameter hole cut in the cap (Fisher Scientific, Ottawa, Canada). A 47 mm 0.45 μm nylon membrane (Whatman, Mississauga, Canada) was used to reduce microbial growth in the solution reservoir and a 47 mm diameter 1.5 μm Whatman glass fibre filter (type 934-AH) was used as a substrate for periphyton growth. A 1.27 cm diameter hole was drilled in the bottom of each bottle, fitted with a silicone recessed septum stopper (Fisher Scientific) and sealed with silicone aquarium sealant so that atrazine could be injected into the periphytometers following a period of growth (Fig. 4.1).

Each experiment consisted of four treatments:

1. Control (C): distilled water
2. Nutrients (NUT: 20 mg/L N-nitrate and 1.25 mg/L P-phosphate)
3. Atrazine (ATR): 20 or 200 μg/L atrazine
4. Nutrients + Atrazine (N+A): as described above

The nutrient treatment was prepared from anhydrous sodium nitrate ($NaNO_3$) and disodium hydrogen phosphate heptahydrate (Na_2HPO_4-$7H_2O$) (Sigma-Aldrich, Oakville,

Canada). The atrazine treatment was prepared using the commercial herbicide formulation, Atrazine 480 (United Agri Products Canada Inc., Dorchester, Canada), containing 451 g/L of the active ingredient atrazine (6-chloro-*N*-ethyl-*N*′-1-methylethyl-1,3,5-triazine-2,4-diamine), and 29 g/L of related triazines. Atrazine was either incorporated into the initial solution or injected into each bottle 7 d following deployment (Table 4.1). Both the nutrient and atrazine solutions were dissolved in distilled water. Each experiment (Table 4.1) was composed of four treatments and there were five replicates of each treatment in 2008 (n=20) and six replicates in 2009 (n=24). Additional periphytometers were assembled, deployed, conductivity monitored and the filters subsequently preserved for algal counts but these samples are not discussed in this chapter.

Periphytometers were assembled in the lab on day 0 of each experiment. The caps were sealed with parafilm and the periphytometers transported to the field in coolers. Once at each field site, either a 1484 ×1081µm grid aluminum screen (2008) or 500 × 500 µm grid Nitex® nylon screen (Wildlife Supply Company, Yulee, USA) (2009) was placed over each cap and secured with cable ties to protect the glass fibre filters from grazers and excessive scouring. The periphytometers were arranged in a randomized block design on floating structures, consisting of 140 × 140 cm PVC frames (7.62 cm diameter PVC) supporting 5.08 × 10.16 cm grid galvanized steel fencing and secured in the stream channel with four cement cinder blocks. Each structure was capable of supporting 48 periphytometers (Fig. 4.1). Periphytometers were oriented parallel to the stream current with the glass fibre filters perpendicular to the water's surface (Matlock et al., 1998).

Periphytometers were retrieved after a period of 14 d. In 2008, periphytometers were also deployed for a 29 d period with a subset of duplicate periphytometers retrieved every

three or four days to monitor periphyton growth as well as nutrient and atrazine diffusion over time. Following each experimental period, glass fibre filters were carefully removed from the bottles and frozen at -30°C until extraction of pigments. Nutrients and atrazine were measured from a subset of duplicate samples on day 0, 7 and 14 of each experiment. For nutrient analysis, solutions in periphytometer reservoirs were transferred to 300 mL PET bottles. For atrazine analysis, 20 mL sub-samples were frozen and stored at -30°C in 40 mL pre-cleaned amber borosilicate vials with PTFE caps until analysis.

4.3.3 Periphyton survey

In 2010, periphyton was collected from 24 sites located throughout the South Nation River watershed. Periphyton was colonized on white high density polyethylene shields used to protect polar organic chemical integrative samplers (POCIS) (Fig. 2.2). Each shield had a total surface area of 372 cm^2. The shields were scuffed with sand paper before deployment to make the surface more suitable for periphyton growth. POCIS were deployed at each field site for a total of 56 d between 18 May and 22 July 2010. Accumulated periphyton was removed from the shields with a nylon brush after 28 d and then allowed to re-colonize the shields for another 28 d. At each site, one upstream and one downstream facing shield was collected. Upon retrieval, the shields were placed in individual polyethylene bags and stored in a freezer at -30°C in darkness until processing. Periphyton was removed from the shields with a plastic putty knife and nylon brush. The material was collected into a plastic tray by rinsing the shield, knife and brush with tap water. Each sample was brought to a final volume of 350 mL and homogenized in a blender. A 5-20 mL sub-sample was filtered through a 1.5 μm Whatman glass fiber filter (type 934-AH) and frozen at -30°C until

extraction of pigments. Triplicate sub-samples were processed for six shields, bringing the total number of samples to 60 (24 sites × 2 shields per site + 6 × 2 additional shields).

4.3.4 Physical and chemical characteristics of field sites

Strahler stream order was determined from data provided by the South Nation River Conservation Authority and Ontario Ministry of Natural Resources as part of the Water Resources Information Project (WRIP). Data were produced by the South Nation Conservation Authority under license with the Ontario Ministry of Natural Resources (© Queen's Printer, 2013). Stream width was measured in triplicate at each site along a 20 m stream length. Stream depth was measured in triplicate during base flow in August 2010. Maximum depth was measured mid-channel, while average depth was measured mid-channel as well as halfway between the mid-channel and each bank. Surface velocity was estimated by measuring the time for an orange wiffle golf ball to travel 1 m in triplicate in June and July 2010. Dissolved oxygen, pH, temperature and conductivity were measured with a HydroLab 4a Sonde (Hach Hydromet, Loveland, USA). Measurements were taken once in May, June and July 2010 at all sites. Duplicate mid-channel, integrated water samples were taken in 1 L Nalgene polypropylene bottles in May, June and July 2010 for turbidity and in-stream planktonic chlorophyll *a* analysis. All bottles were rinsed 3× with stream/river water at each site. Samples were taken using a pole sampler to collect water upstream of each canoe/wading location to avoid disturbance and contamination of water and sediments. Turbidity was measured with a portable turbidity meter (LaMotte, Chestertown, USA). Water samples (500 mL) for chlorophyll *a* analysis were filtered

through a 1.5 μm Whatman glass fiber filter and frozen at -30°C until extraction of algal pigments.

Field sites were characterized by in-stream nitrogen and phosphorus concentrations, with elevated concentrations representing agricultural contamination from synthetic fertilizers and manure (Dubrovsky et al., 2010). Mid-channel, integrated water samples were collected in 300 mL PET bottles for nutrient analysis in May, June and July 2010 with duplicate samples collected for approximately 10% of all samples. Time-weighted-average atrazine concentrations (56 d) were determined by passive sampling with polar organic chemical integrative samplers (POCIS), deployed in triplicate for two consecutive 28 d periods between 18 May and 22 July 2010 as described in Dalton et al. (2014).

4.3.5 Chemical analysis

Nutrients from periphytometers and in-stream sampling were analyzed at the Robert O. Pickard Environmental Centre Laboratory of the City of Ottawa (Canada) following established methods of the Ontario Ministry of the Environment (2007a, b). Nitrate (NO_3^-) and nitrite (NO_2^-) were measured with an ion chromatograph system (Dionex® DX100, Thermo Fisher Scientific Inc. Sunnyvale, USA). Reactive phosphate (RP), and ammonia + ammonium ($NH_3 + NH_4^+$) were measured with colorimetric assays at 880 nm and 630 nm respectively using an autoanalyzer (Skalar^{TM} 1070 Autoanalyzer, Skalar, Inc, Brampton, Canada). Total phosphorus (TP) and total Kjeldahl nitrogen (TKN) were converted to RP and NH_3 respectively following an acid digestion and analyzed as above. Dissolved inorganic nitrogen (DIN) was calculated as the sum of NO_3^-, NO_2^- and $NH_3 + NH_4^+$ and total nitrogen (TN) was calculated as the sum of TKN, $NO_3^-$and NO_2^-. Dissolved and particulate

organic nitrogen (DON+PON) were calculated as TKN minus NH_3 + NH_4^+. Method detection limits (MDLs) were 2, 3, 5, 20, 20 and 40 µg/L for RP, NH_3 + NH_4^+, TP, NO_3^-, TKN and NO_2^- respectively.

Atrazine from periphytometers and in-stream passive sampling was measured using LC-MS/MS analyses performed on a high performance liquid chromatograph hyphenated with a tandem mass spectrometer. For the periphytometer experiments, a sub-sample was taken from periphytometer solution reservoirs and diluted as required to give a final solution in 1:1 methanol:water. The diluted samples were filtered through a 0.45 µm PVDF membrane (Acrodisc®, Pall Canada Ltd., Mississauga, Canada). The injection volume was 10 µL with atrazine separated by an Atlantis® dC18 guard column (2.1 × 10 mm, average particle size 3 µm) (Waters, Mississauga, Canada) and Atlantis® dC_{18} analytical column (2.1 × 50 mm, average particle size 3 µm) (Waters) at a column temperature of 35°C. Atrazine periphytometer samples from 2008 were analyzed with a Waters Alliance 2995 HPLC (flow rate of 200 µL/min flow rate, mobile phase of 40% A: 10 mM ammonium acetate containing 0.05% formic acid and 60% B: methanol) coupled to a Quattro-Ultima triple quadropole tandem mass spectrometer (Waters) in multiple reaction monitoring (MRM) mode with positive electrospray ionization (ESI^+). Periphytometer samples from 2009 were analyzed with an Agilent 1200 HPLC (Agilent Technologies, Mississauga, Canada) (flow rate of 600 µL/min, isocratic mobile phase of 70% methanol and 30% 10 mM ammonium acetate containing 0.05% formic acid) coupled to an API 5000 Triple Quadropole Mass Spectrometer (AB Sciex, Concord, Canada) in MRM mode with ESI^+. Atrazine was monitored using the transition 216>174 for the 2008 subset of samples as well as 216>65 for all other samples. Atrazine was quantified using external calibration with a 6-point

calibration curve (0.5-20 pg/µL) for the 2008 subset and with an 8-point calibration curve (0.005-1.0 pg/µL) for all other samples (R^2>0.999). The 2010 atrazine POCIS samples were also analyzed with LC-MS/MS (Agilent HPLC 1200 series, Agilent Technologies coupled with a MS/MS (3200 QTRAP, AB Sciex). Details are described in Dalton et al. (2014).

4.3.6 Extraction and quantitation of algal pigments

In-stream concentrations of total chlorophyll *a* (2010) and periphyton colonization on periphytometers over a 29 d period (2008) were measured via spectrophotometry. Thawed glass fiber filters were heated in 4 mL dimethyl sulfoxide for 10 min at 65°C and pigments extracted with the addition of 90% acetone to a final volume of 15-18 mL (Burnison, 1980). Optical density was measured with a spectrophotometer (Pye Unicam SP8-100 UV-Visible spectrophotometer, Thermo Fisher Scientific Inc., Waltham, USA or Varian Cary100 UV-Visible spectrophotometer, Agilent Technologies, Mississauga, Canada) at 630, 647, 664 and 750 nm and chlorophyll *a* calculated using a trichromatic equation (Jeffrey and Humphrey, 1975).

HPLC was used to identify algal pigments for chemotaxonomic determination of the contribution of major algal groups to chlorophyll *a* for both the periphytometer experiments and the survey of periphyton across 24 sites (Table 4.2). Pigment extraction was modified from Buffan-Dubau and Carman (2000) with all steps conducted in darkness or low light to prevent photodegradation of pigments. Glass fibre filters were freeze dried for 48-72 hours (Super Modulyo freeze dryer, Fisher Scientific) in 15 mL polypropylene centrifuge tubes. Samples were sonicated in 5 mL (periphytometer samples) or 10 mL (periphyton survey samples) HPLC grade acetone (Fisher Scientific) for 30 s (3 × 10 s) and the centrifuge tubes

purged with nitrogen. Algal pigments were extracted in acetone for 24 hrs at -30°C, centrifuged at 4000 rpms at 4°C for 15 min and filtered through 0.2 μm 13 mm diameter PTFE syringe filters (Fisher Scientific). Periphyton survey samples from 2010 were diluted in acetone to achieve approximately 40 ng chlorophyll *a* on column, as determined from test samples. Pigments were separated following Zapata et al. (2000) using a Waters HPLC (626 pump, 717 plus autosampler and 600s controller) with a 2996 photodiode array and a 2475 multi-wavelength fluorescence detector with a Symmetry C_8 analytical column (3.6×150 mm, average particle size 4 μm, mobile phase A: methanol: acetonitrile: aqueous pyridine solution (50:25:25), B: methanol: acetonitrile: acetone (20:60:20), 25 μL injection). Pigments were quantified using Waters Empower-2 Software.

4.3.7 CHEMTAX analysis

Potential algal classes for CHEMTAX analysis were determined by reviewing algal classes found in freshwater and associated with the measured pigments (Table 4.2; Jeffrey et al., 2011). An initial pigment to chlorophyll *a* ratio matrix was created by averaging ratios from culture and field samples for relevant groups of algae using data summarized from a large number of studies in Higgins et al. (2011). A preliminary CHEMTAX analysis using periphyton data from 24 field sites (2010 survey) was conducted to assess the suitability of the algal classes and pigments selected. The number of algal classes and pigments were subsequently reduced if necessary. CHEMTAX is sensitive to the initial pigment ratio matrix and running multiple, randomized ratios is recommended to optimize the pigment ratio matrix and ensure a global (as opposed to local) minimum solution is reached (Higgins et al., 2011).

An additional 60 ratio matrices were created by multiplying each cell of the initial matrix by a randomly determined factor *F* calculated as

$$F = 1 + S \times (R - 0.5) \quad (1)$$

where *S* is a scaling factor of 0.7 and *R* is a random number between 0 and 1 (Higgins et al. 2011).

The best 10% (n=6) of the 61 solutions, having the lowest root mean square (RMS) residual, were averaged and the resulting pigment ratios used to further optimize subsequent CHEMTAX analyses. Pigment ratio matrices were optimized separately for the 2010 periphyton study across 24 field sites and for each of the five periphytometer experiments using data from all treatments. In each instance, 61 pigment ratio matrices were run in CHEMTAX, the solution with the lowest RMS residual used to calculate algal class abundances and the best six solutions used to calculate average pigment ratios and their standard deviations for the final pigment ratio matrix. The ratio limit of chlorophyll *a* was set to 100 and all other pigments were set to 500. The initial step size was 10 with a step ratio 1.3 and the number of iterations was set to 500.

4.3.8 Statistics

Univariate statistical analyses were performed using SPSS v21 (IBM Corp., Armonk, USA). Differences in periphyton colonization over time were assessed using a general linear model (GLM) with day as a covariate, treatment as a fixed factor and chlorophyll *a* as the dependent variable. In-stream concentrations of atrazine, NO_3^-, RP and DIN:RP were compared across experiments with a 1-way analysis of variance (ANOVA). The diffusion of NO_3^-, RP and atrazine from periphytometers over time was modelled separately using GLMs

with day as a covariate and experiment as a fixed factor. Nitrate, RP and atrazine concentrations for each day were transformed into percentage of day 0 concentrations. The diffusion rates for atrazine were modelled for both the first 7 d of all five experiments and over a period of 14 d for the three experiments where atrazine data were available for this time period. Two-way ANOVAs were used to examine the effects of periphytometer treatment (control, nutrient, atrazine, nutrient + atrazine) and algal class (Bacillariophyta and Chlorophyta as well as Cryptophyta and Euglenophyta for the 2008 experiment) on chlorophyll *a* biomass. Algal class and treatment were treated as fixed factors in factorial 2-way ANOVAs for each periphytometer experiment.

Differences in physical and chemical variables, atrazine concentrations, major nutrient forms and chlorophyll *a* concentrations were compared between low and high agriculture sites using paired t-tests. Linear regression was used to assess effects of atrazine, NO_3^-, RP and DIN:RP (separately) on periphyton biomass. Pearson's correlations were subsequently used to assess the correlation between atrazine, NO_3^-, RP and DIN:RP. A factorial 2-way ANOVA was used to examine effects of algal class (Bacillariophyta, Chlorophyta, Cryptophyta and Euglenophyta) and agriculture impact (low or high) on chlorophyll *a* biomass. A GLM was used to assess effects of algal class (fixed factor) and atrazine, nitrate, phosphate and DIN:RP (covariates) on chlorophyll *a* biomass. General linear model assumptions of normality and heterogeneity of variance were assessed using Shapiro-Wilk's and Levene's tests respectively where appropriate. Data were transformed if necessary to best meet these assumptions. The assumption of homogeneity of slopes was also assessed when covariates were included in models with fixed factors.

Canonical correspondence analysis (CCA) was used to ordinate algal class data and sites relative to measures nutrient and atrazine concentrations, after accounting for variation in periphyton composition due to intrinsic physico-chemical differences between field sites. Of the potential environmental variables, stream order, stream width, average depth, maximum depth, surface velocity, conductivity and turbidity were included as environmental variables in an initial CCA. Variables were normalized if necessary following an assessment of normality using Shapiro-Wilk's tests and then standardized to a mean of 0 and standard deviation of 1 (z-score transformation). Temperature and planktonic chlorophyll *a* concentrations were not included because they were not likely drivers of periphyton across the watershed. Dissolved oxygen concentrations and pH were excluded because although they may influence periphyton communities, they may also be affected by periphyton. Symmetric biplot scaling was used with no down-weighting. Stepwise regression and Monte Carlo permutations were used to test the significance of the environmental variables. Significant environmental variables were then used as covariables in a subsequent partial CCA where the ordination was constrained to variation in the algal class data explained by measures of nutrient and atrazine concentrations. Multivariate analyses were conducted using CANOCO v.4.5 (Plant Research International, Wageningen, The Netherlands).

4.4 Results

4.4.1 CHEMTAX analysis

Several measured pigments and potential algal classes (Table 4.2) were eliminated from CHEMTAX analysis. The pigments α-carotene, echinenone and peridinin were not detected in any samples and were removed (Table 4.3). Diatoxanthin and zeaxanthin were

present in only a few samples and were removed. Chlorophyll c_2 was present in most samples, but at low concentrations (Table 4.3) and has ambiguous taxonomic resolution (Mackey et al., 1996) so it was also removed. The Dinophyta were removed because peridinin was not detected (Table 4.3), the remaining pigment markers are variable (Table 4.2) and the majority of dinoflagellates are free-living (Jeffrey et al., 2011). The Cyanophyta and Rhodophyta were included in preliminary analysis. However, these two classes could not be distinguished from one another and it was unclear if they were properly distinguished from other β-carotene-containing classes. In addition, no definitive method currently exists for identifying biomass of red algae (Jeffrey et al., 2011). Since the marker pigment zeaxanthin was detected in only a few samples, it was concluded that the Cyanophyta and Rhodophyta likely contributed little to the overall periphyton community and these algal classes were removed. Similarly, low detection of zeaxanthin meant that the Bacillariophyta could not be distinguished from the Chrysophyta and the Chrysophyta were also eliminated from the analysis. Initial analyses were conducted using chlorophyll *a*, chlorophyll *b*, β-carotene, alloxanthin, diadinoxanthin, fucoxanthin and lutein to identify the contribution of the Bacillariophyta, Chlorophyta, Cryptophyta and Euglenophyta to total biomass.

Data summarized from Higgins et al. (2011) were used to create the initial pigment to chlorophyll *a* ratio matrix since site-specific data were lacking (Table 4.4). The matrix was then optimized for the 2010 dataset of periphyton located across 24 field sites and for the 2008 South Castor periphytometer experiment (Table 4.4). Pigment concentrations were very low in the 2009 periphytometer experiments, limiting the ability of CHEMTAX to reliably distinguish between multiple algal classes (Table 4.3). Of the marker pigments, fucoxanthin was most abundant and was used along with chlorophyll *b* and lutein to

characterize the abundance of the most dominant algal classes, the Bacillariophyta and Chlorophyta (Table 4.5). Data from the 2008 periphytometer experiment were re-analyzed with the two dominant algal classes to compare experiments (Table 4.5).

4.4.2 Periphytometer experiments

Proof of concept

In 2008, colonization of periphyton onto periphytometer substrates was monitored over a 29 d period to assess the performance of the periphytometers and the suitability of a 14 d experimental period (Fig. 4.2). Biomass reached a maximum of ~75 mg/m^2 chlorophyll *a* after 22 d of colonization (Fig. 4.2). No significant difference in biomass was observed between periphytometers filled with distilled water (control) and a mixture of NO_3^-, RP and atrazine (20 µg/L atrazine spiked on day 7) over the course of the experiment (Fig. 4.2; $F=0.000$; $df=1,31$; $p=0.997$; $R^2=0.664$).

In-stream agrochemicals

In-stream concentrations of NO_3^- ranged from 317 to 2207 µg/L and differed significantly between experiments ($F=10.697$; $df=4,35$; $p<0.001$; $R^2=0.550$). Concentrations of NO_3^- tended to be higher in the South Castor River compared to the North Branch of the South Nation River (Table 4.6). In-stream RP concentrations ranged from 9-40 µg/L and did not differ significantly between sites and experiments (Table 4.6; $F=1.597$; $df=4,35$; $p=0.197$; $R^2=0.154$). The ratio of DIN:RP ranged from 9-88 and differed significantly between experiments (Table 4.6; $F=18.658$; $df=4,35$; $p<0.001$; $R^2=0.681$). Total nitrogen ranged from 811-2937 µg/L and was similar between sites and time periods (Table 4.6;

F=2.097; df=4,35; p=0.102; R^2=0.193). Total phosphorus ranged from 15-66 μg/L and differed significantly between experiments (Table 4.6; F=3.432; df=4,35; p=0.018; 0.282). In-stream atrazine concentrations were low at both sites and ranged from below quantitation (20 ng/L) to 34 ng/L (Table 4.6).

Experimental treatments

Concentrations of NO_3^-, RP and atrazine were measured in the periphytometer solution reservoirs from samples taken on days 0, 7 and 14. Nitrate and RP were not detected in any of the carboys of distilled water (N=4). Similarly, atrazine was not detected in distilled water (N=1). Measured concentrations of NO_3^- and RP in solutions used for nutrient enriched treatments were 99.5 ± 0.57% and 106.2 ± 2.6% of nominal concentrations respectively (N=8). In 2008, measured concentrations of atrazine (average 30.2 μg/L) were higher than the nominal concentration of 20 μg/L, ranging from 136.5-165.5% (average 151.0) of the nominal concentration (N=2). In 2009, measured concentrations of atrazine were 102.3 ± 6.0% of nominal concentrations (N=10). Atrazine concentrations were at or below quantitation for nutrient and distilled water periphytometers over the course of all experiments. Concentrations of NO_3^- and RP increased over time in control periphytometers (average 690.1 ± 479.8 and 1.7 ± 2.1 μg/L respectively (N=37) as stream water diffused into periphytometers.

The diffusion of NO_3^-, RP and atrazine from periphytometers over time was compared between experiments (Fig. 4.3) to assess whether exposure of periphyton to these chemicals was comparable between experimental periods. There was an overall significant difference in the diffusion of NO_3^- from periphytometers (Fig. 4.3; F=13.214; df=4,14;

$p<0.001$; $R^2=0.891$), although diffusion was similar between all but one experiment (Fig. 4.3). The rate of diffusion of RP from periphytometers was similar for all experiments (Fig. 4.3; $F=2.477$; $df=4,14$; $p=0.092$; $R^2=0.865$). Atrazine was added to periphytometers either on day 0 or 7 and atrazine diffusion rates were compared after 7 d for all experiments as well as after 14 d where applicable. Overall significant differences in diffusion between experiments were observed after 7 d ($F=6.856$; $df=4,5$; $p=0.029$; $R^2=0.846$) and after 14 d ($F=6.649$; $df=2,8$; $p=0.020$; $R^2=0.877$). However, diffusion was generally comparable between most experiments (Fig. 4.3).

Periphyton biomass and communities

The effects of algal class and experimental treatment on periphyton biomass were examined by conducting a factorial two-way ANOVA for each experiment Biomass was an order of magnitude higher in 2008 (experiment 1) compared to 2009 (experiments 2-5) (Fig. 4.4). Total rainfall in 2009 was substantially higher than in 2008 (334 mm versus 139 mm respectively during July and August at the Ottawa Macdonald-Cartier International Airport) (Environment Canada, 2014). In 2008, the Cryptophyta and Euglenophyta represented 4.0 and 1.5% of total chlorophyll *a* biomass respectively (Fig. 4.4). Subsequent analyses examined the contributions of only the Bacillariophyta and Chlorophyta to biomass. Biomass of the Bacillariophyta was significantly higher than biomass of the Chlorophyta for all experiments (Fig. 4.4; $F\geq205.152$; $p\leq0.001$). Overall, periphytometer biomass consisted of an average of 90.4% Bacillariophyta with samples ranging from 69.9-99.4% Bacillariophyta. Conversely, periphytometer biomass was on average 8.8% Chlorophyta with samples ranging from 0.6-30.1% Chlorophyta. No interaction between algal class and

periphytometer treatment was observed for any experiment (Fig. 4.4; $F \leq 0.303$; $p \geq 0.825$). Biomass was similar across treatments for experiments 1 (2008 SC 7), 2 (2009 NB 7) and 3 (2009 NB 0) (Fig. 4.4; $F \leq 0.490$; $p \geq 0.656$). Significant differences between treatments were observed for experiment 4 (2009 SC 7) (Fig. 4.4; $F=2.875$; $df=7,40$; $p=0.048$; $R^2=0.869$) and experiment 5 (2009 SC 0) (Fig. 4.4; $F=3.163$; $df=7,40$; $p=0.035$; $R^2=0.844$). Biomass of the control was significantly higher than the atrazine treatment in experiment 4 (2009 SC 7) ($p=0.042$) and significantly higher than all experimental treatments in experiment 5 (2009 SC 0) ($p \leq 0.021$) (Fig. 4.4).

4.4.3 Periphyton communities across atrazine and nutrients gradients

Site characteristics

Physical and chemical characteristics varied across 24 field sites in 2010 but were typically similar between paired sites (Table 4.7). Strahler stream order ranged from 3 near the headwaters to 6 along the main branch of the South Nation River (Table 4.7). Across the watershed, sites differed in terms of stream width, baseflow depth (both average and maximum) and surface velocity. However, paired sites were similar in terms of these physical characteristics, as well chemical characteristics including temperature, conductivity and planktonic chlorophyll *a* (Table 4.7). High agriculture sites were more turbid, more alkaline and had a higher concentration of dissolved oxygen compared to low agriculture sites (Table 4.7).

Atrazine, estimated from 56 d time-weighted-average concentrations, ranged from 4-412 ng/L and was significantly higher at high agriculture sites (Table 4.8). Concentrations of RP, TP, ON and NH_3 + NH_4^+ were similar between paired sites between May and July

2010 (Table 4.8). Nitrite concentrations were typically below detection and were not reported. The range of NO_3^- concentrations at field sites varied by three orders of magnitude (Table 4.8). Although NO_3^- tended to be higher at high agriculture sites compared to low agriculture sites, the trend was statistically significant only for data averaged from May, June and July samples (Table 4.8). Total nitrogen followed similar trends, driven by changes in NO_3^- (Table 4.8). Ratios of nitrogen (N) to phosphorus (P) also varied and the ratio of DIN:RP was significantly higher at high agriculture sites in data averaged from May to July samples (Table 4.8).

Periphyton biomass

The average coefficient of variation between six triplicate periphyton sub-samples was 8.2% and ranged from 2.6-14.0%. Periphyton biomass ranged from 1.6-138.5 mg/m^2 chlorophyll *a* across 24 field sites with an average of 37.2 ± 38.7 mg/m^2 chlorophyll *a* (Fig. 4.5). Biomass was similar between paired low and high agriculture sites (Fig. 4.5). Linear regression was used to examine the relationship between periphyton biomass and an agrochemical gradient, as measured by concentrations of atrazine, NO_3^-, RP and DIN:RP. All measures showed a general trend of increasing periphyton biomass as agrochemicals increased (Fig. 4.6; Appendix C Fig. C.1). The trends were generally stronger when data from samples collected in June were used (Fig. 4.6) compared to data averaged from samples collected in May, June and July (Appendix C Fig. C.1). Both atrazine and NO_3^- concentrations had a significant positive effect on periphyton biomass, whereas RP concentrations and DIN:RP had no effect (Fig. 4.6). Atrazine concentrations were correlated with NO_3^- (Pearson correlation coefficient (PCC)=0.561; p=0.004) and DIN:RP

(PCC=0.636; p=0.001). Nitrate concentrations were correlated with RP (PCC=0.407; p=0.048) and DIN:RP (PCC=0.823; p<0.001).

Periphyton communities

Across the watershed, the periphyton community was composed of an average of 60.9 ± 12.9% Bacillariophyta (26.1-80.8%), 28.1 ± 15.0% Chlorophyta (2.3-64.8%), 6.9 ± 5.4% Cryptophyta (0.0-15.7%) and 4.1 ± 8.4% Euglenophyta (0.0-11.9%). A factorial two-way ANOVA was used to examine effects of algal class and agricultural impact (low versus high) on biomass. Biomass differed between algal classes ($F=18.936$; $df=3,88$; $p<0.001$; $R^2=0.393$), with higher biomass observed for the Bacillariophyta and Chlorophyta compared to the Cryptophyta and Euglenophyta (Fig. 4.7). Biomass did not differ between low and high agriculture sites ($F=0.158$; $df=1,88$; $p=0.692$; $R^2=0.393$) and no interaction was observed between algal class and level of agricultural impact ($F=0.038$; $df=3,88$; $p=0.990$; $R^2=0.393$) (Fig. 4.7). Changes in periphyton biomass along a gradient of agrochemical stress were assessed using a GLM examining effects of algal class, atrazine and June values of NO_3^-, RP and DIN:RP. Interactions between measures of agrochemical stress were included due to the observed correlation between variables. However, no significant interactions were observed between agrochemical variables ($F\leq2.618$; $df=1,85$; $p\geq0.109$; $R^2=0.538$). The contribution of algal classes to periphyton biomass differed significantly ($F=24.016$; $df=3,85$; $p<0.001$, $R^2=0.538$) between all classes ($p\leq0.32$), except between the Cryptophta and Euglenophyta which had similar biomass ($p=0.956$). Nitrate had a significant positive effect on overall biomass ($F=6.205$; $df=1,85$; $p=0.015$; $R^2=0.538$), whereas RP ($F=1.572$; $df=1,85$; $p=0.213$; $R^2=0.538$) and atrazine had no effect ($F=0.691$;

df=1,85; p=0.408; R^2=0.538). Significant effects of nitrate were also observed in a subsequent GLM examining the effects of only algal class and NO_3^- on biomass (F=14.876; df=1,88; p≤0.001; R^2=0.491) and no interaction between algal class and nitrate was observed (F=0.760; df=3,88; p=0.520; R^2=0.491), indicating that the algal classes responded similarly to nitrate.

An initial CCA indicated that of the environmental variables, turbidity, surface velocity and average depth explained a significant amount of variation in periphyton community structure between field sites (Fig. 4.8). Turbid sites were associated with the Bacillariophyta, high velocity with the Euglenophyta and deep sites with the Cryptophyta (Fig. 4.8). Turbidity, surface velocity and average depth were included as covariables in a subsequent CCA to examine the influence of an agrochemical gradient on periphyton communities, after accounting for physical and chemical differences between field sites. Maximum depth was also included as a covariable because it contributed to the significance of average depth (i.e average depth no longer explained a significant amount of variation once maximum depth was excluded). A partial CCA was conducted using turbidity, surface velocity, average depth and maximum depth as covariables and constrained to variation in algal class data explained by linear combinations of atrazine, June NO_3^-, June RP and June DIN:RP. Atrazine, NO_3^-, and DIN:RP were closely related and were explained by Axis 1 which accounted for 22.5% of the variation in periphyton communities between sites (Fig. 4.9). Reactive phosphate was associated with Axis 2 which explained 5.0% of the variation in periphyton communities between sites (Fig. 4.9). Of the agrochemical variables, only DIN:RP explained a significant amount of variation (Fig. 4.9). Sites enriched with nitrogen relative to phosphorus were associated with higher Chlorophyta biomass, whereas sites less

enriched with nitrogen relative to phosphorus were associated with higher Bacillariophyta biomass (Fig. 4.9).

4.5 Discussion

4.5.1 Chemotaxonomic characterization of freshwater periphyton

Improvements in the separation of algal pigments with HPLC have led to increased use in chemotaxonomic methods of characterizing algal communities. Of the available methods, including multiple regression, inverse simultaneous equations and matrix factorization (CHEMTAX), the software CHEMTAX is best suited for handling marker pigments that are shared among taxonomic groups (Wright and Jeffrey, 2006). Despite this potential, CHEMTAX has been used primarily for classification of marine phytoplankton (Higgins et al., 2011 and references therein). However, it has also been successful in the classification of freshwater algae. For example, CHEMTAX was used to characterize phytoplankton in nine North American (USA) lakes (Descy et al., 2000) as well as both phytoplankton and benthic algae in three Danish lakes and three streams (Lauridsen et al., 2011). To my knowledge, the present study was the first to use CHEMTAX to characterize freshwater periphyton communities at the watershed scale.

Periphyton communities were classified into four broad taxonomic groups: the Bacillariophyta, Chlorophyta, Cryptophyta and Euglenophyta. Marker pigments were too low to include the Cyanophyta, Rhodophyta and Chrysophyta, although these groups may occur in periphyton communities (Allan, 1995). Cyanophyta and Rhodophyta have previously been found in periphyton communities in agricultural landscapes (Munn et al., 2002) and in southern Ontario and western Quebec rivers (Chételat et al., 1999). One

limitation of chemotaxonomy is that interpretation of the absence of pigments is less reliable than the presence of pigments because minor pigments may be unidentified or unreported (Higgins et al., 2011). For example, in the present study 2010 periphyton survey samples were diluted to achieve 40 ng chlorophyll *a* on column to avoid saturation of the HPLC-PDA detector. At this high concentration of chlorophyll *a*, the concentration of a number of present pigments was still very low and it was unclear whether absent pigments and their related algal classes were truly absent or simply below the limit of detection. A further limitation is that pigment ratios must be constant across samples (Mackey et al., 1996). Pigment ratios are known to vary between species and even strains (Jeffrey and Wright, 1994) and may be altered by light and nutrient regimes (Higgins et al., 2011). Despite these limitations, CHEMTAX was able to converge to stable solutions following optimization of pigment ratios and appeared to provide a reasonable characterization of dominant algal classes based on the pigment markers present.

4.5.2 Experimental evidence for effects of nutrients and atrazine on periphyton

Previous studies have used a periphytometer approach to assess nutrient limitation (Matlock et al., 1998; Carey et al., 2007; Ludwig et al., 2008) and effects of herbicides (Kish, 2006) on periphyton. None examined changes in periphyton community structure, although Kish (2006) measured chlorophyll *b* and *c* with a spectrophotometer. In the present study, periphytometers were used to assess the combined effects of nutrient enrichment and atrazine contamination on periphyton communities. Successful colonization of periphyton onto periphytometer substrates over a four week period in 2008 demonstrated that the substrates were suitable for growth. Diffusion of nutrients and atrazine from

periphytometers were generally comparable between time periods and field sites. Ranges of biomass (measured as mg/m^2 chlorophyll *a*) from earlier 14 d studies were 12.9-34.4 mg/m^2 (Matlock et al., 1998), 0.3-69.3 mg/m^2 (Carey et al., 2007) and 2.6-34.4 mg/m^2 (Ludwig et al., 2008). In the present study, biomass averaged 24.7 mg/m^2 across treatments in 2008, comparable to values reported in the literature. However, biomass was an order of magnitude lower in 2009 (average 2.6 mg/m^2), likely due to high rainfall limiting colonization and increasing scouring.

In general, nutrient enrichment and atrazine contamination had no effect on periphyton biomass in the periphytometer experiments. In 2008, nutrient enrichment and the addition of 20 µg/L atrazine clearly had no effect on periphyton biomass or community composition, indicating that the South Castor River was not nutrient limited and that 20 µg/L atrazine did not inhibit periphyton growth. In contrast, Kish (2006) observed an increase in chlorophyll *c* relative to chlorophyll *a* at 12 µg/L atrazine in a periphytometer experiment, suggesting that chlorophyll *c* containing Bacillariophyta were more atrazine tolerant compared to the Chlorophyta (Kish, 2006). Similarly, Detenbeck et al., (1996) observed reductions in periphyton gross productivity in multi-species stream mesocosms at concentrations as low as 15 µg/L atrazine and a shift in dominance from atrazine sensitive Chlorophyta to more tolerant Bacillariophyta. The sensitivity of algal species to atrazine varies. For example, Fairchild et al. (1998) observed 50% inhibition concentrations (IC_{50}s) ranging from 94 µg/L for *Chlorella sp*. (Chlorophyta) to >3000 µg/L for *Anabaena sp.* (Cyanophyta). A review of toxicity data found the lowest reported IC_{50} for the Chlorophyta was <1 µg/L (35 spp.) compared to 19 µg/L for the Bacillariophyta (46 spp.) (US EPA, 2012d). Increased sensitivity of the Chlorophyta to atrazine compared to the Bacillariophyta

has been associated with higher rates of atrazine accumulation in the Chlorophyta (Tang et al., 1998).

In 2009, the North Branch of the South Nation River did not appear to be nutrient limited. Atrazine (200 μg/L) had no effect on periphyton biomass when injected on day 7 (intended to reduce existing biomass) or day 0 (intended to inhibit colonization). Biomass was higher in the control treatment in the South Castor River (day 0 atrazine injection). However, this was likely due to chance since a similar increase in biomass would be expected in the nutrient treatment. A reduction in biomass in the atrazine treatment in the South Castor River (day 7 atrazine injection) may also be due to chance since no difference was observed between the nutrient and atrazine treatment. Observed ratios of DIN:RP >16 suggest that the sites were phosphorus limited (Redfield, 1958) but the lack of increase in biomass following nutrient enrichment provided evidence that the field sites were not strongly nutrient limited. The lack of response to atrazine contamination suggested the periphyton communities were not inhibited by atrazine at 200 μg/L. However, in 2009 the sensitivity of the periphytometers and their ability to detect effects of nutrient enrichment and atrazine contamination may have been limited by the overall low biomass of periphyton.

4.5.3 Changes in periphyton across nutrient and atrazine gradients

Increased dissolved oxygen and alkalinity at high compared to low agriculture sites suggested higher rates of photosynthesis, producing dissolved oxygen and increasing pH through uptake of CO_2. Since periphyton biomass did not differ between paired sites, it is plausible that increased productivity at high agriculture sites may be attributed to macrophytes and metaphyton. However, further research would be needed to verify that

observed results were not due to a decrease in microbial respiration at high agriculture sites. Within a given tributary, periphyton growth was likely limited by similar physical and chemical constraints such as hydrology, nutrient and light availability as well as invertebrate grazing. Sites were generally dominated by the Bacillariophyta and Chlorophyta to a lesser degree, with minor contributions of the Cryptophyta and Euglenophyta, and had similar compositions between low and high agriculture sites.

Changes in periphyton biomass were observed across gradients of agrochemical contamination with a general trend towards increased biomass with increasing agrochemical concentration. Concentrations of RP had no significant effect on biomass, whereas both atrazine and NO_3^- had significant effects. However, atrazine and NO_3^- were correlated and no effect of atrazine was observed once effects of NO_3^- on biomass were considered. Both a GLM and CCA indicated that NO_3^- was the main driver in changes in periphyton biomass. Specific effects on community structure were only apparent in the CCA when differences in physical and chemical characteristics, such as stream velocity, depth and turbidity were considered. High agriculture sites were more turbid and high turbidity was associated with the Bacillariophyta. High DIN:RP ratios were related to high NO_3^- concentrations and were associated with the Chlorophyta. In contrast, Munn et al. (2002) found that U.S. agricultural streams were dominated by either Bacillariophyta or Cyanophyta with Chlorophyta representing only 1% of total periphyton relative abundance. Nelson et al. (2013) found that the Bacillariophyta, Chlorophyta and Cyanophyta showed no consistent response to nutrient augmentation. However, specific diatom species associated with low nutrient conditions decreased in response to nutrient augmentation (Nelson et al., 2013).

The influence of nutrients on periphyton biomass and community structure was examined further by comparing differences in the distribution of P and N. Measures of RP varied across the watershed but were similar between paired sites. In contrast, NO_3^- concentrations varied by three orders of magnitude across the watershed and high agriculture sites tended to have higher concentrations of NO_3^-. In a survey of U.S. stream sites, Dubrovsky et al. (2010) estimated that background concentrations of RP and NO_3^- due to natural processes were 10 and 240 μg/L respectively. Sites within the South Nation River watershed generally exceeded these values and were characterized by both N and P enrichment. The Redfield ratio, which predicts P limitation at N:P ratios of >16:1 and N limitation at ratios <16:1 (Redfield, 1958), suggests that field sites within the South Nation River watershed were generally N enriched and P limited. However, nutrient limitation within streams and rivers is difficult to assess and is a function of both N:P ratios, as well as absolute concentrations of N and P. Keck and Lepori (2012) examined 382 stream/ river nutrient enrichment experiments and found that nutrient limitation was difficult to predict except at extreme N:P ratios of <1:1 and >100:1. Eutrophication may be better predicted by absolute nutrient concentrations. For example, Dodds et al. (1998) proposed that streams were likely to be oligotrophic at concentrations of planktonic chlorophyll *a* <10 μg/L, periphyton chlorophyll *a* <20 mg/m^2, TN <700 μg/L and TP <25 μg/L and eutrophic at concentrations of planktonic chlorophyll *a* >30 μg/L, periphyton chlorophyll *a* >70 mg/m^2, TN >1500 μg/L and TP > 75 μg/L. Based on this classification, sites within the South Nation River watershed were generally oligotrophic in terms of phytoplankton, mesotrophic in terms of periphyton, eutrophic in terms of TN and mesotrophic in terms of TP. However, in Canada streams are considered eutrophic at a lower threshold (TP >35 μg/L)

(Canadian Council of Ministers of the Environment, 2004) and sites within the South Nation River watershed would generally be eutrophic based on this guideline. Keck and Lepori (2012) observed a wide transition in the probability of N limitation, which did not support Redfield's theory (1958) that N limitation occurs at a precise tipping point. Keck and Lepori (2012) proposed that while individual species may be limited by specific optimal N:P ratios, communities may be able to take advantage of additions of either N or P through increased acquisition and reduced loss of the scarcest nutrient and shifts in community structure to species with different optimal N:P ratios. In the present study, an increase in periphyton biomass under N enriched conditions was observed as well as evidence that N was the driver in increased biomass.

Atrazine concentrations were higher at high agriculture sites compared to low agriculture sites but all sites had time-weighted-average concentrations well below Canadian water quality guidelines for the protection of aquatic life (1.8 μg/L) (Canadian Council of Ministers of the Environment, 1999). A risk assessment concluded that atrazine was not likely to have direct toxic effects on periphyton at environmentally relevant concentrations (Solomon et al. 1996). However, inhibitory effects on algal communities have been observed at concentrations <5 μg/L (DeNoyelles et al., 1982; Pannard et al., 2009). Nutrients and atrazine were expected to have contrasting effects on periphyton with nutrients stimulating growth and atrazine inhibiting growth. However, atrazine can both stimulate periphyton (10 μg/L) (Murdock and Wetzel, 2012) and inhibit periphyton (100 μg/L) (Guasch et al., 2007). In addition, the response of periphyton to interactions between nutrients and atrazine is complex and conflicting trends have been observed in a comparison between field and laboratory studies (Murdock et al., 2013). Prior exposure to atrazine may

also be a factor in complicating the response of algae to agrochemicals because it tends to increase tolerance towards atrazine (Guasch et al., 1998; Guasch et al., 2007) and favour the Bacillariophyta compared to the Chlorophyta (Weiner et al., 2004; Lockert et al., 2006). Interactions between nutrients and atrazine complicate and potentially mask the relative strength of their effects on periphyton communities. In the present study, effects of NO_3^- on periphyton were greater than any observable effects of atrazine and RP. Nitrate enrichment appeared to result in an increase in periphyton biomass and an increase in the Chlorophyta.

4.6 Acknowledgements

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Table 4.1 Overview of periphytometer experiments conducted in the South Nation River watershed in 2008 and 2009

Experiment Code	Site	Dates	Duration (d)	Nutrient addition	Atrazine addition
1 (2008 SC 7)	South Castor River	24 July - 7 Aug. 2008 24 July - 22 Aug. 2009	14 & 29		20 µg/L spiked on d 7
2 (2009 NB 7)	North Branch South Nation	14 - 28 July 2009	14	20 mg/L N	200 µg/L spiked on d 7
3 (2009 NB 0)	North Branch South Nation	6 - 20 Aug. 2009	14	1.25 mg/L P	200 µg/L spiked on d 0
4 (2009 SC 7)	South Castor River	9 - 23 July 2009	14		200 µg/L spiked on d 7
5 (2009 SC 0)	South Castor River	5 -19 Aug. 2009	14		200 µg/L spiked on d 0

Table 4.2 Pigments measured with HPLC and their presence in various algal classes. Data are from Jeffrey et al. (2011) with symbols representing ■ dominant pigments, □ significant pigments not found in all species, ● minor pigments and ○ minor pigments not found in all species

Pigment	Cyanophyta	Rhodophyta	Bacillariophyta	Chrysophyta	Cryptophyta	Dinophyta	Chlorophyta	Euglenophyta
Chlorophylls								
Chlorophyll *a*	■	■	■	■	■	■	■	■
Chlorophyll *b*						□	■	■
Chlorophyll c_2			■	□	■	□		
Carotenes								
α-carotene					●	○	●	
β-carotene	■	■	●	●		○	■	●
Xanthophylls								
Alloxanthin					■	□		
Diadinoxanthin			■			□		■
Diatoxanthin			●			○		●
Echinenone	●							
Fucoxanthin			■	■		□		
Lutein							■	
Peridinin						□		
Zeaxanthin	■	■		■		○	●	

Table 4.3 Pigment biomass (mg/m^2) from empirical and experimental studies. Average values (± standard deviation) are shown with minimum and maximum values in brackets

Pigment	2010 periphyton at 24 sites (n=60)	2008 periphytometer experiment (South Castor River) (n=20)	2009 periphytometer experiments (North Branch South Nation and South Castor R.) (n=96)[a]
Chlorophylls			
Chlorophyll *a*	38.00 ± 38.93 (1.07-152.21)	24.74 ± 7.22 (10.96-38.66)	1.82 ± 1.09 (0.35-5.07)
Chlorophyll *b*	4.53 ± 7.38 (0.11-51.25)	0.90 ± 0.26 (0.37-1.51)	0.04 ± 0.02 (0.01-0.11)
Chlorophyll c_2	0.25 ± 0.26 (0.01-1.05)	0.14 ± 0.03 (0.08-0.21)	0.004 ± 0.004 (0.000-0.026)
Carotenes			
α-carotene	0	0	0
β-carotene	1.34 ± 1.98 (0.00-10.24)	2.23 ± 0.18 (1.94-2.59)	0.08 ± 0.11 (0.00-0.41)
Xanthophylls			
Alloxanthin	0.87 ± 1.21 (0.00-5.62)	0.31 ± 0.11 (0.17-0.62)	0.08 ± 0.11 (0.00-0.42)
Diadinoxanthin	1.46 ± 2.50 (0.00-11.85)	1.23 ± 0.34 (0.73-2.04)	0.04 ± 0.10 (0.00-0.47)
Diatoxanthin	0.10 ± 0.37 (0.00-2.13)	0	0.04 ± 0.10 (0.00-0.38)
Echinenone	0	0	0
Fucoxanthin	11.14 ± 12.98 (0.22-53.28)	10.21 ± 3.15 (5.02-17.55)	0.97 ± 0.60 (0.10-2.67)
Lutein	1.30 ± 1.78 (0.00-10.93)	0.39 ± 0.12 (0.17-0.67)	0.07 ± 0.11 (0.00-0.45)
Peridinin	0	0	0
Zeaxanthin	0.09 ± 0.34 (0.00-1.91)	0	0.02 ± 0.06 (0.00-0.22)

[a] Values and ranges shown are for all treatments of the four 2009 experiments (each n=24)

Table 4.4 Pigment to chlorophyll *a* ratios optimized with CHEMTAX for four algal classes

Algal Class	Chlorophyll *b*	β-carotene	Alloxanthin	Fucoxanthin	Diadinoxanthin	Lutein
			Initial pigment ratios[a]			
Bacillariophyta	0	0.023	0	0.699	0.163	0
Cryptophyta	0	0	0.316	0	0	0
Euglenophyta	0.354	0.021	0	0	0.232	0
Chlorophyta	0.325	0.063	0	0	0.000	0.172
			2010 Periphyton at 24 sites			
Bacillariophyta	0	0.019 ± 0.003	0	0.458 ± 0.019	0.007 ± 0.002	0
Cryptophyta	0	0	0.361 ± 0.051	0	0	0
Euglenophyta	0.211 ± 0.035	0.025 ± 0.005	0	0	0.427 ± 0.055	0
Chlorophyta	0.399 ± 0.048	0.038 ± 0.006	0	0	0	0.120 ± 0.014
			2008 South Castor experiment			
Bacillariophyta	0	0.082 ± 0.014	0	0.542 ± 0.040	0.058 ± 0.008	0
Cryptophyta	0	0	0.361 ± 0.120	0	0	0
Euglenophyta	0.213 ± 0.088	0.023 ± 0.005	0	0	0.268 ± 0.083	0
Chlorophyta	0.202 ± 0.036	0.180 ± 0.073	0	0	0	0.099 ± 0.020

[a]Data from Higgins et al. 2011

Table 4.5 Pigment to chlorophyll *a* ratios calculated with CHEMTAX for two dominant algal classes

Algal Class	Chlorophyll *b*	Fucoxanthin	Lutein
	1) 2008 South Castor experiment (Root mean square residual *(RMS) 0.039 ± 0.0000)*		
Bacillariophyta	0	0.474 ± 0.001	0
Chlorophyta	0.288 ± 0.004	0	0.127 ± 0.002
	2) 2009 North Branch experiment (atrazine injected on day 7) (RMS 0.131 ± 0.0003)		
Bacillariophyta	0	0.434 ± 0.004	0
Chlorophyta	0.153 ± 0.011	0	0.720 ± 0.049
	3) 2009 North Branch experiment (atrazine injected on day 0) (RMS 0.058 ± 0.0000)		
Bacillariophyta	0	0.546 ± 0.006	0
Chlorophyta	0.626 ± 0.139	0	0.015 ± 0.002
	4) 2009 South Castor experiment (atrazine injected on day 7) (RMS 0.123 ± 0.0001)		
Bacillariophyta	0	0.633 ± 0.002	0
Chlorophyta	0.151 ± 0.008	0	0.560 ± 0.030
	5) 2009 South Castor experiment (atrazine injected on day 0) (RMS 0.174 ± 0.0002)		
Bacillariophyta	0	0.681 ± 0.005	0
Chlorophyta	0.089 ± 0.005	0	0.320 ± 0.018

Table 4.6 Average ± standard deviation of in-stream concentrations of nutrients and atrazine during periphytometer experiments conducted in the South Nation River watershed in 2008 and 2009. Minimum and maximum values are in brackets. Values followed by different letters are significantly different ($p<0.05$).

Experiment Code[a]	Nitrate (NO_3^-) (µg/L)	Reactive phosphate (RP) (µg/L)	Dissolved inorganic nitrogen: reactive phosphate (DIN:RP)	Total nitrogen (TN) (µg/L)	Total phosphorus (TP) (µg/L)	Atrazine (ng/L)
1 (2008 SC 7) (n=16)	870 ± 467 (379-1961) ab	21 ± 11 (9-40) a	44 ± 13 (30-71) a	1572 ± 520 (1020-2759) a	37 ± 11 (23-55) ab	<20
2 (2009 NB 7) (n=6)	507 ± 150 (317-640) bc	16 ± 4 (11-21) a	35 ± 5 (30-42) a	1370 ± 383 (811-1781) a	32 ± 12 (15-44) b	<20
3 (2009 NB 0) (n=6)	304 ± 80 (223-401) c	28 ± 9 (20-40) a	14 ± 6 (9-21) b	1435 ± 147 (1275-1597) a	50 ± 9 (42-66) a	<20
4 (2009 SC 7) (n=6)	1470 ± 236 (1183-1717) a	19 ± 6 (11-24) a	88 ± 38 (51-141) c	2064 ± 258 (1753-2363) a	34 ± 3 (29-37) ab	32 ± 2 (29-34)
5 (2009 SC 0) (n=6)	1114 ± 860 (386-2207) ab	21 ± 4 (18-26) a	49 ± 30 (22-88) a	1820 ± 880 (1057-2937) a	39 ± 5 (32-44) ab	<20 (<20-27)

[a]Experiments were conducted in the South Castor R (SC), North Branch South Nation R (NB) with atrazine injected on day 0 or day 7 of each experiment.

Table 4.7 Physical and chemical characteristics of 12 paired (24 in total) stream/ river sites in 2010 in the South Nation River watershed, Canada.

Variable	Units	Average ± standard deviation (range in brackets) Low agriculture sites	Average ± standard deviation (range in brackets) High agriculture sites	n	Paired t-test comparing average values (df=11)
Physical stream characteristics					
Strahler stream order	n/a	4.5 ± 0.90 (3-6)	4.7 ± 0.89 (4-6)	24	Wilcoxon p=0.157[a]
Stream width	m	14.2 ± 7.6 (4.7-32.6)	16.5 ± 11.2 (5.6-48.5)	72	t=-0.919; p=0.378
Average baseflow depth	cm	66 ± 30 (33-115)	66 ± 45 (18-182)	216	t=0.063; p=0.951
Maximum baseflow depth	cm	89 ± 44 (40-190)	90 ± 67 (22-258)	72	t=-0.036; p=0.972
Surface velocity	m/s	0.091 ± 0.086 (0.024-0.321)	0.094 ± 0.055 (0.009-0.202)	144	t=-0.096; p=0.926
Water chemistry					
pH	n/a	7.85 ± 0.24 (7.42-8.08)	8.06 ± 0.24 (7.75-8.63)	72	**t=-3.862; p=0.003***
Dissolved oxygen	mg/L	5.93 ± 1.80 (2.21-8.25)	7.74 ± 2.20 (4.96-12.55)	72	**t=-3.220; p=0.008***
Temperature	°C	20.95 ± 1.90 (18.42-24.08)	21.90 ± 1.62 (18.46-23.94)	72	t=-2.021; p=0.068
Conductivity	µS/cm	521.4 ± 136.4 (343.9-788.7)	532.2 ± 100.2 (366.5-690.0)	72	t=-0.748; p=0.470
Turbidity	NTU	9.3 ± 5.5 (2.3-18.1)	17.2 ± 13.6 (2.4-50.9)	144	**t=-2.294; p=0.043***
Planktonic chlorophyll *a*	µg/L	7.2 ± 4.1 (2.6-15.1)	8.5 ± 4.1 (3.8-16.2)	144	t=-1.052; p=0.315

* Significant at $p<0.05$

[a]Non-parametric Wilcoxon signed rank test p-value

Table 4.8 Concentrations of atrazine and major nutrient forms and their ratios averaged from May, June and July 2010 samples

Chemical	Abbreviation	Units	Time	Low agriculture sites	High agriculture sites	Paired t-test (df=11)
Atrazine	ATR	µg/L	May-July	98 ± 102 (4-364)	178 ± 128 (14-412)	**t=-2.480; p=0.031**
Reactive phosphate	RP	µg/L	June	35 ± 20 (10-69)	40 ± 27 (4-102)	t=-0.781; p=0.451
			May-July	54 ± 29 (20-110)	59 ± 25 (22-103)	t=-0.872; p=0.402
Total phosphorus	TP	µg/L	June	55 ± 20 (30-85)	57 ± 20 (24-102)	t=-0.305; p=0.766
			May-July	81 ± 32 (39-131)	86 ± 28 (48-139)	t=-0.574; p=0.577
Ammonia + Ammonium	$NH_3 + NH_4^+$	µg/L	June	69 ± 51 (16-150)	49 ± 34 (10-112)	t=1.004; p=0.337
			May-July	74 ± 41 (25-152)	67 ± 39 (13-151)	t=0.441; p=0.668
Nitrate	NO_3^-	µg/L	June	1356 ± 1476 (3-4235)	2379 ± 1829 (117-5404)	t=-2.087; p=0.061
			May-July	643 ± 689 (8-1784)	1271 ± 836 (70-2503)	**Wilcoxon p=0.004** [a]
Dissolved + particulate	DON+PON	µg/L	June	840 ± 162 (540-1062)	743 ± 163 (494-1026)	t=1.993; p=0.072
organic nitrogen			May-July	916 ± 177 (648-1161)	831 ± 138 (639-1018)	t=1.688; p=0.120
Total nitrogen	TN	µg/L	June	2286 ± 1413 (851-5019)	3204 ± 1740 (1027-6144)	t=-1.955; p=0.076
			May-July	1649 ± 611 (895-2704)	2194 ± 772 (1155-3447)	**Wilcoxon p=0.012** [a]
Dissolved inorganic nitrogen:	DIN: RP	n/a	June	41 ± 47 (2-157)	113 ± 157 (3-564)	t=-2.181; p=0.052
reactive phosphate			May-July	20 ± 18 (2-55)	51 ± 54 (2-190)	**t=-2.598; p=0.025**
Total nitrogen: phosphorus	TN: TP	n/a	June	42 ± 23 (15-85)	64 ± 43 (16-150)	t=-1.901; p=0.084
			May-July	27 ± 9 (13-41)	35 ± 18 (12-65)	t=-1.720; p=0.113

* Significant at $p \leq 0.05$

[a]Non-parametric Wilcoxon signed rank test p-value

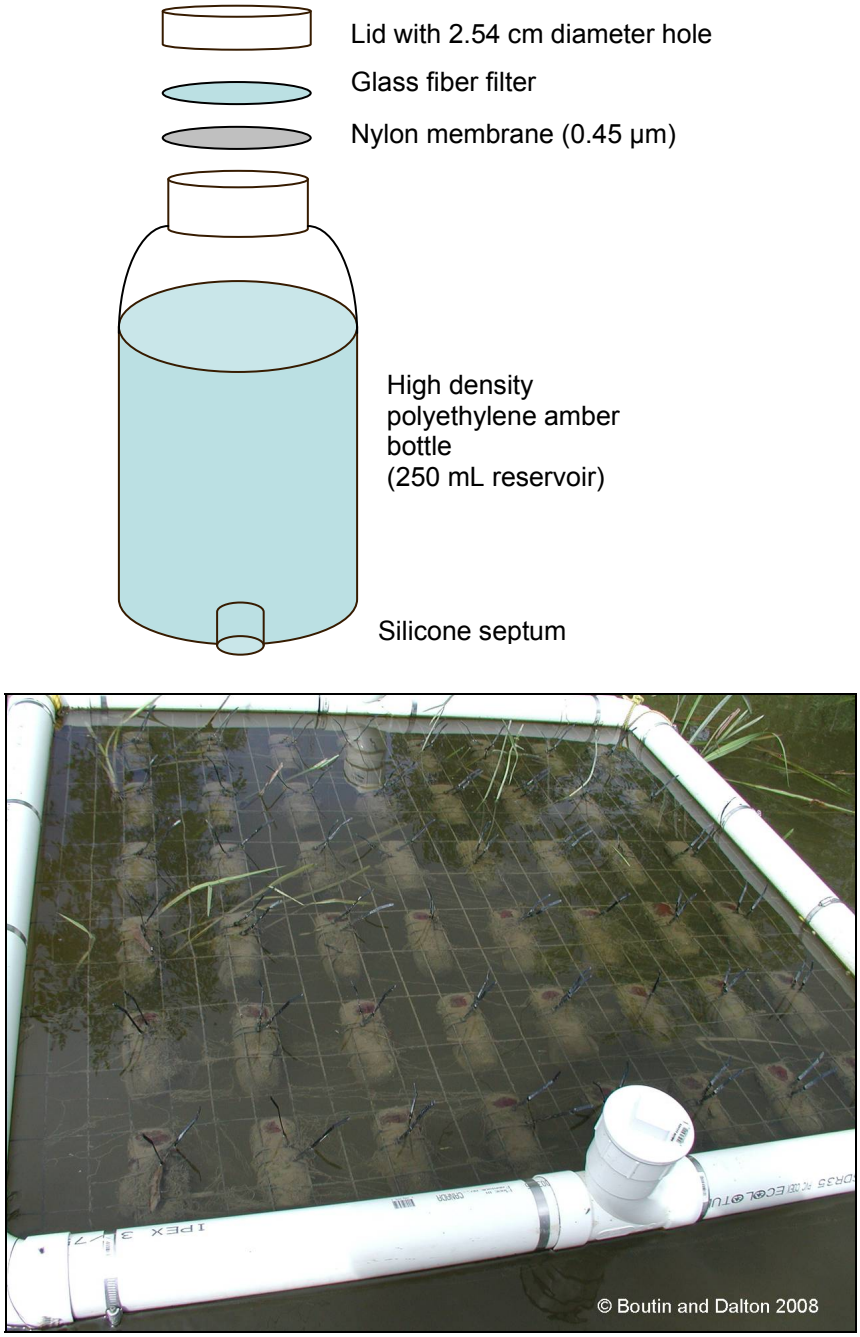

Fig. 4.1 Passive diffusion periphytometers modified from Matlock et al. (1998) and Kish (2006) and support frame for periphytometers

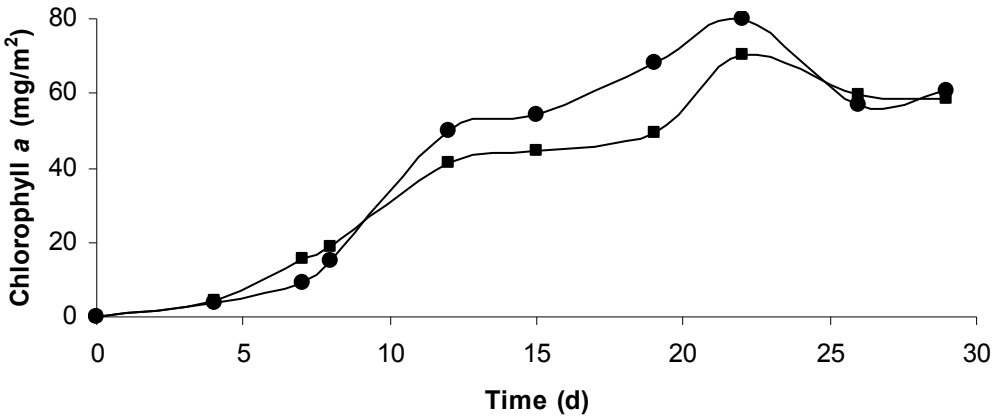

Fig. 4.2 Colonization of periphyton over 29 d in the South Castor River (2008) for ■ control (distilled water) and ● nitrate (NO_3^-), reactive phosphate (RP) and atrazine (spiked on day 7) containing periphytometers. Data were averaged from duplicate samples.

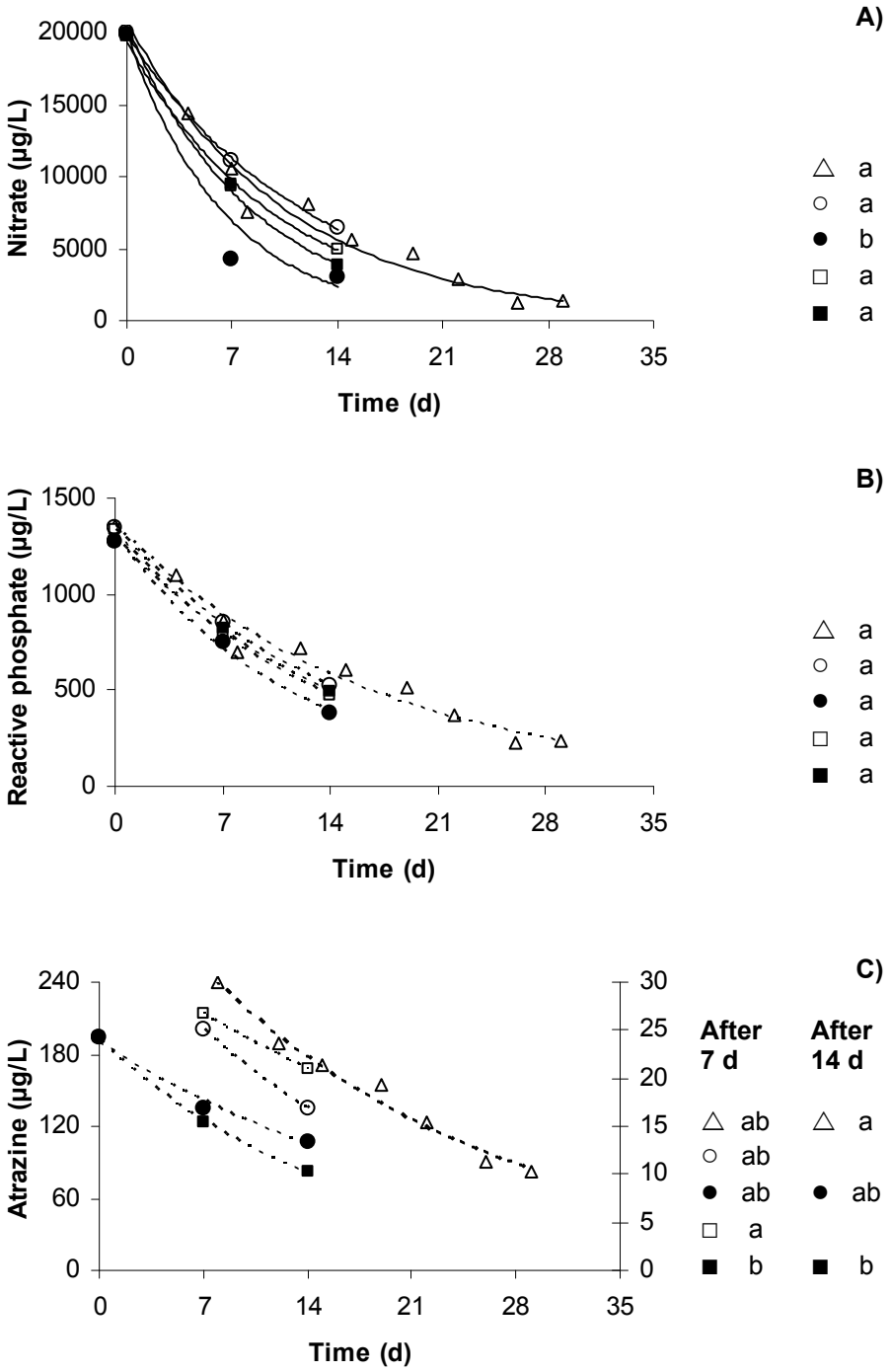

Fig. 4.3 Diffusion of A) nitrate (NO_3^-), B) reactive phosphate (RP) and C) atrazine from duplicate periphytometers over △ 29 d in 2008 in the South Castor R and over 14 d in 2009 in ○, ● the South Castor River and □, ■ the North Branch South Nation River. Atrazine was injected on day 7 (*open symbols*) or day 0 (*closed symbols*). Nominal atrazine concentrations were 20 µg/L in 2008 and 200 µg/L in 2009. Curves with different letters are significantly different ($p<0.05$).

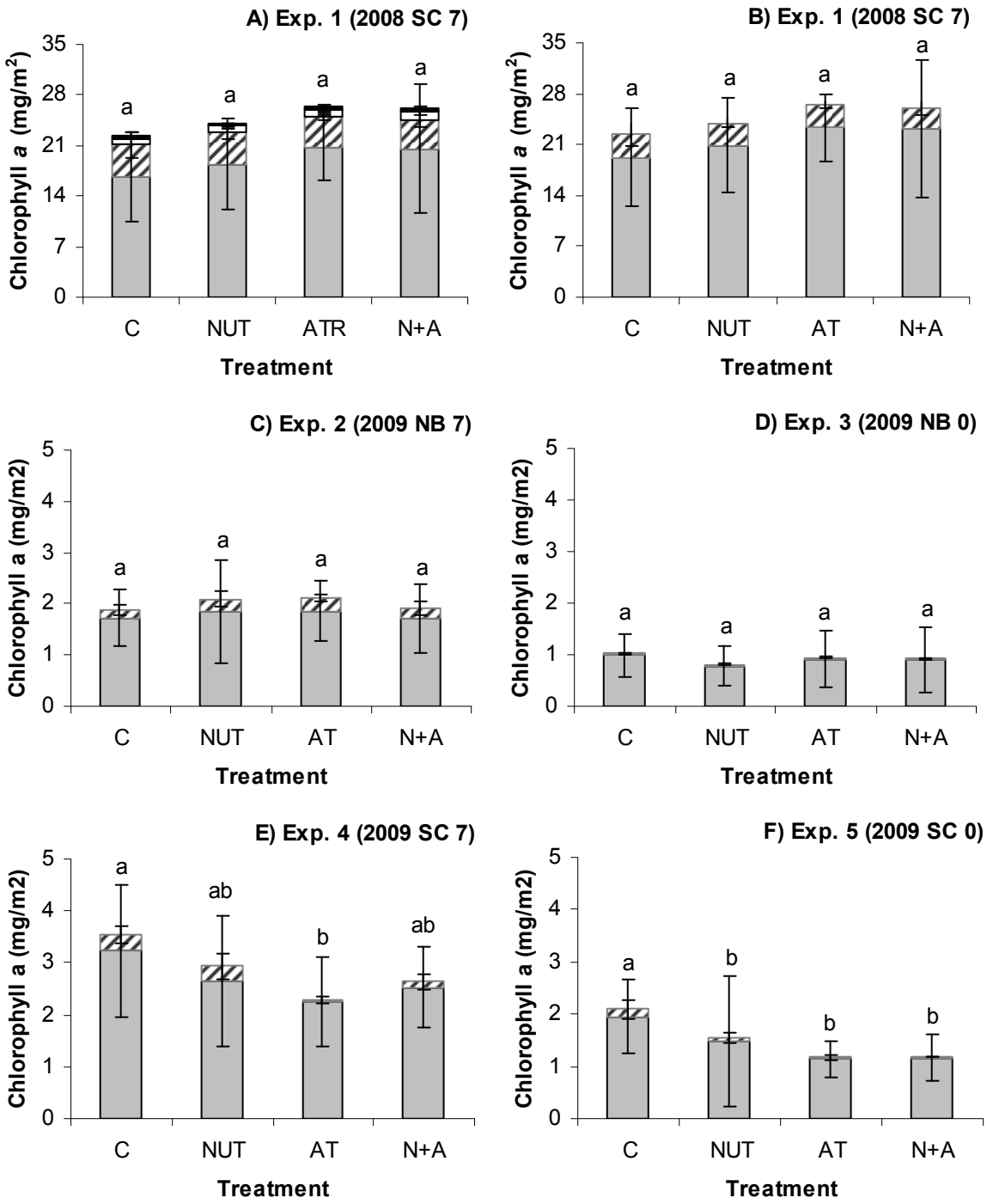

Fig. 4.4 Average contribution (± standard deviation) of the ▮ Bacillariophyta and ▨ Chlorophyta to chlorophyll *a* (mg/m^2) in periphyton colonized on periphytometers for 14 d in the South Castor R (SC) or North Branch South Nation R (NB). The □ Cryptophyta and ■ Euglenophyta are also shown in A). Treatments consisted of a distilled water control (C), nitrate and reactive phosphate nutrients (NUT), atrazine (ATR) or both nutrients and atrazine (N+A). Atrazine was spiked on day 0 or day 7. Treatments followed by different letters are statistically different ($p<0.05$), n= 20 (A,B) or n= 24 (C-F). Experiment numbers correspond to Table 4.1.

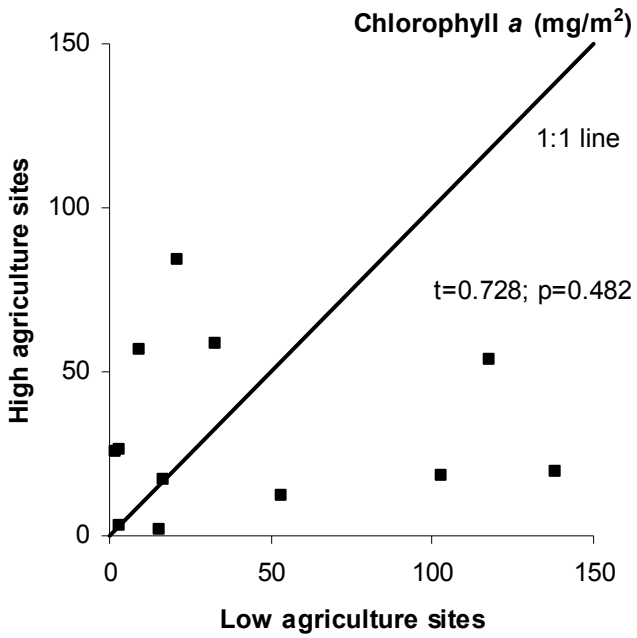

Fig. 4.5 Comparison of periphyton biomass (mg/m^2 chlorophyll *a*) at 12 paired sites (24 in total) surrounded by low and high agriculture. Paired t-test statistics and 1:1 line are shown.

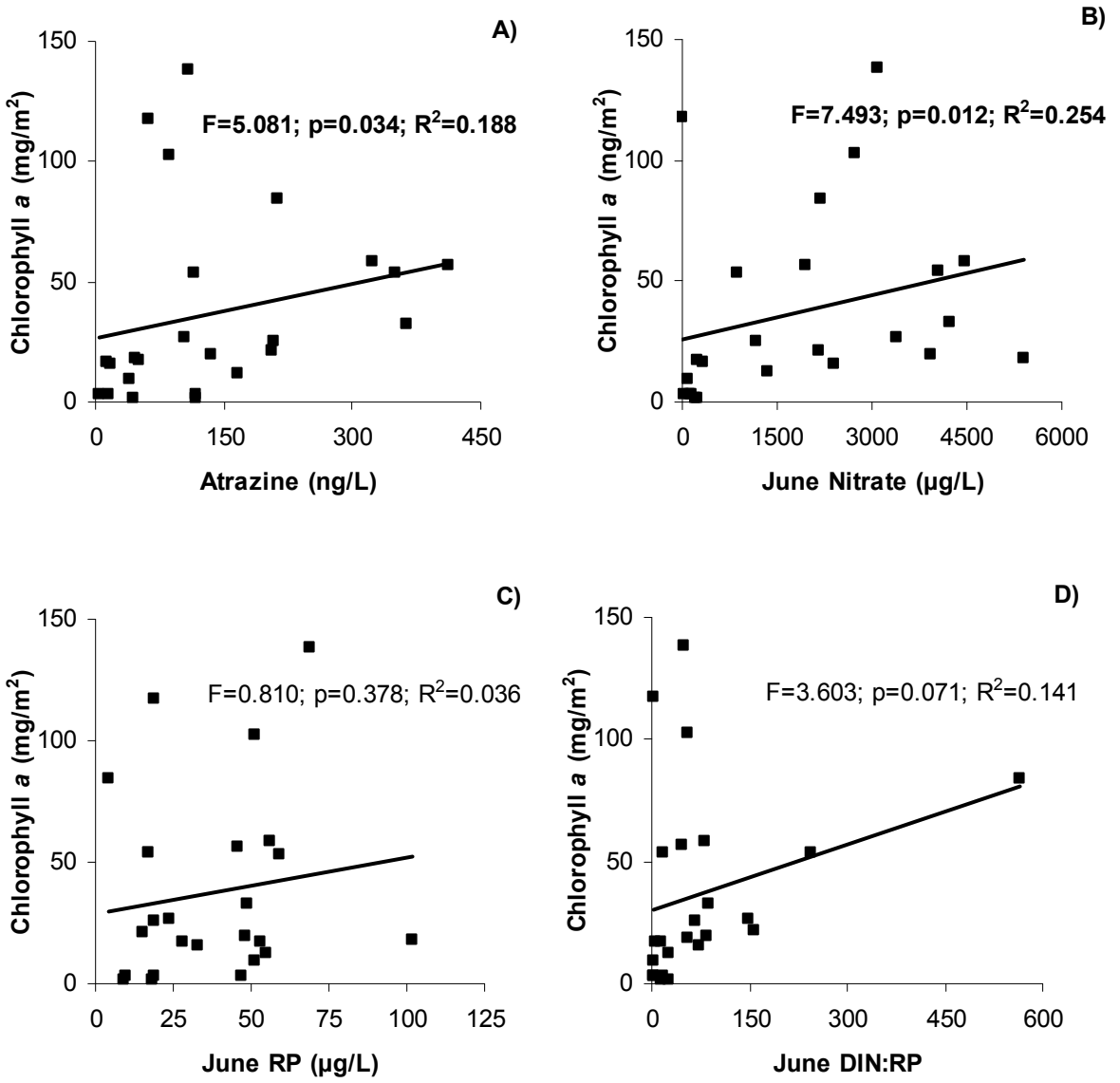

Fig. 4.6 Relationship between periphyton biomass (mg/m^2 chlorophyll *a*) at 12 paired sites (24 in total) and A) atrazine, B) June nitrate (NO_3^-), C) June reactive phosphate (RP) and D) June ratio of dissolved inorganic nitrogen to reactive phosphate (DIN:RP). Regression statistics and trend lines are shown.

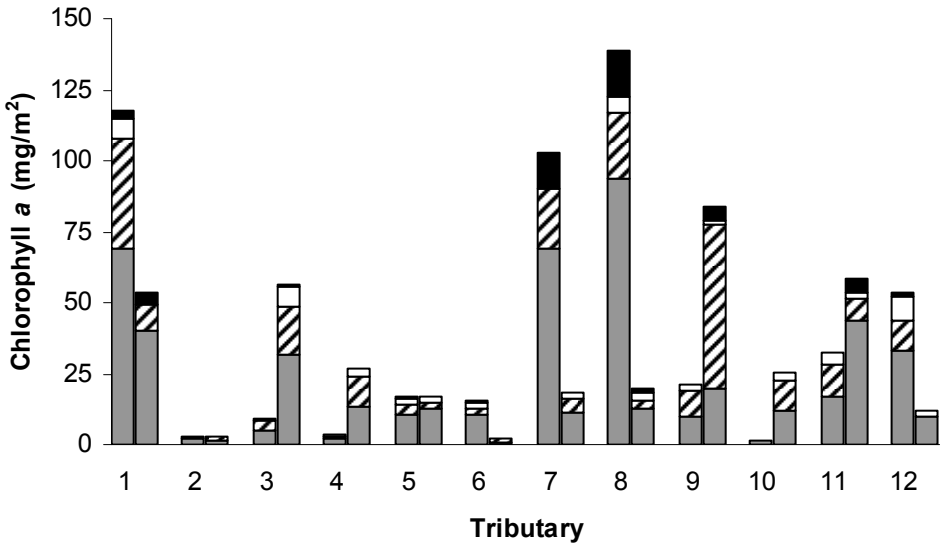

Fig. 4.7 Biomass (mg/m^2 chlorophyll *a*) of the ▯ Bacillariophyta, ▨ Chlorophyta, ■ Cryptophyta and □ Euglenophyta at 12 paired sites (24 in total). Sites were paired along tributaries. Low agriculture sites (left column) were located upstream of high agriculture sites (right column).

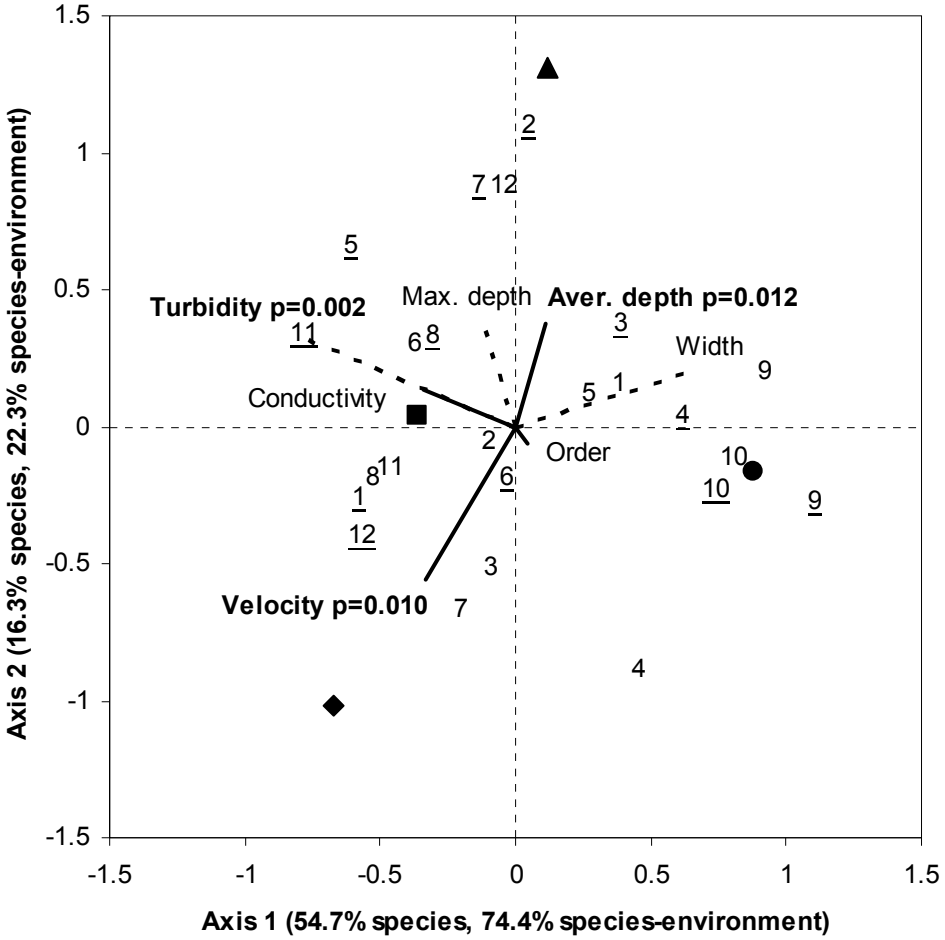

Fig. 4.8 Canonical correspondence analysis (CCA) ordinating field sites by biomass of the ■ Bacillariophyta, ● Chlorophyta, ▲Cryptophyta and ◆ Euglenophyta, constrained to variation explained by stream order, stream width, maximum depth, average depth, surface velocity, conductivity and turbidity. Twelve paired sites (24 in total) are shown with paired sites represented by the same number and high agriculture sites underlined.

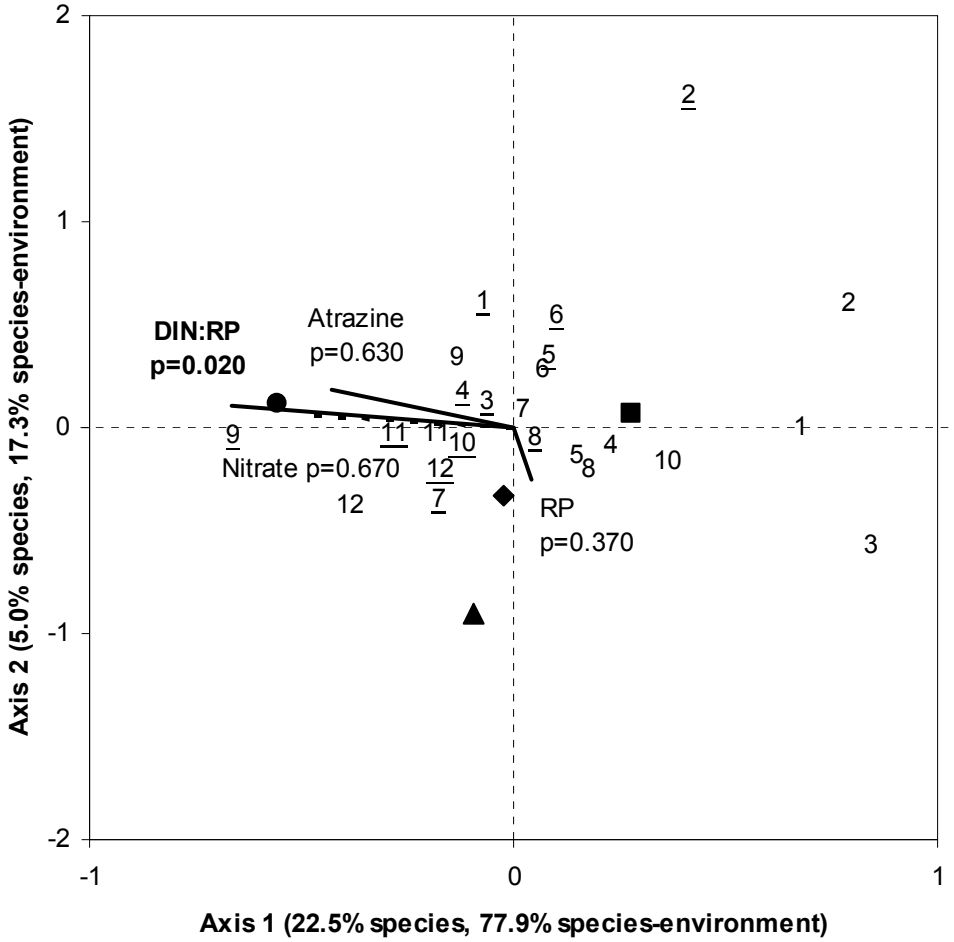

Fig. 4.9 Partial canonical correspondence analysis (partial CCA) ordinating field sites by biomass of the ■ Bacillariophyta, ● Chlorophyta, ▲Cryptophyta and ◆ Euglenophyta, constrained to variation explained by June nitrate, June reactive phosphate (RP), June ratio of dissolved inorganic nitrogen to reactive phosphate (DIN:RP) and atrazine concentrations using surface velocity, turbidity, average depth and maximum depth as covariables. Twelve paired sites (24 in total) are shown with pairs represented by the same number and high agriculture sites underlined.

Chapter 5: Comparing the sensitivity of geographically distinct *Lemna minor* populations to atrazine

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5.1 Abstract

The objectives of this study were to compare the sensitivities of field populations and a laboratory culture of a duckweed species (*Lemna minor*) to the herbicide atrazine using three different endpoints and to determine whether sensitivity to atrazine was affected by past exposure to the herbicide. *L. minor* cultures were purchased commercially or collected from field sites within an agricultural watershed and exposed to atrazine for 7 days under greenhouse conditions. Populations differed significantly in their sensitivity to atrazine. Biomass was more sensitive than frond number, while chlorophyll fluorescence was not a sensitive endpoint. Overall, the sensitivity of the various populations to atrazine was not strongly related to measures of past exposure to agriculture stressors. Positive correlations between biomass twenty-five percent inhibition concentrations (IC_{25}s), biomass estimated marginal means and in-stream atrazine concentrations were observed, providing evidence that atrazine exposure is linked to a decrease in sensitivity to atrazine. However, IC_{25}s generated for each population were similar, ranging from 19-40 µg/L and 57-92 µg/L atrazine for biomass and frond data respectively, and likely do not represent biologically significant differences in atrazine sensitivity. Given the small range in sensitivity observed between populations, commercial laboratory cultures appear to provide a good estimate of the sensitivity of field populations of *L. minor* to atrazine and should continue to be used in regulatory phytotoxicity testing.

Key words: duckweed; herbicide; population sensitivity; phytotoxicity testing

5.2 Introduction

An important aim in ecotoxicology is to determine the effects of toxic chemicals on populations and communities and ultimately on the health and functioning of ecosystems. Concentration-response studies with representative species are often used to address this goal. Members of the Lemnaceae (duckweed family) have been used extensively in toxicity testing for several decades (reviewed in Wang, 1990) and are also currently required in regulatory phytotoxicity testing for the registration of pesticides in several jurisdictions (OECD, 2002; US EPA, 2012b). *Lemna* spp. are ideal test species due to their cosmopolitan distribution (Gleason and Cronquist, 1991; Crow and Hellquist, 2000) and because they are easy to grow and manipulate as a result of their small size, rapid growth rate and structural simplicity (Hillman, 1961). As almost all reproduction is vegetative, *Lemna* spp. have an additional advantage in that genetic variability within experiments can be eliminated with the use of a single clone (Hillman, 1961). Data from toxicity tests with *Lemna* spp. are critical because they are used to predict the risk a given chemical poses to all macrophytes in the aquatic environment. There is an ongoing debate over whether *Lemna* spp. are representative of all submerged, floating, emergent and rooted macrophytes (e.g. Hanson et al., 2007; Arts et al., 2010). Perhaps, even more fundamental, is the question of whether commercial laboratory cultures are representative of populations found in the aquatic environment.

Previous studies have shown that despite their primarily vegetative reproduction, *L. minor* L. populations possess considerable genotypic and phenotypic variation (Landholt, 1986; Landholt, 1987; Vasseur and Aarssen, 1992; Vasseur et al., 1993). Current phytotoxicity guidelines do not require that a specific clone of *Lemna gibba* L. or *L. minor*

be used, the rationale being that while there is considerable genetic variability in the response of *Lemna* spp. to toxins, there is not enough information on the source of that variation (OECD, 2002). If substantial differences between populations exist, extrapolation between laboratory testing and actual field conditions may not be possible.

Extrapolation between testing with one clone to other clones and environmental conditions may be further complicated because past exposure to stressors may leave an imprint long after the stress occurred. Ecological communities retain information about events in their history at a number of different levels of organization and this information may not always be measurable at a given point in time (Matthews et al., 1996; Landis et al., 1996; Landis et al., 1997). In the context of chemical stressors, the pollution-induced-community-tolerance concept predicts that a toxic agent will exert selection pressure towards a global increase in tolerance to that particular toxic agent (Blanck et al., 1988). This concept has been well documented for the evolution of herbicide resistant weed species, which are now represented by over 200 herbicide resistant plant species worldwide, of which 69 are resistant to photosystem II inhibitors (Heap, 2011).

Of the photosystem II inhibitor herbicides, the triazine atrazine is of particular interest because it has been studied extensively and is a common contaminant in surface waters of North America (Solomon et al., 1996). Phytoplankton communities have been shown to develop reduced sensitivity to atrazine following exposure in both mesocosms (DeNoyelles, 1982; Knauer et al., 2010) and along a natural gradient of atrazine contamination in the field (Dorigo et al., 2004) through changes in community structure. It is currently unknown whether the sensitivity of *Lemna* spp. populations to atrazine is modified following exposure as a result of species level acclimation.

In the present study, I compared the sensitivity of a commercially available laboratory culture of *L. minor* and six different field populations to the herbicide atrazine. The field populations were collected from tributaries in the South Nation River watershed in Eastern Ontario, Canada, comprising 3915 km^2. The watershed is predominately agricultural with a sparse population and no major industry. The land use gradient ranges from approximately 37% agriculture near the headwaters close to Spencerville in the southwest of the watershed to 77% agriculture near Winchester and Chesterville in the center of the watershed (Statistics Canada, 2006). The dominant crops in the watershed are corn (*Zea mays* L.) and soybean (*Glycine max* L. (Merr.)). Atrazine is used extensively on corn in Ontario, where it is the second most commonly used herbicide after glyphosate with an estimated 448,071 kg of active ingredient applied annually (McGee et al., 2010). Usage of atrazine in the South Nation River watershed is typical of usage in other agricultural watersheds and atrazine has been detected within surface waters of the watershed between mid-April and late October (Fischer et al., 1995).

The objectives of this study were: (1) to compare the sensitivities of *L. minor* field populations and a commercially available laboratory culture to atrazine using three different endpoints (biomass, frond number and chlorophyll fluorescence), and (2) to determine whether the sensitivity of field populations was affected by prior exposure to atrazine and other agricultural stressors. Several endpoints were selected because two measures of growth, biomass and frond number, are recommended in current *Lemna* spp. inhibition test guidelines (OECD 2002; Environment Canada, 2007; US EPA, 2012b). The chlorophyll fluorescence parameter Fv/Fm is a measure of the maximum achievable efficiency of photosystem II, with values for healthy plants typically ranging from 0.79-0.84 (Rosenqvist

and van Kooten, 2003) and was selected because chlorophyll fluorescence is considered to be a rapid, non-invasive and sensitive measure of photosynthetic performance (Lichtenthaler and Rinderle, 1988; Schreiber et al., 1998). It was hypothesized that the sensitivity of *L. minor* populations to atrazine would decrease with increasing past exposure to atrazine.

5.3 Material and Methods

5.3.1 *Lemna minor* populations

Lemna minor plants were collected from tributaries representing six different hydrologically independent sub-watersheds of the South Nation River watershed, Ontario, Canada during the first week of September, 2008 (Table 5.1). The streams were chosen to reflect a gradient of agricultural intensity and likely past exposure to the herbicide, atrazine (6-chloro-*N*-ethyl-*N*′-(1-methylethyl)-1,3,5-triazine-2,4-diamine). At each 20 m long open canopy field site, at least 50 plants were collected in the stream channel and near the water's edge along both sides of the stream bank. In addition to field-collected populations, an aseptic culture of *L. minor* (CPCC 490) was purchased commercially (Canadian Phycological Culture Centre (CPCC), Canada). CPCC 490 was initially collected and isolated from Wainfleet, ON in 1977 and deposited to CPCC in 1999.

The streams were characterized in terms of their surrounding agricultural land use as well as their nitrate and atrazine concentrations. Agricultural intensity at each field site was determined by calculating the percentage of annual cropland in a zone (500 m radius) surrounding each site as well as the percentage of land in perennial crops and pasture. The percentage of undisturbed natural habitat was estimated similarly by calculating the

percentage of wetland, forest, exposed land and shrub land surrounding each site. The remaining land area consisted of land with urban or residential development. Calculations were made from data provided by Agriculture and Agri-Food Canada (2008).

In-stream nitrate concentrations provided an additional measure of agricultural intensity. Elevated concentrations of nitrate have been shown to be a good indicator of agricultural contamination from synthetic fertilizers and manure (Dubrovsky et al., 2010) and seasonal sampling in the South Nation River watershed has also shown that nitrate is a good indicator of agricultural intensity. Duplicate mid-channel integrated water samples (300 mL in PET bottles) were taken twice at each site between 5 and 25 June 2008. Samples were taken using a pole sampler to collect water upstream of the wading location to avoid disturbance and contamination of water and sediments. Samples were sent to the Robert O. Pickard Environmental Centre Laboratory of the City of Ottawa (Canada) for nitrate analysis following established methods of the Ontario Ministry of the Environment (2007a). Briefly, nitrate was measured by ion chromatography using a carbonate/bicarbonate eluent on an ion exchange resin. Nitrate was converted to its conductive acid form and the eluent into carbonic acid in a chemical suppressor. Conductivity was subsequently used to quantify nitrate concentrations with an ion chromatograph system (Dionex® DX100, USA). The method detection limit was 4 μg/L. The average in-stream nitrate concentration for each site was calculated by averaging duplicate values from both sampling days.

The likelihood and degree of past exposure to atrazine at the field sites was estimated by measuring in-stream atrazine concentrations using microtiter plate format enzyme-linked immunosorbent assays (ELISA) (Abraxis LLC, USA). Previous work has shown that although ELISA may overestimate atrazine concentrations, likely due to cross-reactivity

with similar pesticides, concentrations obtained with ELISA are strongly correlated with those obtained from LC-MS/MS (Černoch et al., 2011) and GC-MS (Byer et al., 2011). Duplicate mid-channel integrated water samples for atrazine analysis were collected in 1 L amber borosilicate bottles twice at each site between 5 and 25 June 2008. Bottles were rinsed 3× with stream water at each site and mid-channel, integrated samples collected using a pole sampler. Twenty mL sub-samples were frozen and stored at -20°C in 40 mL amber borosilicate vials with PTFE caps. Atrazine concentrations were quantified with a Spectramax® Plus UV/VIS spectrophotometer with a microplate reader using Softmax® Pro V. 3.1 (Molecular Devices, USA). Atrazine in each sample was measured in duplicate wells and on two different days. Atrazine values for each site were averaged from these values for both sampling days. The method detection limit was 0.050 µg/L.

5.3.2 Growth conditions

Field-collected plants were stored at 4°C in stream water for several days. They were then rinsed with tap water and transferred to 500 ml Erlenmeyer flasks containing 200 ml sterile Hoagland's No. 2 basal salt mixture (Sigma-Aldrich, Canada). Following a period of acclimation (~ one week), field-collected plants were sterilized in a 0.5% sodium hypochlorite solution for at least one minute and subsequently rinsed thoroughly with sterile media to eliminate algae associated with the plants.

Cultures of all seven populations were maintained in a greenhouse at the Center for Advanced Research in Environmental Genomics (University of Ottawa, Canada). Cultures were grown with an ambient air temperature of 25°C (±2°C) and 12 hours of artificial lighting (400 W high pressure sodium lights) supplementing natural sunlight. Average

photosynthetic photon flux density ranged from 159 μmol/m^2/s (cloudy day) to 1,750 μmol/m^2/s (sunny day at noon) (LI-250A light meter, LI-COR Biosciences, USA). Cultures were transferred to fresh medium every 7-10 days as outlined in the Environment Canada protocol EPS1/RM/37 (Environment Canada 2007).

5.3.3 Atrazine sensitivity range-finding experiment

All experiments were conducted using the commercial herbicide formulation, Atrazine 480 (United Agri Products Canada, Inc., Canada), containing 451 g/L of the active ingredient (a.i.) atrazine and 29 g/L of related triazines.

A sensitivity range-finding concentration-response test was conducted using the culture collection strain CPCC 490. Methods followed guidelines outlined by Environment Canada (2007). The test consisted of eight concentrations of Atrazine 480 following a geometric progression and a control with five replicates each. A working stock solution of 96 μg/ml of the active ingredients of Atrazine 480 (atrazine+related triazines) was dissolved in distilled water and used to yield final nominal concentrations of 0, 7.5, 15, 30, 60, 120, 240, 480 and 960 μg/L a.i. Plants were grown in 250 ml Erlenmeyer flasks containing 100 ml of spiked Hoagland's media. Two healthy, three-frond *L. minor* plants with rootlets were transferred from 7-10 day old cultures into each experimental flask aseptically and grown in the greenhouse as described above for seven days between 3 and 10 February 2009.

5.3.4 Endpoints

Plants were harvested after a 7-day test period and biomass, frond number and chlorophyll fluorescence were measured. Although no measure of initial biomass was made,

all experimental flasks began each experiment with six fronds. All plants from each flask were placed in 47 mm diameter plastic petri dishes containing sufficient volumes of distilled water to float the plants. The total number of fronds was counted. Plants were subsequently dark-adapted for a minimum of 30 min. Chlorophyll fluorescence was measured using a kinetic fluorescence charge-coupled device (CCD) camera (Photon Systems Instruments, Czech Republic). Measuring flashes were approximately 0.03 μmol/(m^2 s^1) in intensity and 10 ms in duration. Saturating light pulses, generated from a 250 W halogen bulb were 1.6 s in duration at an intensity of 2,000 μmol/(m^2 s^1). Actinic light, generated from two LED panels was 60 s in duration at an intensity of 175 μmol/(m^2 s^1) and had a maximum wavelength of 620 nm. Plants were then dried at 60°C for 24 hours and weighed to determine biomass.

5.3.5 Comparison of atrazine sensitivity between populations

Following the sensitivity range-finding experiment with CPCC 490, a concentration-response test consisting of three concentrations of Atrazine 480 and a control with six replicates each was conducted separately for all seven *L. minor* populations. Nominal concentrations of 20, 80 and 160 μg/L a.i of Atrazine 480 were selected based on biomass data from the sensitivity range-finding experiment. These concentrations were intended to encompass the twenty-five percent inhibition concentration (IC_{25}), defined as a point estimate of the concentration required to cause a 25% reduction in a given endpoint compared to the controls, for all seven populations. A single working stock solution, containing 16 μg/mL of the active ingredients of Atrazine 480 (atrazine + related triazines) dissolved in distilled water, was prepared and used for all treatment flasks for all seven

populations. Plants were grown and treated as described above with all tests conducted between 17 February and 26 March 2009. Following a 7-day experimental period, frond number, chlorophyll fluorescence and biomass were measured as described above. Chlorophyll fluorescence was measured for three replicates only, whereas six replicates were measured for biomass and frond number.

5.3.6 Statistics

A twenty-five percent inhibition concentration was calculated for each endpoint of the sensitivity range-finding experiment. Twenty-five percent inhibition concentrations were chosen because they represented biologically significant reductions in biomass, frond number and chlorophyll fluorescence. Concentration-response curves were fit individually using non-linear regression with one of five models: linear, exponential, logistic, gompertz or hormetic (SYSTAT® 11, USA) (Environment Canada, 2005). Data were transformed if necessary to best meet the assumptions of normality of residuals and homogeneity of variance, which were evaluated with Shapiro-Wilk's and Levene's tests respectively. For the sensitivity range-finding experiment, data were fit as follows: biomass data were square root transformed and fit with a hormetic model, frond data were $\log_{10}$ transformed and fit with a logistic model, and Fv/Fm data were cube transformed and fit with a hormetic model. For the population experiment, IC_{25}s were calculated separately for each population and an overall IC_{25} was calculated using data for all seven populations. The overall best transformation and model was selected and used for all seven populations to avoid bias due to transformation and model selection and to allow for comparison between population IC_{25}s. Biomass, frond and Fv/Fm data were all fit with the gompertz model. Frond data

were square root transformed while general linear model assumptions were best met using untransformed biomass and Fv/Fm data.

For each endpoint, two one-way analysis of variances (ANOVAs) were conducted to determine whether population or test start date had significant effects on the control values of the populations. A two-way ANOVA was then conducted to statistically determine if the sensitivity of the seven *L. minor* populations to atrazine differed. Population and atrazine concentration were treated as fixed factors using Type III sums of squares (SPSS® 20, USA). Each value was represented as a percentage of the average of the control values to eliminate effects due to intrinsic differences in growth rates between populations. Percent of control values were calculated by dividing each individual value by the average value of the six replicate control flasks. Assumptions of normality of residuals and homogeneity of variance were evaluated with Shapiro-Wilk's and Levene's tests respectively. Data were transformed to best meet these assumptions. Biomass data were not transformed, frond data were square-root transformed and Fv/Fm data were cube transformed. Following ANOVA, estimated marginal means were calculated by averaging the modeled percent of control values from all three treatment concentrations (for each endpoint separately). Significant statistical differences between populations were then evaluated using Sidak pairwise comparisons.

Pearson's correlations were used to assess whether there was a significant relationship between population sensitivity to atrazine and measures of agriculture intensity at the different field sites. The correlation between IC_{25}s and in-stream nitrate, in-stream atrazine, percentage of annual crops and percentage of natural vegetation were examined (SPSS® 20, USA). Correlations were not conducted for IC_{25}s generated from Fv/Fm

because these were outside of the range of atrazine concentrations used. The relationship between variables was illustrated using simple linear regression and 95% confidence intervals (based on individual predicted values) where the correlations were statistically significant. Correlations were similarly conducted between population estimated marginal means and measures of agricultural intensity. Correlations were also conducted between test start dates, average control values of biomass, average control values of frond number and their respective IC_{25}s, estimated marginal means and measures of agricultural intensity.

5.4 Results

5.4.1 Characteristics of field sites

Annual crops constituted a substantial portion of overall land use at several field sites where *L. minor* plants were collected (Table 5.1). The average percentage of land in annual crops for all six sites was 51.6% with a range from 6.9 to 83.9% (Table 5.1). Sites were more similar in terms of percentage of perennial crops and pastureland with an average of 13.0% and range from 7.2 to 26.0% land area (Table 5.1). Many regions of the South Nation River watershed have sparse urban and residential development and this trend was also observed at the six field sites. Only two sites, the East Castor River and the South Nation River headwaters, contained land with urban or residential development within the 500 m radius zone examined and residential development comprised only 3.3 and 2.9% of total land use at these sites respectively. Sites with high percentages of land in annual crops tended to have low percentages of natural habitat and vice versa (Table 5.1).

June in-stream nitrate concentrations were used as a proxy of fertilizer use and a measure of agricultural intensity. Nitrate concentrations spanned a wide range of values

from below detection (4 μg/L) to 5,795 μg/L. Sites with high nitrate concentrations tended to have lower percentages of surrounding natural habitat (Table 5.1). Nitrate concentrations were higher later in June (18-25 June 2008) compared to the earlier sampling period (5-10 June 2008) for all but one field site but this overall trend was not significant ($t=0.264$; $p=0.803$).

June in-stream atrazine concentrations were used as a measure of historical atrazine exposure for the field-collected *L. minor* populations. Atrazine concentrations varied from below detection (0.050 μg/L) to 0.916 μg/L. Sites with lower percentages of surrounding natural habitat tended to have higher atrazine concentrations. Similarly to nitrate, atrazine concentrations were highest during the second sampling period in June (18-25 June 2008) for all but one field site but this overall trend was not significant ($t=1.190$; $p=0.287$).

5.4.2 Sensitivity of *Lemna minor* to atrazine

A sensitivity range-finding concentration-response experiment was conducted using the culture collection strain CPCC 490 to determine the sensitivity of *L. minor* to atrazine. Non-linear regression was used to calculate IC_{25}s for three endpoints: biomass, frond number and chlorophyll fluorescence (Fv/Fm). Biomass was the most sensitive endpoint whereas chlorophyll fluorescence was the least sensitive endpoint (Fig. 5.1). Variability between replicates tended to be highest at low concentrations of atrazine, except for chlorophyll fluorescence (Fig. 5.1).

5.4.3 Comparison of atrazine sensitivity between populations

ANOVA indicated that the control values of the seven different populations of *L. minor* differed significantly in terms of biomass, frond number and Fv/Fm ($F \geq 10.9$; $p < 0.001$). The starting date of each test was a significant factor in explaining differences between population controls ($F \geq 4.7$; $p \leq 0.014$). Overall, average biomass for the controls was 4.7 mg and ranged between 3.7 and 5.9 mg. Of the three endpoints, frond number varied most widely with an overall control average of 75 and a range between 60 and 97 fronds. Fv/Fm varied least widely with an overall control average of 0.828 and a range between 0.816 and 0.833. Initial, intrinsic differences between populations and temporal differences between tests were controlled in subsequent analysis by using percentage of control values.

A significant effect of atrazine concentration was observed for all three endpoints ($F \geq 96.1$; $p < 0.001$). Significant differences between populations were also observed for all three endpoints (Table 5.2; $F \geq 15.3$; $p < 0.001$). In addition, significant interactions between concentration and population were observed for biomass ($F = 2.9$; $p = 0.001$) and Fv/Fm ($F = 3.4$; $p = 0.002$) but not for frond number ($F = 1.0$; $p = 0.442$). This suggests that the response of the different populations to atrazine differs depending on concentration for biomass and chlorophyll fluorescence but not for frond number. As with the sensitivity range-finding experiment, biomass was the most sensitive endpoint and chlorophyll fluorescence was the least sensitive endpoint. Overall, reductions of 53.9, 44.6 and 2.3% compared to control values were observed for biomass, frond number and chlorophyll fluorescence respectively (Table 5.2). Sidak pair-wise comparisons demonstrated that

populations had both similarities and significant differences in their response to atrazine (Table 5.2).

The relative sensitivities of the six field populations and one commercial culture of *L. minor* to atrazine were examined further using non-linear regression to estimate IC_{25}s. Again, biomass was the most sensitive endpoint, followed by frond number (Table 5.3). When biomass from all populations was pooled, an overall IC_{25} value of 30.2 μg/L was calculated compared to 75.0 μg/L when frond data were used (Table 5.3). Twenty-five percent inhibition concentrations for individual populations ranged from 19 to 40 μg/L for biomass and from 57 to 92 μg/L for frond number (Table 5.3). In contrast, Fv/Fm was not a sensitive endpoint and yielded IC_{25}s outside of the range of concentrations tested (Table 5.3).

5.4.4 Atrazine sensitivity and exposure to agricultural stressors

Pearson's correlations were conducted to determine whether there was a relationship between the sensitivity of the various populations to atrazine (measured using IC_{25}s generated from biomass and frond data) and exposure to agricultural stressors at the sites where each field population was initially collected (measured using in-stream atrazine concentrations, in-stream nitrate concentrations, percentage of annual crops and percentage of natural habitat). There were no significant correlations between IC_{25}s generated from frond data and the four indicators of agriculture (Fig. 5.2; Pearson correlation coefficient (PCC)$\leq$0.603; p$\geq$0.206). Similarly, there were no significant correlations between IC_{25}s generated from biomass data and nitrate concentration, percentage of annual crops or percentage of natural habitat (Fig. 5.2; PCC$\leq$0.798; p$\geq$0.057). However, there was a significant correlation between in-stream atrazine concentrations and IC_{25}s calculated from

biomass data (Fig. 5.2; PCC=0.832; p= 0.040). Correlations between estimated marginal means and measures of agricultural intensity yielded similar results in terms of the significance, strength and direction of the correlations. The only significant correlation was between biomass estimated marginal means and in-stream atrazine concentrations (PCC=0.863; p=0.027).

No significant correlations were observed between biomass and frond average control values or test start date and measures of agricultural stressors (PCC≤|0.435|; p≥ 0.389). Similarly, no significant correlations were observed between biomass and frond average control values or test start date and their respective IC_{25}s and estimated marginal means (PCC≤0.362; p≥ 0.481). Although differences in growth between populations were controlled using percent control data, these correlations suggest that initial differences in growth between populations were not contributing factors to observed patterns of sensitivity between populations.

5.5 Discussion

5.5.1 Characteristics of field sites

The level of agricultural intensity surrounding the field sites where *L. minor* populations were collected varied from 6.9 to 83.9 % of land in annual crops, with four of the six sites surrounded by over 50% annual crops (Table 5.1). Similarly, sites varied in terms of in-stream nitrate concentrations, with sites with the highest nitrate levels likely representing areas exposed to high levels of fertilizer run-off from surrounding agricultural fields. In a comprehensive survey of streams in North America, Dubrovsky et al. (2010) estimated background concentrations of nitrate due to natural processes to be 240 µg/L and

found that over 90% of 190 urban and agricultural streams exceeded this value. In the present study, three sites exceeded this estimate of background concentration. All but one field site showed evidence that *L. minor* field populations had been previously exposed to atrazine. Overall, in-stream atrazine concentrations were far below peak concentrations of 20 µg/L that have been observed in some North America streams (Solomon et al., 1996), but a gradient of atrazine contamination in the South Nation River watershed was clearly observed.

5.5.2 Atrazine sensitivity and past exposure to agricultural stressors

Significant statistical differences were observed between populations. However, the sensitivity of the various populations was not strongly related to measures of past agricultural stressors, except for significant positive correlations between biomass measures and in-stream atrazine concentrations. Changes in sensitivity of *L. minor* populations following exposure to atrazine have not been well documented in the literature. However, changes in sensitivity of duckweeds following exposure to other chemicals have been observed. Kováts et al. (2011) demonstrated that sensitivity of *L. minor* collected along a gradient of eutrophication showed differences in sensitivity to lyophilized *Microcystis aeruginosa* Kutz.em. Elenkin, a cyanobacterium capable of producing the hepatotoxin microcystin. However, their data did not reveal a consistent pattern of sensitivity to different concentrations of microcystin (Kováts et al., 2011) and negative effects of microcystins on *Lemna* spp. have also been questioned (LeBlanc et al., 2005). Kiss et al. (2001) reported an inverse relationship between growth rate and sensitivity to potassium chromate for five populations of *L. minor* and observed that a population previously exposed to leachate from

a waste disposal site had a 50% higher lethal concentration compared to other populations. The most convincing evidence that the sensitivity of different *L. minor* clones may be affected by exposure to contaminants is found in studies of trace metals. Kanoun-Boulé et al. (2009) found that copper uptake and sensitivity was higher in a *L. minor* population originating from an uncontaminated pond compared to a population originating from an abandoned uranium mine and concluded that *L. minor* is capable of developing some capacity to limit uptake of trace metal contaminants. Support for this conclusion can be found from an earlier study where a population of *L. minor* exposed to heavy metals showed an increase in esterase activity and the presence of three esterase isozymes not found in an unexposed population (Mukherjee et al., 2004). Similarly to the present study, Mazzeo et al. (1998) observed an overall lack of variability in the response of fourteen clones of *L. gibba* to the triazine simazine. Although previous exposure to simazine was not known, Mazzeo et al. (1998) attributed the general lack of difference in response to there being few molecular modifications for the site of herbicide action and a large amount of pheonotypic plasticity in the various clones.

Phenotypic plasticity has been previously studied in Lemnaceae. In a study with eight genotypes of *L. minor* from four continents, the trait best representing fitness (biomass) was the least plastic, whereas the trait least related to fitness (root length) showed the most plasticity (Vasseur and Aarssen, 1992). No relationship was found between genetic and phenotypic divergence and the authors concluded that plasticity may not be adaptive in *L. minor*. These findings were consistent with those of Landolt (1957) who studied 60 clones comprising 12 Lemnaceae species and found that differences between clones of the same species did not appear to be ecologically significant and may be the result of chance,

perpetuated by vegetative growth. In the present study, phenotypic plasticity in the populations of *L. minor* in terms of biomass, frond number and chlorophyll fluorescence may have reduced observable differences in sensitivity to atrazine.

There are also environmental considerations as to why the populations of *L. minor* in the present study generally responded similarly to atrazine. The first is that in-stream concentrations measured at the field sites were well below the IC_{25}s and may not have been sufficiently high to induce a dramatic increased tolerance to atrazine. The second explanation is that in-stream herbicide concentrations are highly variable and occur in pulses following rain events. It is possible that the *L. minor* populations are not exposed to atrazine for a sufficient duration to induce increased tolerance. It is interesting to note that despite factors limiting differences between *L. minor* populations, a general trend of decreasing sensitivity to atrazine with increasing levels of past exposure to intense agricultural was observed. This trend was only statistically significant for correlations between biomass IC_{25}s, biomass estimated marginal means and in-stream atrazine concentrations, suggesting that past atrazine exposure and not other agricultural stressors is linked to a decrease in the sensitivity of *L. minor* populations to atrazine. However, given the small range in sensitivities, it is unlikely that the observed decrease in sensitivity with past exposure to atrazine represents a biologically meaningful reduction in the overall sensitivity of the populations to atrazine.

5.5.3 Endpoint sensitivity

Biomass was the most sensitive endpoint and chlorophyll fluorescence the least sensitive endpoint in both the atrazine sensitivity range-finding experiment and in the

experiment conducted to compare field populations. The results of the present study were in contrast to Kirby and Sheahan (1994) who found no major differences in the sensitivity of *L. minor* to atrazine when chlorophyll *a* concentration, fresh weight and frond production were compared. However, they did find that the sensitivity of frond production was variable, being more sensitive than fresh weight for mecoprop but less sensitive than fresh weight for isoproturon. In general, using frond number may underestimate the toxicity of a chemical compared to biomass measurements because chemical stress may result in the production of numerous small buds that are considered equal to larger, healthier fronds (Wang, 1990).

Chlorophyll fluorescence is regarded as a rapid, non-invasive and sensitive measure of photosynthetic performance and the overall physiological status of plants (Lichtenthaler and Rinderle, 1988; Schreiber et al., 1998). However, the success of the chlorophyll fluorescence parameter Fv/Fm in assessing the toxicity of atrazine to *Lemna* spp. has been variable in previous studies. Küster and Altenburger (2007) found Fv/Fm to be a very effective endpoint for *L. minor* and observed up to 100% inhibition of Fv/Fm following 1 and 24 hr exposure to atrazine (15-1,920 µg/L), resulting in IC_{50}s of 69 and 28 µg/L respectively. Kumar and Han (2010) also found Fv/Fm to be a sensitive endpoint for field-collected *Lemna* spp. and estimated a 50% effective concentration of 69 µg/L following 7 days of exposure to atrazine (25-800 µg/L). In the sensitivity range-finding experiment, Fv/Fm showed a fairly narrow range of response to atrazine, decreasing by only 55% of control values at 960 µg/L and resulting in an IC_{25} of 206 µg/L. Similarly to the present study, Frankart et al. (2003) detected a significant but weak effect of atrazine (100 µg/L) on Fv/Fm in *L. minor* plants following 48 hours of exposure. The reason for conflicting results between these few studies is not entirely clear but could possibly be the result of differences

between genotypes of *Lemna* spp. In a study of fourteen clones of *L. gibba*, total chlorophyll per unit biomass increased in response to the triazine simazine (0.1 to 1 mg/L) for some but not all clones (Mazzeo et al., 1998). Overall, it appears that chlorophyll fluorescence may be useful as an early indicator of herbicide damage but is not a sensitive endpoint for 7-day experiments.

5.5.4 Atrazine sensitivity and test system variability

Twenty-five percent inhibition values generated from biomass data were 52 and 33 µg/L atrazine for the range-finding and population experiments respectively. Values generated with frond data differed more widely, with IC_{25}s of 167 and 76 µg/L atrazine for the range-finding and population experiments respectively. Production of numerous small fronds in some replicates of the range-finding experiment may have contributed to the high variability observed at low concentrations of atrazine and inflated the IC_{25}. Growth rates of *L. gibba* L. were found to vary by a factor of two over the course of a year in a study that conducted 35 different 7-day experiments (Scherr et al., 2008). The authors found a poor correlation between environmental conditions such as temperature and relative humidity and hypothesized that the differences in growth rates may be the result of endogenic periodicities in *L. gibba*. In the present study, average biomass of the controls was 3.9 mg for both experiments, whereas frond number was 60 for the sensitivity range-finding experiment and 78 for the comparison of populations. Endogenic periodicities may have contributed to differences in frond production between the two test periods. Differences in IC_{25}s may also be attributable to differences in model selection, data transformation and number of concentrations between the two experiments.

5.5.5 Suitability of commercial cultures in risk assessment

A comparison of IC_{25} values between CPCC 490 and field-collected populations of *L. minor* illustrated that the sensitivities of the various populations to atrazine were quite similar. Values of IC_{25}s generated from biomass data ranged from 19 to 40 µg/L, while values generated from frond data were slightly higher, ranging from 57 to 92 µg/L. The commercial laboratory culture CPCC 490 had IC_{25}s falling within the middle of this range (33 and 76 µg/L atrazine for biomass and frond number respectively), suggesting that this culture provided a very good estimate of the sensitivity of *L. minor* to atrazine and should continue to be used in regulatory phytotoxicity testing.

5.6 Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

Table 5.1 Characteristics of field sites in the South Nation River watershed, Canada where populations of *Lemna minor* L. were collected

Site	Geographical location	Sample collection dates (2008)	Percentage of land in annual crops (%)	Percentage of land in perennial crops and pasture (%)	Percentage of undisturbed natural habitat (%)	Vegetation immediately surrounding site	In-stream nitrate (µg/L) n= 4	In-stream atrazine (µg/L) n=4
Black Creek	44º55'29.36"N 75º22'57.21"W	10 June 23 June 4 Sept.	70.4	7.2	22.4	Mainly corn, periodically soy crops	2,444 (1,872 - 3,017)	0.627 (0.304 - 0.950)
East Branch of Scotch River	45º20'00.88"N 74º49'49.25"W	5 June 18 June 7 Sept.	21.4	14.2	64.4	Natural vegetation	98 (31 - 165)	<0.050 (below detection)
East Castor River	45°8'58.61"N 75°21'39.10"W	10 June 25 June 2 Sept.	57.7	26.0	13.0	Mainly corn, periodically soy or wheat crops	5,795 (4,932 - 6,658)	0.916 (0.272 - 1.560)
Payne River	45º10'30.52"N 75º06'16.17"W	5 June 25 June 2 Sept.	83.9	12.6	3.5	Natural vegetation at immediate site but intense agriculture nearby	2,557 (1,410 - 3,704)	0.481 (0.255 - 0.707)
South Branch of South Nation	44º53'12.12"N 75º25'12.16"W	10 June 2008 23 June 2008 4 Sept. 2008	68.9	5.1	26.0	Mainly corn and soy, periodically wheat crops	157 (69 - 244)	0.384 (<0.050 - 0.755)
South Nation River headwaters	44º46'51.08"N 75º35'06.61"W	10 June 2008 23 June 2008 4 Sept. 2008	6.9	12.8	77.4	Natural vegetation	<4 (<4 - 5)	0.173 (<0.050 - 0.298)

Land use in a 500 m radius zone surrounding the sites was characterized by the percentage of total land area in: annual crops, perennial crops and pasture, and undisturbed natural habitat (composed of wetland, forest, exposed land and shrub land). In-stream nitrate and atrazine concentrations were measured from duplicate samples taken during two time periods in June 2008 (n= 4). Minimum and maximum concentrations (averaged from duplicate samples) are shown in brackets. *L. minor* populations were collected in September 2008.

Table 5.2 Comparison of the sensitivity of seven different *Lemna minor* L. populations to atrazine for three different endpoints

Endpoint	Population	Estimated marginal means (% control) (95% CI)
Biomass	CPCC 490	42.7 (39.8-45.6) AB
	Black Creek	52.9 (50.0-55.8) C
	East Branch of Scotch River	36.9 (34.0-39.8) A
	East Castor River	53.0 (50.0-55.9) C
	Payne River	47.6 (44.6-50.5) BC
	South Branch of South Nation	43.2 (40.3-46.1) AB
	South Nation River headwaters	46.5 (43.6-49.4) BC
	Overall	46.1 (40.5-51.7)
Frond number	CPCC 490	55.7 (53.5-57.9) B
	Black Creek	53.9 (51.8-56.2) AB[a]
	East Branch of Scotch River	49.8 (47.7-51.9) A
	East Castor River	62.1 (59.7-64.6) C[a]
	Payne River	57.3 (55.0-59.6) BC
	South Branch of South Nation	49.4 (47.3-51.5) A
	South Nation River headwaters	62.0 (59.7-64.4) C
	Overall	55.4 (51.1-59.8)[a]
Chlorophyll fluorescence (Fv/Fm)	CPCC 490	98.5 (97.8-99.2) CD
	Black Creek	96.2 (95.5-96.9) AB
	East Branch of Scotch River	95.0 (94.2-95.7) A
	East Castor River	97.1 (96.4-97.8) BC
	Payne River	98.8 (98.1-99.5) D
	South Branch of South Nation	98.7 (98.0-99.4) CD[a]
	South Nation River headwaters	99.2 (98.5-99.8) D
	Overall	97.7 (97.0-98.3)[a]

Estimated marginal means, representing the overall percentage of control values averaged from all three atrazine treatments, are shown as a measure of the overall effect of atrazine on each population. Confidence Intervals (95% CI) are shown in brackets. ANOVA was followed by Sidak pairwise comparisons. Means followed by the same letter are not significantly different, means followed by different letters are significantly different at $p<0.05$

[a] Outlier removed

Table 5.3 Twenty-five percent inhibition concentrations (IC_{25}s) comparing the sensitivity of seven different *Lemna minor* L. populations to atrazine for three different endpoints

Endpoint	Population	IC_{25} (95% CI) (µg/L)	Model parameter estimates			Degrees of freedom (regression, error)	Adjusted R^2
			g	x	b		
Biomass	CPCC 490	33 (28-40)	3.846	1.520	5.956	3, 20	0.986
	Black Creek	32 (18-56)	3.797	1.499	4.528	3, 20	0.845
	East Branch of Scotch River	19 (16-23)	4.512	1.285	3.912	3, 20	0.984
	East Castor River	40 (31-52)	4.836	1.607	5.732	3, 20	0.961
	Payne River	32 (25-41)	4.795	1.509	5.054	3, 20	0.971
	South Branch of South Nation	27 (21-35)	5.428	1.433	4.559	3, 20	0.965
	South Nation River headwaters	32 (26-39)	5.891	1.509	5.115	3, 20	0.980
	Overall	30 (25-36)	4.725	1.480	4.878	3, 164	0.874
Frond number	CPCC 490	76 (68-86)	8.787	1.883	4.488	3, 20	0.982
	Black Creek	69 (58-81)	7.732	1.838	3.813	3, 19[a]	0.975
	East Branch of Scotch River	57 (47-68)	7.766	1.754	3.433	3, 20	0.971
	East Castor River	92 (81-105)	8.587	1.965	5.504	3, 19[a]	0.966
	Payne River	82 (73-92)	9.002	1.914	4.902	3, 20	0.979
	South Branch of South Nation	61 (50-74)	9.775	1.787	3.948	3, 20	0.967
	South Nation River headwaters	88 (78-100)	8.927	1.945	5.435	3, 20	0.971
	Overall	75 (66-85)	8.654	1.875	4.445	3, 162 [a]	0.846
Chlorophyll fluorescence (Fv/Fm)	CPCC 490	>160	0.832	2.912	6.853	3, 8	0.669
	Black Creek	>160	0.818	2.798	5.180	3, 8	0.935
	East Branch of Scotch River	>160	0.830	3.396	2.864	3, 8	0.951
	East Castor River	>160	0.822	2.706	7.153	3, 8	0.872
	Payne River	>160	0.831	3.009	7.517	3, 8	0.959
	South Branch of South Nation	>160	0.833	2.864	7.260	3, 7 [a]	0.980
	South Nation River headwaters	>160	0.833	4.285	4.464	3, 8	0.913
	Overall	>160	0.828	3.009	5.540	3, 79 [a]	0.503

Confidence Intervals (95% CI) are shown in brackets. IC_{25}s were estimated using a gompertz model, $Y = g \times e^{((\log(1-p)) \times (C/x)^b)}$, where g is the control response (y-intercept), p is the percent inhibition/100, C is the $\log_{10}$ concentration of atrazine, x is the $\log_{10}$ ICp and b is a scale parameter.

[a] Outlier removed

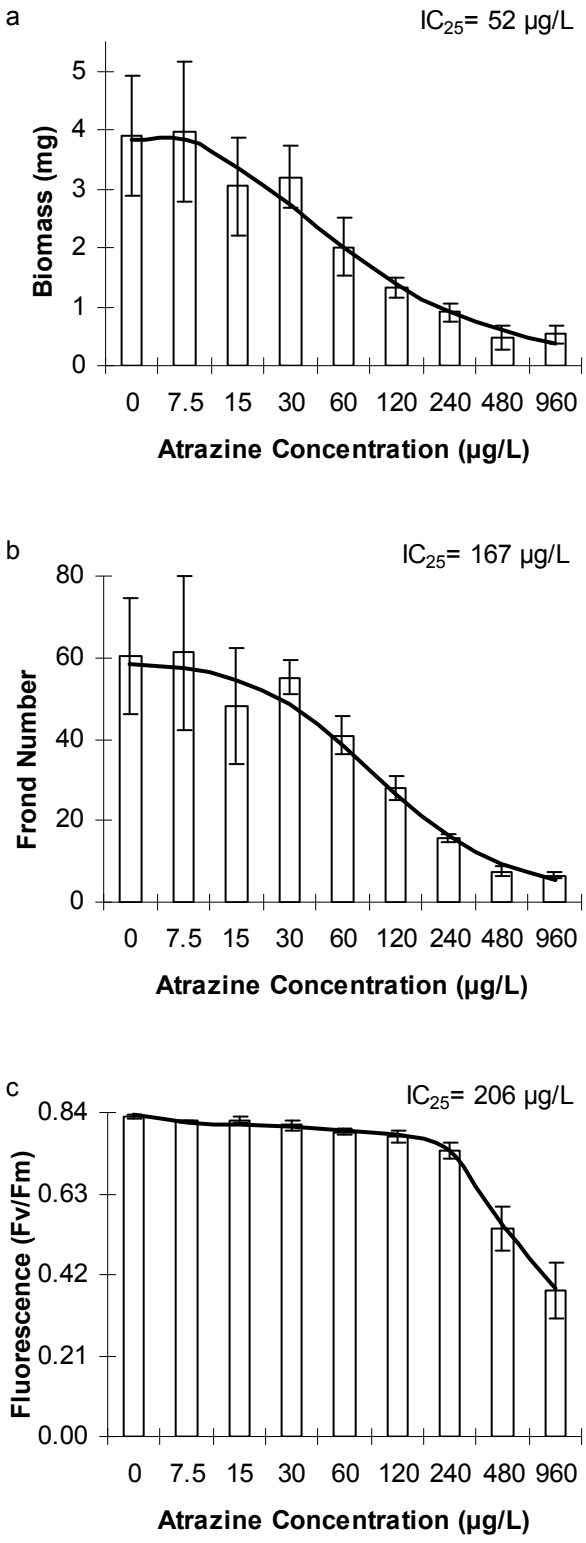

Fig. 5.1 Response of *Lemna minor* L. (CPCC 490) to atrazine. Average values ± standard deviation and the modelled response (*solid line*) are shown for **a** biomass, **b** frond number and **c** chlorophyll fluorescence (Fv/Fm). Twenty-five percent inhibition concentrations (IC_{25}s) are shown. Biomass data and Fv/Fm data were fit with hormetic models ($Y = (t \times (1 + h \times C)) / (1 + ((p + h \times C) / (1 - p)) \times (C / x)^b)$) after being square root and cube transformed respectively whereas frond data were $\log_{10}$ transformed and fit with a logistic model ($Y = t / (1 + (p / (1 - p)) \times (C / x)^b)$), where t is the control response (y-intercept), h is the hormetic effect, C is the $\log_{10}$ concentration of atrazine, p is the percent inhibition/100, x is the ICp and b is a scale parameter.

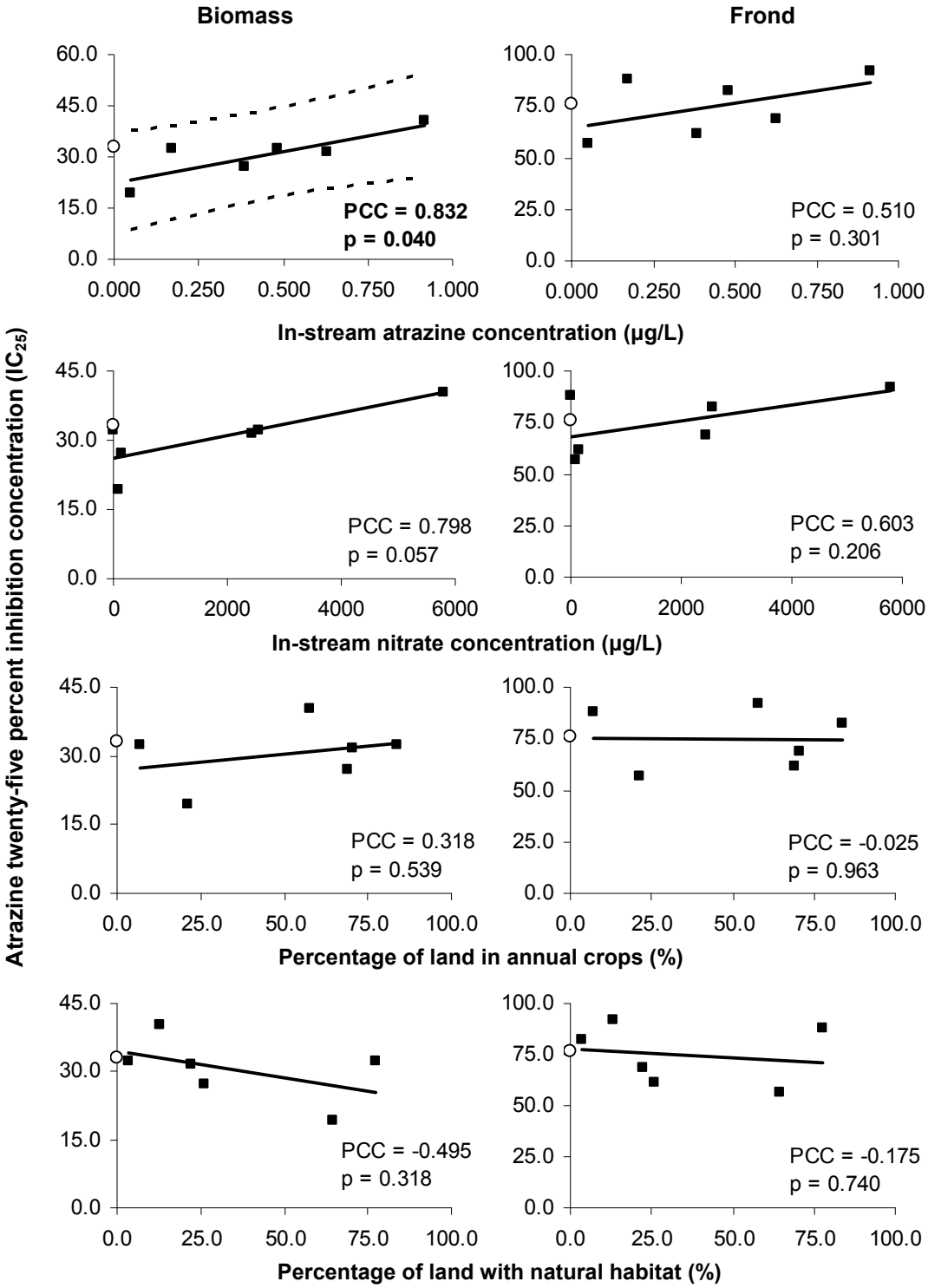

Fig. 5.2 Correlations between atrazine sensitivity (25% inhibition concentrations (IC_{25}s) calculated from *Lemna minor* L. biomass and frond data) and four measures of past exposure to agriculture (in-stream atrazine concentration, in-stream nitrate concentration, percentage of land in annual crops, and percentage of surrounding natural habitat). *Open circles* represent the IC_{25}s generated for CPCC 490 (not used in analysis). The *line* of best fit was illustrated using linear regression with 95% confidence intervals calculated for significant relationships. Pearson's correlation coefficients (PCC) and *p* values are shown.

Chapter 6: Conclusions

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6.1 General discussion and significance of research

The overall objective of this thesis was to assess effects of agrochemicals (fertilizers and the herbicide atrazine) on riparian and aquatic wetland primary producers in river systems. Effects of agrochemicals on primary producers were studied at several scales of observation ranging from empirical studies at the watershed scale (Chapters 3, 4) following characterization of atrazine contamination (Chapter 2), *in-situ* experimental manipulations in two temperate streams (Chapter 4) and laboratory concentration-response experiments (Chapter 5).

A key objective was to estimate time-weighted average atrazine concentrations across a large agricultural watershed using polar organic chemical integrative samplers (POCIS). This study was important in characterizing atrazine contamination because unlike traditional grab samples, passive sampling integrates trace level concentrations of contaminants over time, integrates pulses in concentrations and improves understanding of actual exposures to biota. A clear gradient of atrazine contamination was observed across the South Nation River watershed with 56 d time-weighted-average atrazine concentrations >100 ng/L at more than half the field sites. The hypothesis that atrazine concentrations were correlated with surrounding land use was accepted.

Most other POCIS studies have focused on laboratory calibrations or deployments at a few field sites (reviewed in Harman et al., 2012; Morin et al., 2012). Alvarez et al. (2004) deployed POCIS at eight stream/river sites during development and proof-of-concept field deployments of POCIS and Li et al. (2010) deployed POCIS more recently at 11 sites in Lake Ontario. To my knowledge, the research presented in this thesis represented the most comprehensive spatial coverage with POCIS to date.

POCIS remains poorly characterized in terms of modeling effects of environmental factors on contaminant uptake and sampling rates (Harman et al., 2012). Calibration studies using a performance reference compound (PRC) were novel because they represented the first use of a PRC approach to evaluate spatial and temporal differences in sampling rates. Sampling rates did not differ between field sites, suggesting that comparison of time-weighted-average atrazine concentrations between field sites within a single time period was reasonable, even without site-level correction of sampling rates. This finding has important implications for future work because it demonstrated that a PRC approach can be used to evaluate whether sampling rates are comparable between field sites and a single sampling rate adequate for estimating analyte concentrations, or whether site-specific corrections are necessary.

A number of studies have demonstrated that sampling rates are affected by factors such as temperature, stream velocity, biofouling and pH (Harman et al. 2012) but calibration studies in this thesis found only temperature affected sampling rates in the field. This finding highlighted the importance of studying factors affecting sampling rates under actual field conditions. Although sampling rates have been determined under laboratory conditions for over 200 compounds, only three studies have calculated *in-situ* sampling rates (Morin et al, 2012), likely because of the amount of time required to calibrate sampling rates with frequent monitoring of the uptake of target analytes over time. The incorporation of a PRC into POCIS is less time intensive and allows *in-situ* sampling rates to be calculated from PRC concentrations measured only at the beginning and end of the deployment period. While further understanding is needed regarding the behaviour of PRCs in relation to target analytes and their interactions with POCIS sorbent for quantitative correction of sampling

rates, PRCs allow comparison of sampling rates between field sites and provide insight into factors affecting field sampling rates. Overall, the calibration studies illustrated that incorporating a PRC into POCIS is a promising alternative to traditional *in-situ* calibration and for developing future improvements in the quantitative use of POCIS.

A substantial portion of the thesis focused on empirical field studies comparing the community structure of riparian and aquatic plants and periphyton across gradients of agrochemical contamination. Similarly to the gradient observed for atrazine, a clear gradient of surrounding land use was observed, with the percentage of annual crops surrounding field sites ranging from less than 10% to almost 100%. Gradients in nutrient enrichment were also observed across the watershed. In particular, nitrate concentrations spanned three orders of magnitude and ranged from below detection to >5 mg/L, with over half the sites considered to be eutrophic (TN >1.5 mg/L) (Dodds, et al., 1998). Phosphorus concentrations were high throughout the watershed with all 24 sites considered to be eutrophic or hyper-eutrophic (TP > 35 μg/L) (Canadian Council of Ministers of the Environment, 2004).

Atrazine and the percentage of surrounding annual crops had no direct effects on riparian and aquatic plant community structure. In contrast, an increase in the percentage of non-native species, a decrease in submerged macrophytes and a decrease in the overall floristic quality index of the plant communities was observed along a gradient of increasing nitrate. Although species richness ranged from 43-107 species per site, no difference in species richness was observed between paired sites with low and high levels of surrounding agriculture. With the exception of species richness, observed changes in plant communities in response to agrochemicals were in agreement with initial predictions. Bowers and Boutin (2008) also found that species richness was a poor measure of anthropogenic perturbation of

riparian plant species compared to the percentage of non-native species and the floristic quality index. The number of species present within a given tributary appeared to be constrained by physical and chemical characteristics at the tributary scale. A shift in community structure was observed with increasing agricultural impact (i.e. nitrate). Species with medium to high coefficients of conservation were replaced with non-native species and species with low coefficients of conservation. Species positively associated with nitrate were riparian species, whereas species negatively associated with nitrate included riparian, emergent, floating-leaved and submerged species. Fast growing, emergent, shade resistant species appeared to thrive under nitrate enriched conditions, whereas these conditions led to a loss of submerged macrophytes. Declines in submerged species have been previously associated with light limitation as nutrient enrichment stimulates growth of macrophyte forms best able to access light and phytoplankton growth further reduces light availability by increasing turbidity (Phillips et al. 1978; Irvine et al. 1989; Scheffer et al. 1993; reviewed in Hilton et al., 2006). McJannet et al. (1995) found that fast-growing annual wetland plants had low internal nitrogen and phosphorus contents and hypothesized that they were able to efficiently exploit nutrients through rapid production of new tissues, a conclusion supported by the observed changes in community structure.

The research presented in this thesis represented a comprehensive study of the effects of agrochemicals (nutrients and atrazine) on riparian and aquatic plant community structure. Such information is lacking in North America and in Canada in particular. Although a substantial amount of work has been done in Europe, riparian species are not typically included in assessments of stream/river quality (Dawson, 2002). A limitation of this thesis was the inability to separate out effects of atrazine and nutrients due to the strength of the

nitrate signal, the correlation between these agrochemicals and the lack of comparable sites with only high atrazine or high nutrient concentrations. One approach to improving the detection of atrazine effects on plant community structure might be to examine traits of atrazine-resistant plant species. An increase in the presence of these particular species or species with traits characteristic of atrazine-resistant species would indicate a shift in community structure in response to atrazine exposure.

The other major group of primary producers in the watershed were periphytic algae. Across the watershed, periphyton communities colonized on artificial substrates were typically dominated by the Bacillariophyta and by the Chlorophyta to a lesser degree, while the Cryptophyta and Euglenophyta were minor contributors to total biomass. Nitrate had a significant positive effect on biomass, while few direct effects of reactive phosphate or atrazine were observed. The Chlorophyta were associated with high nitrate, a finding in agreement with previous studies associating high nitrogen concentrations with periphyton communities dominated by the chlorophyte *Cladophora sp.* (Dodds, 1991; Chételat, et al. 1999). While no effects of atrazine were detected, the results supported the hypothesis that periphyton communities would be dominated by the Chlorophyta in nutrient enriched sites. Although nitrogen to phosphorus ratios suggested that most field sites were phosphorus limited, overall high nutrient concentrations and a trend towards increasing biomass with increasing nitrate suggested that sites were generally not phosphorus limited. Keck and Lepori (2012) proposed that algal communities may be able to take advantage of additions of either nitrogen or phosphorus through increased acquisition and reduced loss of the scarcest nutrient and shifts in community structure to species with different optimal N: P ratios.

In contrast to results of the periphyton field study, no clear response of periphyton to nutrient enrichment or atrazine contamination was observed in the periphytometer experiments. The lack of response to nutrient enrichment suggested that the two streams selected for study were overall not phosphorus or nitrogen limited, a result supported by trends observed in the field study. In 2008, periphyton communities were not adversely affected by exposure to 20 µg/L atrazine. Atrazine was increased 10 fold in 2009 to 200 µg/L atrazine to induce a response. The lack of response in 2009 was surprising because reductions in periphyton gross productivity in multi-species stream mesocosms had previously been observed at concentrations as low as 15 µg/L atrazine (Detenbeck et al., 1996). However, Detenbeck et al. (1996) also observed a shift in dominance from atrazine sensitive Chlorophyta to more tolerant Bacillariophyta. In the periphytometer study, communities were composed primarily of the Bacillariophyta and may have been generally tolerant to atrazine. High rainfall in 2009 may also have limited overall periphyton colonization, reducing observable effects. Periphytometers are a useful tool for evaluating nutrient limitation and responses to contaminants. However, future studies may be improved by ensuring the substrates are adequately protected from scouring and by efforts to better understand the influence of environmental conditions on diffusion from the solution reservoirs to the substrates.

Despite some limitations with the periphytometers, both the empirical and *in-situ* experimental studies with periphyton were a valuable contribution to the novel application of a chemotaxonomic approach (CHEMTAX) to freshwater algae and highlighted its potential in future work. CHEMTAX has been used to characterize periphyton in only one earlier study, which also determined pigment to chlorophyll *a* ratios for 15 phytoplankton species in

culture (Lauridsen et al., 2011). The accuracy and sensitivity of CHEMTAX is limited by the consistency of pigment ratios across samples (Mackey et al., 1996). Differences in species composition as well as light and nutrient regimes can alter pigment ratios (Jeffrey and Wright 1994; Higgins et al., 2011). Samples representing algal communities originating from similar environmental conditions can be grouped and pigment ratios optimized in separate CHEMTAX analyses to ensure the accuracy of the resulting pigment ratios. This approach requires an adequate sample size (5-10) for each group. Future studies would benefit from the collection of multiple samples from a subset of environmental conditions so that the need for grouping samples could be assessed. Pigment ratios have been well characterized for marine algae and this has allowed for broad taxonomic groups to be sub-divided. For example, the Haptophyta can be divided into eight pigment classes (Higgins et al., 2011). Future studies that characterize pigment ratios in freshwater periphyton would allow for greater taxonomic resolution.

In both major field studies, community structure was compared across agrochemical gradients but also between paired sites in tributaries of the South Nation River watershed. Paired sites were selected because physical, chemical and land use characteristics differed across the 3915 km^2 watershed. Factors controlling primary production in one tributary were likely to be different than factors controlling primary production in another. By pairing sites along tributaries and studying tributaries across the watershed, it was possible to examine changes in primary production at both the tributary and watershed scale. This approach was important because there is no single scale at which ecological phenomena should be studied (Levin, 1992) and in the field studies, both the tributary and watershed scales were important. For example, declines in the number of submerged species and

overall floristic quality of riparian and aquatic plants with increasing nitrate were only observed at the watershed scale, whereas a reduction in the relative frequency of submerged macrophytes was only observed between paired sites at the tributary scale. In the periphyton field study, an increase in biomass with increasing nitrate was only observed at the watershed scale and an association of the Chlorophyta with nitrate was only apparent once differences in physical and chemical characteristics were taken into account.

The lack of significant direct effects of atrazine on primary producers could be due to prior selection, acclimation or adaptation in this watershed. This was tested in the laboratory experiment where effects of atrazine on duckweed (*Lemna minor*) were examined at the population scale using greenhouse concentration-response studies. Field-collected populations of duckweed with likely past exposure to atrazine were generally more tolerant than populations from streams characterized by low in-stream atrazine concentrations, supporting the hypothesis that atrazine exposure exerted selection pressure towards increased atrazine tolerance. However, this response was not observed for all endpoints and 25% inhibition concentrations generated from biomass data were similar between populations. The overall lack of response may be attributed to the presence of relatively few molecular modifications for the site of herbicide action and a large amount of pheonotypic plasticity in the various clones (Mazzeo et al., 1998). The sensitivity of the commercial laboratory culture was similar to that of field populations, evidence that it provided a good estimate of the sensitivity of *L. minor* to atrazine, is a suitable proxy for field populations in phytotoxicity testing and useful in testing hypotheses generated from patterns observed in the field.

6.2 Conclusions and recommendations

The research presented in this thesis clearly demonstrated that atrazine contamination is widespread across the South Nation River watershed. This situation is likely to be similar in other North American agricultural watersheds, particularly those dominated by corn crops. While concentrations of atrazine were well below the Canadian water quality guidelines for the protection of aquatic life (1.8 μg/L) (Canadian Council of Ministers of the Environment, 1999), contamination was observed over a significant time period and at concentrations known to affect sensitive endpoints in amphibians and fish (Hayes et al., 2003; Tillitt et al., 2010). Laboratory concentration-response studies with duckweed populations illustrated a decline in sensitivity to atrazine with previous exposure to atrazine in the field. These subtle effects, observed under controlled laboratory conditions, would not be apparent through observations in the field and highlighted the need for studies along multiple scales of study. Furthermore, results from this thesis indicate that the current atrazine guideline for the protection of aquatic life may be too high for the protection of sensitive organisms and that efforts to reduce inputs of atrazine to surface waters are needed.

Field studies clearly illustrated a gradient of nutrient contamination and evidence that although nutrients were not generally limiting plant or periphyton biomass they were affecting community structure. The watershed was characterized by high concentrations of both phosphorus and nitrogen that are indicative of eutrophic conditions according to guidelines established by the Canadian Council of Ministers of the Environment (2004, 2012). Nitrate concentrations were associated with a loss of submerged macrophytes, a shift towards fast-growing emergent and riparian plant species, an increase in non-native species, an overall reduction in floristic quality and an increase in periphyton biomass. The current

Canadian water quality guideline for nitrate (3 mg/L for long-term exposure) is based on protection from direct toxic effects (Canadian Council of Ministers of the Environment, 2012). This guideline is inadequate for protection against effects of nitrate on riparian and aquatic primary communities.

This thesis provided evidence of multiple negative effects of agrochemicals on riparian and aquatic primary producers. However, the effects of nutrients, specifically nitrate, superseded any observable direct effects of the herbicide atrazine and highlighted a need to control nitrogen in aquatic systems. Currently, significant management efforts are placed on regulating phosphorus in freshwater systems but this thesis demonstrated that efforts towards regulating nitrogen are also necessary if native plant biodiversity conservation is an important goal. Finally, nitrogen loading to freshwater and marine ecosystems is clearly affecting many ecosystem services globally (Carpenter et al., 1998; Camargo and Alonso, 2006) and warrants further environmental regulation consideration.

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Appendix A. Statement of contributions of collaborators

This thesis was my original work, conducted under the supervision of Dr. Céline Boutin and Dr. Frances Pick and under the guidance of advisory committee members Dr. Antoine Morin and Dr. Pierre Mineau. Chapters 1 and 6 consist of a General Introduction and Conclusions respectively. Chapters 2 to 5 are a series of articles that have been or will be submitted to peer reviewed journals for publication consideration. Contributions of co-authors and collaborators are outlined below.

Chapter 2

Dr. Ammar Saleem (University of Ottawa) developed the initial LC-MS/MS method for atrazine. He also provided training on the operation of the LC-MS/MS as well as guidance on sample preparation and quantitation methods.

Chapter 3

The 2010 riparian plant survey and subsequent plant identifications were largely conducted by members of Dr. Boutin's lab (Environment Canada) as part of a collaborative project examining the seedbank and bank vegetation at field sites within the South Nation River watershed.

Chapter 4

Periphytometer atrazine samples were analyzed by France Maisonneuve (Environment Canada). Periphyton pigments were analyzed via HPLC in Dr. Gregory-Eaves' lab (McGill University). I completed the pigment extractions, assisted with some of the HPLC analyses and integrated the chromatograms. The Gregory-Eaves' lab was responsible for the HPLC method (adapted from the literature) and for running a number of the samples.

Chapter 5

This work was conducted in conjunction Christina Nussbaumer, a 4th year Honours student supervised by Dr. Pick, who was responsible for data collection and maintenance of duckweed cultures. I was responsible for experimental design and providing training for culturing techniques and chlorophyll fluorescence measurements. I also prepared atrazine solutions, analyzed in-stream measurements of atrazine, analyzed the data and wrote the published manuscript.

Appendix B. Supplementary data for Chapter 3

Table B.1 Land use 1 km upstream of 12 paired (24 in total) stream/ river sites in the South Nation River watershed, Canada (100 m wide zone). Statistics in **bold** are significant at $p \leq 0.05$

Land use	Low agriculture sites	High agriculture sites	Paired t-test (df=11)
Annual crops (%)	21.2 ± 25.8 (2.7-98.2)	73.5 ± 23.7 (43.0-100.0)	**t=-5.559; p<0.001**
Perennial crops and pasture (%)	16.6 ± 16.3 (0.0-45.8)	9.7 ± 13.5 (0.0-37.7)	t=1.290; p=0.223
Natural habitat[a] (%)	61.8 ± 26.0 (1.8-90.0)	16.6 ± 15.3 (0.0-45.3)	**t=4.729; p=0.001**
Developed land[b] (%)	0.5 ± 1.1 (0.0-2.7)	0.3 ± 0.8 (0.0-2.7)	Wilcoxon p=1.000[c]

[a]forest, wetland, grassland, shrub land, water, rock, soil and sediments

[b]suburban development, roads, buildings, parks, farmsteads, golf courses

[c]Non-parametric Wilcoxon signed rank test

Table B.2 Riparian and aquatic plants identified in the South Nation River watershed

Species	Authority	Family	Native Status	Coefficient of conservation (CC)[a]	Wetness category (Index)[b]	Number sites found (%)	Average frequency/ site (%)
Acalypha rhomboidea	Raf.	Euphorbiaceae	Native	0	FACU (3)	9 (37.5)	16.7
Acer negundo	L.	Aceraceae	Native	0	FACW- (-2)	24 (100.0)	39.8
Acer rubrum	L.	Aceraceae	Native	4	FAC (0)	1 (4.2)	6.3
Acer saccharinum	L.	Aceraceae	Native	5	FACW (-3)	2 (8.3)	12.5
Achillea millefolium	L.	Asteraceae	Non-Native	-1	FACU (3)	10 (41.7)	12.5
Ageratina altissima	(L.) King & H. Rob.	Asteraceae	Native	5	FACU (3)	2 (8.3)	6.3
Agrostis gigantea	Roth	Poaceae	Non-Native	-2	FAC (0)	13 (54.2)	18.3
Agrostis stolonifera	L.	Poaceae	Native	0	FACW (-3)	2 (8.3)	15.6
Alisma gramineum	Lej.	Alismataceae	Native	6	OBL (-5)	3 (12.5)	11.2
Alisma subcordatum	Raf.	Alismataceae	Native	3	OBL (-5)	1 (4.2)	6.3
Alisma triviale	Pursh	Alismataceae	Native	3	OBL (-5)	17 (70.8)	13.7
Alliaria petiolata	(M. Bieb.) Cavara & Grande	Brassicaceae	Non-Native	-3	FAC (0)	1 (4.2)	6.3
Alnus incana	(L.) Moench	Betulaceae	Native	6	OBL (-5)	3 (12.5)	12.5
Ambrosia artemisiifolia	L.	Asteraceae	Native	0	FACU (3)	15 (62.5)	16.3
Ambrosia trifida	L.	Asteraceae	Native	0	FAC+ (-1)	10 (41.7)	15.0
Amphicarpaea bracteata	(L.) Fernald	Fabaceae	Native	4	FAC (0)	18 (75.0)	39.2
Anemone canadensis	L.	Ranunculaceae	Native	3	FACW (-3)	19 (79.2)	32.2
Anthemis arvensis	L.	Asteraceae	Non-Native	-1	UPL (5)	1 (4.2)	6.3
Apios americana	Medik.	Fabaceae	Native	6	FACW (-3)	2 (8.3)	43.8
Apocynum androsaemifolium	L.	Apocynaceae	Native	3	UPL (5)	6 (25.0)	36.5
Arctium minus	Bernh.	Asteraceae	Non-Native	-2	UPL (5)	9 (37.5)	11.1
Artemisia biennis	Willd.	Asteraceae	Non-Native	-1	FACW- (-2)	5 (20.8)	15.0
Artemisia vulgaris	L.	Asteraceae	Non-Native	-1	UPL (5)	10 (41.7)	21.9
Asclepias incarnata	L.	Asclepiadaceae	Native	6	OBL (-5)	16 (66.7)	15.6
Asclepias syriaca	L.	Asclepiadaceae	Native	0	UPL (5)	13 (54.2)	24.0
Atriplex patula	L.	Chenopodiaceae	Native	0	FACW- (-2)	11 (45.8)	14.2
Barbarea vulgaris	W.T. Aiton	Brassicaceae	Non-Native	-1	FAC (0)	16 (66.7)	21.1
Bidens cernua	L.	Asteraceae	Native	2	OBL (-5)	19 (79.2)	29.8
Bidens connata	Muhl. ex Willd.	Asteraceae	Native	4	FACW (-3)	1 (4.2)	8.3

Species	Authority	Family	Native Status	Coefficient of conservation (CC)[a]	Wetness category (Index)[b]	Number sites found (%)	Average frequency/ site (%)
Bidens frondosa	L.	Asteraceae	Native	3	FACW (-3)	22 (91.7)	26.4
Bidens vulgata	Greene	Asteraceae	Native	5	FACW (-3)	3 (12.5)	14.6
Boehmeria cylindrica	(L.) Sw.	Urticaceae	Native	4	OBL (-5)	6 (25.0)	18.8
Bromus inermis	Leyss.	Poaceae	Non-Native	-3	UPL (5)	18 (75.0)	67.4
Butomus umbellatus	L.	Butomaceae	Non-Native	-2	OBL (-5)	11 (45.8)	52.0
Callitriche palustris	L.	Callitrichaceae	Native	6	OBL (-5)	1 (4.2)	5.0
Calystegia sepium	L.	Convolvulaceae	Native	2	FAC (0)	19 (79.2)	51.3
Campanula aparinoides	Pursh	Campanulaceae	Native	7	OBL (-5)	3 (12.5)	8.3
Capsella bursa-pastoris	(L.) Medik.	Brassicaceae	Non-Native	-1	FAC- (1)	1 (4.2)	6.3
Carex bebbii	Olney ex Fernald	Cyperaceae	Native	3	OBL (-5)	1 (4.2)	31.3
Carex crinita	Lam.	Cyperaceae	Native	6	FACW+ (-4)	4 (16.7)	17.2
Carex lupulina	Muhl. Ex Willd.	Cyperaceae	Native	6	OBL (-5)	3 (12.5)	8.3
Carex sp.	L.	Cyperaceae	Native	7	FAC+ (-1)	9 (37.5)	13.9
Carex stipata	Muhl. Ex Willd.	Cyperaceae	Native	3	OBL (-5)	1 (4.2)	6.3
Carex vesicaria	L.	Cyperaceae	Native	7	OBL (-5)	1 (4.2)	6.3
Carex vulpinoidea	Michx.	Cyperaceae	Native	3	OBL (-5)	11 (45.8)	11.4
Cerastium arvense	L.	Caryophyllaceae	Non-Native	-1	UPL (5)	2 (8.3)	37.5
Cerastium fontanum	Baumg.	Caryophyllaceae	Non-Native	-1	FACU (3)	6 (25.0)	19.8
Ceratophyllum demersum	L.	Ceratophyllaceae	Native	4	OBL (-5)	12 (50.0)	45.4
Chelone glabra	L.	Scrophulariaceae	Native	7	OBL (-5)	2 (8.3)	7.3
Chenopodium album	L.	Chenopodiaceae	Non-Native	-1	FAC- (1)	12 (50.0)	16.7
Chenopodium glaucum	L.	Chenopodiaceae	Non-Native	-1	FACW (-3)	4 (16.7)	10.9
Cichorium intybus	L.	Asteraceae	Non-Native	-1	UPL (5)	1 (4.2)	12.5
Cicuta bulbifera	L.	Apiaceae	Native	5	OBL (-5)	3 (12.5)	12.5
Cicuta maculata	L.	Apiaceae	Native	6	OBL (-5)	7 (29.2)	17.0
Cirsium arvense	(L.) Scop.	Asteraceae	Non-Native	-1	FACU (3)	20 (83.3)	30.9
Cirsium vulgare	(Savi) Ten.	Asteraceae	Non-Native	-1	FACU- (4)	12 (50.0)	12.5
Cornus sericea	L.	Cornaceae	Native	2	FACW (-3)	1 (4.2)	43.8
Crataegus sp.	L.	Rosaceae	Native	4	UPL (5)	2 (8.3)	6.3
Cyperus dentatus	Torr.	Cyperaceae	Native	9	OBL (-5)	2 (8.3)	12.5
Cyperus diandrus	Torr.	Cyperaceae	Native	6	FACW+ (-4)	1 (4.2)	6.3

Species	Authority	Family	Native Status	Coefficient of conservation (CC)[a]	Wetness category (Index)[b]	Number sites found (%)	Average frequency/ site (%)
Daucus carota	L.	Apiaceae	Non-Native	-2	UPL (5)	13 (54.2)	19.2
Decodon verticillatus	(L.) Elliott	Lythraceae	Native	7	OBL (-5)	1 (4.2)	4.2
Digitaria sanguinalis	(L.) Scop.	Poaceae	Non-Native	-1	FACU (3)	1 (4.2)	6.3
Echinochloa crus-galli	(L.) P. Beauv.	Poaceae	Non-Native	-1	FACW (-3)	11 (45.8)	9.7
Echinochloa muricata	(P. Beauv.) Fernald	Poaceae	Native	4	OBL (-5)	4 (16.7)	7.1
Echinocystis lobata	(Michx.) Torr. & A. Gray	Cucurbitaceae	Native	3	FACW- (-2)	18 (75.0)	20.8
Eleocharis acicularis	(L.) Roem. & Schult.	Cyperaceae	Native	5	OBL (-5)	13 (54.2)	17.4
Eleocharis engelmannii	Steud.	Cyperaceae	Native	9	FACW (-3)	1 (4.2)	18.8
Eleocharis intermedia	Schult.	Cyperaceae	Native	7	FACW (-3)	2 (8.3)	12.5
Eleocharis obtusa	(Willd.) Schult.	Cyperaceae	Native	5	OBL (-5)	4 (16.7)	12.8
Eleocharis ovata	(Roth) Roem. & Schult.	Cyperaceae	Native	8	OBL (-5)	2 (8.3)	6.3
Eleocharis sp.	R. Br.	Cyperaceae	Native	8	FACW+ (-4)	1 (4.2)	6.3
Elodea canadensis	Michx.	Hydrocharitaceae	Native	4	OBL (-5)	11 (45.8)	54.0
Elymus repens	(L.) Gould	Poaceae	Non-Native	-3	FACU (3)	7 (29.2)	14.3
Elymus virginicus	L.	Poaceae	Native	5	FACW- (-2)	11 (45.8)	15.9
Epilobium ciliatum	Raf.	Onagraceae	Native	3	FACU (3)	5 (20.8)	12.5
Equisetum arvense	L.	Equisetaceae	Native	0	FAC (0)	17 (70.8)	47.1
Equisetum fluviatile	L.	Equisetaceae	Native	7	OBL (-5)	10 (41.7)	13.1
Equisetum palustre	L.	Equisetaceae	Native	10	FACW (-3)	2 (8.3)	5.0
Equisetum pratense		Equisetaceae	Native	8	FACW (-3)	6 (25.0)	20.8
Erigeron annuus	(L.) Pers.	Asteraceae	Native	0	FAC- (1)	1 (4.2)	12.5
Erigeron philadelphicus	L.	Asteraceae	Native	1	FACW (-3)	7 (29.2)	14.3
Erigeron strigosus	Muhl. Ex Willd.	Asteraceae	Native	0	FAC- (1)	2 (8.3)	18.8
Erysimum cheiranthoides	L.	Brassicaceae	Non-Native	-1	FACU (3)	19 (79.2)	31.6
Eupatorium perfoliatum	L.	Asteraceae	Native	2	FACW+ (-4)	1 (4.2)	6.3
Eutrochium maculatum	(L.) E.E. Lamont	Asteraceae	Native	3	OBL (-5)	15 (62.5)	30.4
Fragaria virginiana	Duchesne	Rosaceae	Native	2	FAC- (1)	8 (33.3)	12.5
Frangula alnus	Mill.	Rhamnaceae	Non-Native	-3	FAC+ (-1)	1 (4.2)	6.3
Fraxinus americana	L.	Oleaceae	Native	4	FACU (3)	2 (8.3)	6.3
Galium aparine	L.	Rubiaceae	Native	4	FACU (3)	1 (4.2)	12.5
Galium asprellum	Michx.	Rubiaceae	Native	6	OBL (-5)	1 (4.2)	18.8

Species	Authority	Family	Native Status	Coefficient of conservation (CC)[a]	Wetness category (Index)[b]	Number sites found (%)	Average frequency/ site (%)
Galium glaucum	L.	Rubiaceae	Non-Native	-2	FAC (0)	1 (4.2)	25.0
Galium mollugo	L.	Rubiaceae	Non-Native	-2	UPL (5)	17 (70.8)	24.3
Galium obtusum	Bigelow	Rubiaceae	Native	6	OBL (-5)	1 (4.2)	43.8
Galium palustre	L.	Rubiaceae	Native	5	OBL (-5)	24 (100.0)	45.6
Galium tinctorium	(L.) Scop.	Rubiaceae	Native	5	OBL (-5)	1 (4.2)	12.5
Galium trifidum	L.	Rubiaceae	Native	5	FACW+ (-4)	3 (12.5)	14.6
Geum aleppicum	Jacq.	Rosaceae	Native	2	FAC+ (-1)	2 (8.3)	6.3
Geum canadense	Jacq.	Rosaceae	Native	3	FAC (0)	3 (12.5)	14.6
Geum laciniatum	Murray	Rosaceae	Native	4	FACW (-3)	2 (8.3)	9.4
Glechoma hederacea	L.	Lamiaceae	Non-Native	-2	FACU (3)	12 (50.0)	40.1
Glyceria striata	(Lam.) Hitchc.	Poaceae	Native	3	OBL (-5)	6 (25.0)	13.5
Glycine max	(L.) Merr.	Fabaceae	Non-Native	-1	UPL (5)	1 (4.2)	6.3
Gnaphalium uliginosum	L.	Asteraceae	Non-Native	-1	FAC (0)	7 (29.2)	12.5
Helianthus sp.	L.	Asteraceae	Native	2	FACU (3)	1 (4.2)	25.0
Hesperis matronalis	L.	Brassicaceae	Non-Native	-3	UPL (5)	1 (4.2)	6.3
Heteranthera dubia	(Jacq.) MacMill.	Pontederiaceae	Native	7	OBL (-5)	5 (20.8)	35.7
Hydrocharis morsus-ranae	L.	Hydrocharitaceae	Non-Native	-3	OBL (-5)	6 (25.0)	28.8
Hydrophyllum virginianum	L.	Hydrophyllaceae	Native	6	FACW- (-2)	1 (4.2)	18.8
Hypericum perforatum	L.	Clusiaceae	Non-Native	-3	UPL (5)	2 (8.3)	6.3
Impatiens capensis	Meerb.	Balsaminaceae	Native	4	FACW (-3)	24 (100.0)	71.6
Iris versicolor	L.	Iridaceae	Native	5	OBL (-5)	3 (12.5)	10.4
Juncus bufonius	L.	Juncaceae	Native	1	FACW+ (-4)	3 (12.5)	8.3
Juncus compressus	Jacq.	Juncaceae	Non-Native	-1	FACW+ (-4)	1 (4.2)	6.3
Juncus effusus	L.	Juncaceae	Native	4	OBL (-5)	1 (4.2)	6.3
Juncus sp.	L.	Juncaceae	Native	5	FACW+ (-4)	1 (4.2)	6.3
Juncus tenuis	Willd.	Juncaceae	Native	0	FAC (0)	1 (4.2)	6.3
Lactuca hirsuta	Muhl. ex Nutt.	Asteraceae	Native	7	UPL (5)	1 (4.2)	6.3
Lactuca serriola	L.	Asteraceae	Non-Native	-1	FAC (0)	1 (4.2)	6.3
Lactuca sp.	L.	Asteraceae	Native	2	FACU (3)	1 (4.2)	6.3
Laportea canadensis	(L.) Weddell	Urticaceae	Native	6	FACW (-3)	1 (4.2)	12.5
Lathyrus palustris	L.	Fabaceae	Native	6	FACW (-3)	2 (8.3)	21.9

Species	Authority	Family	Native Status	Coefficient of conservation (CC)[a]	Wetness category (Index)[b]	Number sites found (%)	Average frequency/ site (%)
Leersia oryzoides	(L.) Sw.	Poaceae	Native	3	OBL (-5)	24 (100.0)	38.7
Lemna minor	L.	Lemnaceae	Native	2	OBL (-5)	21 (87.5)	67.1
Lemna trisulca	L.	Lemnaceae	Native	4	OBL (-5)	2 (8.3)	17.1
Leucanthemum vulgare	Lam.	Asteraceae	Non-Native	-1	UPL (5)	2 (8.3)	6.3
Linaria vulgaris	Mill.	Scrophulariaceae	Non-Native	-1	UPL (5)	8 (33.3)	16.4
Lindernia dubia	(L.) Pennell	Scrophulariaceae	Native	7	OBL (-5)	7 (29.2)	12.5
Lonicera tatarica	L.	Caprifoliaceae	Non-Native	-3	FACU (3)	1 (4.2)	6.3
Lotus corniculatus	L.	Fabaceae	Non-Native	-2	FAC- (1)	7 (29.2)	28.6
Ludwigia palustris	(L.) Elliott	Onagraceae	Native	5	OBL (-5)	15 (62.5)	18.3
Lycopus americanus	Muhl. ex W.P.C. Barton	Lamiaceae	Native	4	OBL (-5)	16 (66.7)	13.7
Lycopus uniflorus	Michx.	Lamiaceae	Native	5	OBL (-5)	14 (58.3)	11.4
Lycopus virginicus	L.	Lamiaceae	Native	8	OBL (-5)	8 (33.3)	10.9
Lysimachia ciliata	L.	Primulaceae	Native	4	FACW (-3)	4 (16.7)	12.5
Lysimachia nummularia	L.	Primulaceae	Non-Native	-3	FACW+ (-4)	7 (29.2)	13.4
Lythrum alatum	Pursh	Lythraceae	Native	5	OBL (-5)	1 (4.2)	4.8
Lythrum salicaria	L.	Lythraceae	Non-Native	-3	OBL (-5)	23 (95.8)	50.8
Maianthemum racemosum	(L.) Link	Liliaceae	Native	4	FACU (3)	1 (4.2)	6.3
Maianthemum sp.	F.H. Wigg.	Liliaceae	Native	6	FAC (0)	1 (4.2)	18.8
Maianthemum stellatum	(L.) Link	Liliaceae	Native	6	FAC- (1)	2 (8.3)	21.9
Matteuccia struthiopteris	(L.) Todaro	Dryopteridaceae	Native	5	FACW (-3)	2 (8.3)	9.4
Medicago lupulina	L.	Fabaceae	Non-Native	-1	FAC- (1)	12 (50.0)	22.9
Medicago sativa	L.	Fabaceae	Non-Native	-1	UPL (5)	1 (4.2)	6.3
Melilotus officinalis	(L.) Lam.	Fabaceae	Non-Native	-2	FACU (3)	8 (33.3)	18.0
Mentha arvensis	L.	Lamiaceae	Native	3	FACW (-3)	18 (75.0)	20.5
Mentha spicata	L.	Lamiaceae	Non-Native	-1	FACW+ (-4)	4 (16.7)	12.5
Mentha x piperita	L. (pro sp.) [aquatica x spicata]	Lamiaceae	Non-Native	-1	OBL (-5)	3 (12.5)	16.7
Mimulus ringens	L.	Scrophulariaceae	Native	6	OBL (-5)	8 (33.3)	15.6
Muhlenbergia frondosa	(Poir.) Fernald	Poaceae	Native	5	FACW (-3)	2 (8.3)	15.6
Muhlenbergia mexicana	(L.) Trin.	Poaceae	Native	1	FACW (-3)	6 (25.0)	17.7
Myosoton aquaticum	(L.) Moench	Caryophyllaceae	Non-Native	-1	FAC+ (-1)	1 (4.2)	12.5
Myriophyllum sibiricum	Kom.	Haloragaceae	Native	6	OBL (-5)	1 (4.2)	25.0

Species	Authority	Family	Native Status	Coefficient of conservation (CC)[a]	Wetness category (Index)[b]	Number sites found (%)	Average frequency/ site (%)
Myriophyllum spicatum	L.	Haloragaceae	Non-Native	-3	OBL (-5)	1 (4.2)	16.7
Myriophyllum verticillatum	L.	Haloragaceae	Native	7	OBL (-5)	1 (4.2)	10.0
Najas flexilis	(Willd.) Rostk. & Schmidt	Najadaceae	Native	5	OBL (-5)	7 (29.2)	16.3
Neobeckia aquatica	(Eaton) Greene	Brassicaceae	Native	9	OBL (-5)	1 (4.2)	5.0
Nuphar lutea ssp. pumila	(L.) Sm. ssp. (Timm) E.O. Beal	Nymphaeaceae	Native	8	OBL (-5)	4 (16.7)	19.8
Nuphar lutea ssp. variegata	(L.) Sm. ssp. (Durand) E.O. Beal	Nymphaeaceae	Native	4	OBL (-5)	10 (41.7)	18.3
Nymphaea odorata	Aiton	Nymphaeaceae	Native	5	OBL (-5)	7 (29.2)	16.1
Oenothera biennis	L.	Onagraceae	Native	0	FACU (3)	5 (20.8)	6.3
Onoclea sensibilis	L.	Dryopteridaceae	Native	4	FACW (-3)	6 (25.0)	29.2
Oxalis stricta	L.	Oxalidaceae	Native	0	FACU (3)	21 (87.5)	31.3
Panicum capillare	L.	Poaceae	Native	0	FAC (0)	17 (70.8)	15.4
Parthenocissus quinquefolia	(L.) Planch.	Vitaceae	Native	6	FAC- (1)	7 (29.2)	25.0
Pastinaca sativa	L.	Apiaceae	Non-Native	-3	UPL (5)	15 (62.5)	40.8
Penthorum sedoides	L.	Crassulaceae	Native	4	OBL (-5)	4 (16.7)	10.9
Phalaris arundinacea	L.	Poaceae	Native	0	FACW+ (-4)	24 (100.0)	87.2
Phleum pratense	L.	Poaceae	Non-Native	-1	FACU (3)	10 (41.7)	13.8
Physalis heterophylla	Nees	Solanaceae	Native	3	UPL (5)	2 (8.3)	12.5
Physalis pubescens	L.	Solanaceae	Non-Native	-1	UPL (5)	1 (4.2)	6.3
Pilea pumila	(L.) A. Gray	Urticaceae	Native	5	FACW (-3)	24 (100.0)	43.2
Plantago lanceolata	L.	Plantaginaceae	Non-Native	-1	FAC (0)	3 (12.5)	12.5
Plantago major	L.	Plantaginaceae	Non-Native	-1	FAC+ (-1)	22 (91.7)	29.8
Poa compressa	L.	Poaceae	Native	0	FACU+ (2)	12 (50.0)	17.2
Poa palustris	L.	Poaceae	Native	5	FACW+ (-4)	6 (25.0)	26.0
Poa pratensis	L.	Poaceae	Native	0	FAC- (1)	21 (87.5)	29.5
Polygonum amphibium	L.	Polygonaceae	Native	5	OBL (-5)	10 (41.7)	19.2
Polygonum aviculare	L.	Polygonaceae	Non-Native	-1	FAC- (1)	2 (8.3)	6.3
Polygonum convolvulus	L.	Polygonaceae	Non-Native	-1	FAC- (1)	6 (25.0)	14.6
Polygonum hydropiper	L.	Polygonaceae	Native	4	OBL (-5)	19 (79.2)	25.7
Polygonum hydropiperoides	Michx.	Polygonaceae	Native	4	OBL (-5)	9 (37.5)	22.8
Polygonum lapathifolium	L.	Polygonaceae	Native	2	FACW+ (-4)	18 (75.0)	25.3
Polygonum pensylvanicum	L.	Polygonaceae	Native	3	FACW+ (-4)	15 (62.5)	15.9

Species	Authority	Family	Native Status	Coefficient of conservation (CC)[a]	Wetness category (Index)[b]	Number sites found (%)	Average frequency/ site (%)
Polygonum persicaria	L.	Polygonaceae	Non-Native	-1	FACW (-3)	16 (66.7)	9.1
Polygonum sagittatum	L.	Polygonaceae	Native	5	OBL (-5)	8 (33.3)	43.0
Polygonum sp.	L.	Polygonaceae	Native	3	FAC (0)	2 (8.3)	9.4
Pontederia cordata	L.	Pontederiaceae	Native	7	OBL (-5)	4 (16.7)	48.7
Potamogeton amplifolius	Tuck.	Potamogetonaceae	Native	5	OBL (-5)	3 (12.5)	28.1
Potamogeton epihydrus	Raf.	Potamogetonaceae	Native	5	OBL (-5)	3 (12.5)	26.0
Potamogeton foliosus	Raf.	Potamogetonaceae	Native	4	OBL (-5)	3 (12.5)	12.8
Potamogeton natans	L.	Potamogetonaceae	Native	5	OBL (-5)	9 (37.5)	26.3
Potamogeton nodosus	Poir.	Potamogetonaceae	Native	7	OBL (-5)	15 (62.5)	30.0
Potamogeton pusillus	L.	Potamogetonaceae	Native	5	OBL (-5)	2 (8.3)	30.0
Potamogeton richardsonii	(Benn.) Rydb.	Potamogetonaceae	Native	5	OBL (-5)	1 (4.2)	8.3
Potamogeton robbinsii	Oakes	Potamogetonaceae	Native	7	OBL (-5)	1 (4.2)	15.0
Potamogeton zosteriformis	Fernald	Potamogetonaceae	Native	5	OBL (-5)	10 (41.7)	29.4
Potentilla norvegica	L.	Rosaceae	Native	0	FAC (0)	2 (8.3)	6.3
Potentilla recta	L.	Rosaceae	Non-Native	-2	UPL (5)	1 (4.2)	12.5
Prunella vulgaris	L.	Lamiaceae	Non-Native	-1	FAC (0)	1 (4.2)	6.3
Prunus sp.	L.	Rosaceae	Native	1	FACU- (4)	1 (4.2)	12.5
Ranunculus abortivus	L.	Ranunculaceae	Native	2	FACW- (-2)	2 (8.3)	6.3
Ranunculus acris	L.	Ranunculaceae	Non-Native	-2	FACW- (-2)	4 (16.7)	14.1
Ranunculus pensylvanicus	L. f.	Ranunculaceae	Native	3	OBL (-5)	2 (8.3)	9.4
Ranunculus sceleratus	L.	Ranunculaceae	Native	2	OBL (-5)	2 (8.3)	9.4
Ranunculus trichophyllus	Chaix	Ranunculaceae	Native	5	OBL (-5)	1 (4.2)	4.2
Ribes americanum	Mill.	Grossulariaceae	Native	4	FACW (-3)	1 (4.2)	6.3
Rorippa palustris	(L.) Besser	Brassicaceae	Native	3	OBL (-5)	12 (50.0)	9.8
Rosa blanda	Aiton	Rosaceae	Native	3	FACU (3)	1 (4.2)	12.5
Rosa palustris	Marshall	Rosaceae	Native	7	OBL (-5)	2 (8.3)	12.5
Rubus allegheniensis	Porter	Rosaceae	Native	2	FACU+ (2)	1 (4.2)	37.5
Rubus idaeus	L.	Rosaceae	Native	0	FACW- (-2)	8 (33.3)	18.8
Rumex crispus	L.	Polygonaceae	Non-Native	-2	FAC+ (-1)	12 (50.0)	18.8
Rumex maritimus	L.	Polygonaceae	Native	2	FACW+ (-4)	1 (4.2)	12.5
Rumex orbiculatus	A. Gray	Polygonaceae	Native	6	OBL (-5)	1 (4.2)	6.3

Species	Authority	Family	Native Status	Coefficient of conservation (CC)[a]	Wetness category (Index)[b]	Number sites found (%)	Average frequency/ site (%)
Rumex verticillatus	L.	Polygonaceae	Native	7	OBL (-5)	1 (4.2)	10.0
Sagittaria cuneata	Sheldon	Alismataceae	Native	7	OBL (-5)	6 (25.0)	19.7
Sagittaria latifolia	Willd.	Alismataceae	Native	4	OBL (-5)	22 (91.7)	41.3
Sagittaria rigida	Pursh	Alismataceae	Native	6	OBL (-5)	3 (12.5)	15.3
Salix sp.	L.	Salicaceae	Native	4	FACW- (-2)	2 (8.3)	9.4
Schoenoplectus fluviatilis	(Torr.) M.T. Strong	Cyperaceae	Native	7	OBL (-5)	2 (8.3)	47.9
Schoenoplectus tabernaemontani	(C.C. Gmel.) Palla	Cyperaceae	Native	5	OBL (-5)	8 (33.3)	16.5
Scirpus atrovirens	Willd.	Cyperaceae	Native	3	OBL (-5)	1 (4.2)	6.3
Scirpus cyperinus	(L.) Kunth	Cyperaceae	Native	4	OBL (-5)	3 (12.5)	6.3
Scutellaria galericulata	L.	Lamiaceae	Native	6	OBL (-5)	2 (8.3)	6.3
Scutellaria lateriflora	L.	Lamiaceae	Native	5	OBL (-5)	5 (20.8)	8.8
Scutellaria x churchilliana	Fernald	Lamiaceae	Native	5.5	OBL (-5)	1 (4.2)	6.3
Setaria viridis	(L.) P. Beauv.	Poaceae	Non-Native	-1	UPL (5)	3 (12.5)	8.3
Sinapis arvensis	L.	Brassicaceae	Non-Native	-1	UPL (5)	8 (33.3)	21.1
Sisymbrium officinale	(L.) Scop.	Brassicaceae	Non-Native	-1	UPL (5)	2 (8.3)	6.3
Solanum dulcamara	L.	Solanaceae	Non-Native	-2	FAC (0)	13 (54.2)	16.8
Solidago altissima	L.	Asteraceae	Native	1	FACU (3)	6 (25.0)	14.6
Solidago canadensis	L.	Asteraceae	Native	1	FACU (3)	7 (29.2)	41.1
Solidago gigantea	Aiton	Asteraceae	Native	4	FACW (-3)	7 (29.2)	27.7
Solidago sp.	L.	Asteraceae	Native	7	FAC- (1)	4 (16.7)	20.3
Sonchus arvensis	L.	Asteraceae	Non-Native	-1	FAC- (1)	7 (29.2)	13.4
Sonchus asper	(L.) Hill	Asteraceae	Native	1	FAC (0)	10 (41.7)	13.1
Sonchus oleraceus	L.	Asteraceae	Non-Native	-1	FACU (3)	10 (41.7)	23.1
Sonchus sp.	L.	Asteraceae	Non-Native	-1	FAC (0)	3 (12.5)	22.9
Sparganium americanum	Nutt.	Sparganiaceae	Native	6	OBL (-5)	1 (4.2)	5.0
Sparganium emersum	Rehmann	Sparganiaceae	Native	5	OBL (-5)	2 (8.3)	12.5
Sparganium eurycarpum	Engelm.	Sparganiaceae	Native	3	OBL (-5)	17 (70.8)	32.5
Spiraea alba	Du Roi	Rosaceae	Native	3	FACW+ (-4)	2 (8.3)	6.3
Spirodela polyrrhiza	(L.) Schleid.	Lemnaceae	Native	4	OBL (-5)	18 (75.0)	59.1
Stachys palustris	L.	Lamiaceae	Non-Native	-1	OBL (-5)	1 (4.2)	12.5
Stachys tenuifolia	Willd.	Lamiaceae	Native	7	FACW+ (-4)	5 (20.8)	20.0

Species	Authority	Family	Native Status	Coefficient of conservation (CC)[a]	Wetness category (Index)[b]	Number sites found (%)	Average frequency/ site (%)
Stellaria graminea	L.	Caryophyllaceae	Non-Native	-2	UPL (5)	2 (8.3)	9.4
Stellaria longifolia	Muhl. ex Willd.	Caryophyllaceae	Native	2	FACW+ (-4)	1 (4.2)	6.3
Stuckenia pectinata	(L.) Börner	Potamogetonaceae	Native	4	OBL (-5)	12 (50.0)	23.7
Symphyotrichum lanceolatum	(Willd.) G.L. Nesom	Asteraceae	Native	3	FACW (-3)	14 (58.3)	19.6
Symphyotrichum lateriflorum	(L.) Á. Löve & D Löve	Asteraceae	Native	3	FACW- (-2)	8 (33.3)	14.8
Symphyotrichum novae-angliae	(L.) G.L. Nesom	Asteraceae	Native	2	FACW (-3)	6 (25.0)	16.7
Symphyotrichum sp.	Nees	Asteraceae	Native	6	FAC- (1)	2 (8.3)	15.6
Tanacetum vulgare	L.	Asteraceae	Non-Native	-1	UPL (5)	4 (16.7)	15.6
Taraxacum officinale	F.H. Wigg.	Asteraceae	Non-Native	-2	FACU (3)	17 (70.8)	21.3
Thalictrum dioicum	L.	Ranunculaceae	Native	5	FACU+ (2)	1 (4.2)	6.3
Thalictrum pubescens	Pursh	Ranunculaceae	Native	5	FACW- (-2)	7 (29.2)	17.9
Thalictrum sp.	L.	Ranunculaceae	Native	7	FAC+ (-1)	1 (4.2)	12.5
Thlaspi arvense	L.	Brassicaceae	Non-Native	-1	UPL (5)	2 (8.3)	12.5
Toxicodendron radicans	(L.) Kuntze	Anacardiaceae	Native	5	FAC+ (-1)	4 (16.7)	14.1
Tragopogon pratensis	L.	Asteraceae	Non-Native	-1	UPL (5)	4 (16.7)	7.8
Trifolium arvense	L.	Fabaceae	Non-Native	-1	UPL (5)	1 (4.2)	6.3
Trifolium hybridum	L.	Fabaceae	Non-Native	-1	FAC- (1)	4 (16.7)	12.5
Trifolium pratense	L.	Fabaceae	Non-Native	-2	FACU+ (2)	5 (20.8)	25.0
Trifolium repens	L.	Fabaceae	Non-Native	-1	FACU+ (2)	7 (29.2)	23.2
Trifolium sp.	L.	Fabaceae	Non-Native	-1	FACU- (4)	1 (4.2)	6.3
Typha latifolia	L.	Typhaceae	Native	3	OBL (-5)	3 (12.5)	25.0
Urtica dioica	L.	Urticaceae	Native	2	FAC+ (-1)	22 (91.7)	45.7
Utricularia macrorhiza	Leconte	Lentibulariaceae	Native	4	OBL (-5)	3 (12.5)	18.1
Vallisneria americana	Michx.	Hydrocharitaceae	Native	6	OBL (-5)	2 (8.3)	43.8
Verbascum thapsus	L.	Scrophulariaceae	Non-Native	-2	UPL (5)	1 (4.2)	12.5
Verbena hastata	L.	Verbenaceae	Native	4	FACW+ (-4)	18 (75.0)	12.8
Verbena urticifolia	L.	Verbenaceae	Native	4	FAC+ (-1)	6 (25.0)	12.5
Veronica anagallis-aquatica	L.	Scrophulariaceae	Non-Native	-1	OBL (-5)	1 (4.2)	6.3
Veronica peregrina	L.	Scrophulariaceae	Native	0	FACW+ (-4)	1 (4.2)	6.3
Viburnum lentago	L.	Caprifoliaceae	Native	4	FAC+ (-1)	2 (8.3)	6.3
Viburnum nudum	(L.) Torr. & A. Gray	Caprifoliaceae	Native	7	FACW (-3)	1 (4.2)	12.5

Species	Authority	Family	Native Status	Coefficient of conservation (CC)[a]	Wetness category (Index)[b]	Number sites found (%)	Average frequency/ site (%)
Vicia cracca	L.	Fabaceae	Non-Native	-1	UPL (5)	22 (91.7)	51.4
Viola sp.	L.	Violaceae	Native	6	FAC+ (-1)	2 (8.3)	9.4
Vitis riparia	Michx.	Vitaceae	Native	0	FACW- (-2)	6 (25.0)	11.5
Wolffia columbiana	Karst.	Lemnaceae	Native	4	OBL (-5)	1 (4.2)	75.0
Xanthium strumarium	L.	Asteraceae	Native	2	FAC (0)	9 (37.5)	20.0
Zanthoxylum americanum	Mill.	Rutaceae	Native	3	UPL (5)	1 (4.2)	12.5
Zizania aquatica	L.	Poaceae	Native	9	OBL (-5)	7 (29.2)	31.7
Zizania palustris	L.	Poaceae	Native	9	OBL (-5)	3 (12.5)	37.8

[a]**Coefficient of Conservation** (Oldham et al., 1995)
-3 to -1: Non-native species (-3: species that can pose serious problems, -2: species that can be problematic infrequently or in localized areas, -1: species that typically have little or no impact on natural areas)
0 to 3: Species found in a variety of communities, including disturbed areas
4 to 6: Species tolerant of moderate disturbance
7 to 8: Species associated with advanced successional stages and communities that have undergone minor disturbance
9 to 10: Species with a high degree of fidelity to a narrow range of environmental conditions

[b]**Wetland category and Index** (Oldham et al., 1995)
Categories:
OBL (-5): obligate wetland
FACW (-4 to -2): facultative wetland
FAC (-1 to 1): facultative
FACU (2 to 4): facultative upland
UPL (5): obligate upland
+: Higher probability of occurring in a wetland compared to the general indicator for a particular category
-: Lower probability of occurring in a wetland compared to the general indicator for a particular category

Table B.3 Sørensen coefficients of 12 (24 in total) stream/ river sites in the South Nation River watershed, Canada.

Tributary[a]	Sorenson Coefficient
1	27.7
2	32.1
3	38.0
4	37.4
5	38.5
6	35.9
7	37.8
8	38.9
9	39.6
10	33.1
11	40.5
12	40.8
Average	36.7 ± 3.9

[a]Numbers correspond with Chapter 2, Fig. 2.1

Table B.4 Comparison of plant communities at sites surrounded by low and high levels of agriculture and across the watershed with three measures of agricultural impact. Significant trends ($p<0.05$) are indicated in **bold** with the slope of the line designated as positive (+) or negative (-).

Variable	Low vs high agriculture sites (paired t-test; df=11)	Effect of nitrate (regression; df=1,23)	Effect of atrazine (regression; df=1,23)	Effect of % annual crops (regression; df=1,23)
Species richness	t=-0.692; p=0.504	F=0.004; p=0.952; R^2=0.000	F=0.008; p=0.931; R^2=0.000	F=0.350; p=0.560; R^2=0.016
Percentage non-native (%)	t=-1.660; p=0.125	**F=5.786; p=0.025; R^2=0.208 (+)**	F=0.002; p=0.967; R^2=0.000	F=2.616; p=0.120; R^2=0.106
Non-native relative frequency (%)	t=-1.420; p=0.183	**F=5.865; p=0.024; R^2=0.210 (+)**	F=0.168; p=0.686; R^2=0.008	F=2.224; p=0.150; R^2=0.092
Number of aquatic species	t=1.200; p=0.255	**F=6.550; p=0.018; R^2=0.229 (-)**	F=1.044; p=0.318; R^2=0.045	F=2.786; p=0.109; R^2=0.112
Number of emergent species	t=0.440; p=0.669	**F=5.140; p=0.034; R^2=0.189 (-)**	F=0.952; p=0.340; R^2=0.041	F=1.800; p=0.193; R^2=0.076
Number of floating species	t=1.328; p=0.211	F=3.771; p=0.065; R^2=0.146	F=0.542 p=0.469; R^2=0.024	F=1.465; p=0.239; R^2=0.062
Number of submerged species	t=2.085; p=0.061	**F=5.030; p=0.035; R^2=0.186 (-)**	F=0.928; p=0.346; R^2=0.040	F=4.002; p=0.058; R^2=0.154
Emergent relative frequency	**t=-2.677; p=0.022 (low<high)**	F=0.988; p=0.331; R^2=0.043	F=0.418; p=0.525; R^2=0.019	F=2.963; p=0.099; R^2=0.119
Floating relative frequency	t=0.881; p=0.397	F=0.629; p=0.436; R^2=0.028	F=0.007; p=0.936; R^2=0.000	F=0.071; p=0.793; R^2=0.003
Submerged relative frequency	**t=2.406; p=0.035 (low>high)**	F=3.235; p=0.086; R^2=0.128	F=0.754; p=0.394; R^2=0.033	F=2.527; p=0.126; R^2=0.103 (sqrt)
Floristic Quality Index	t=1.717; p=0.114	**F=10.566; p=0.004; R^2=0.324 (-)**	F=0.292; p=0.594; R^2=0.013	**F=6.552; p=0.018; R^2=0.229 (-)**

Appendix C. Supplementary data for Chapter 4

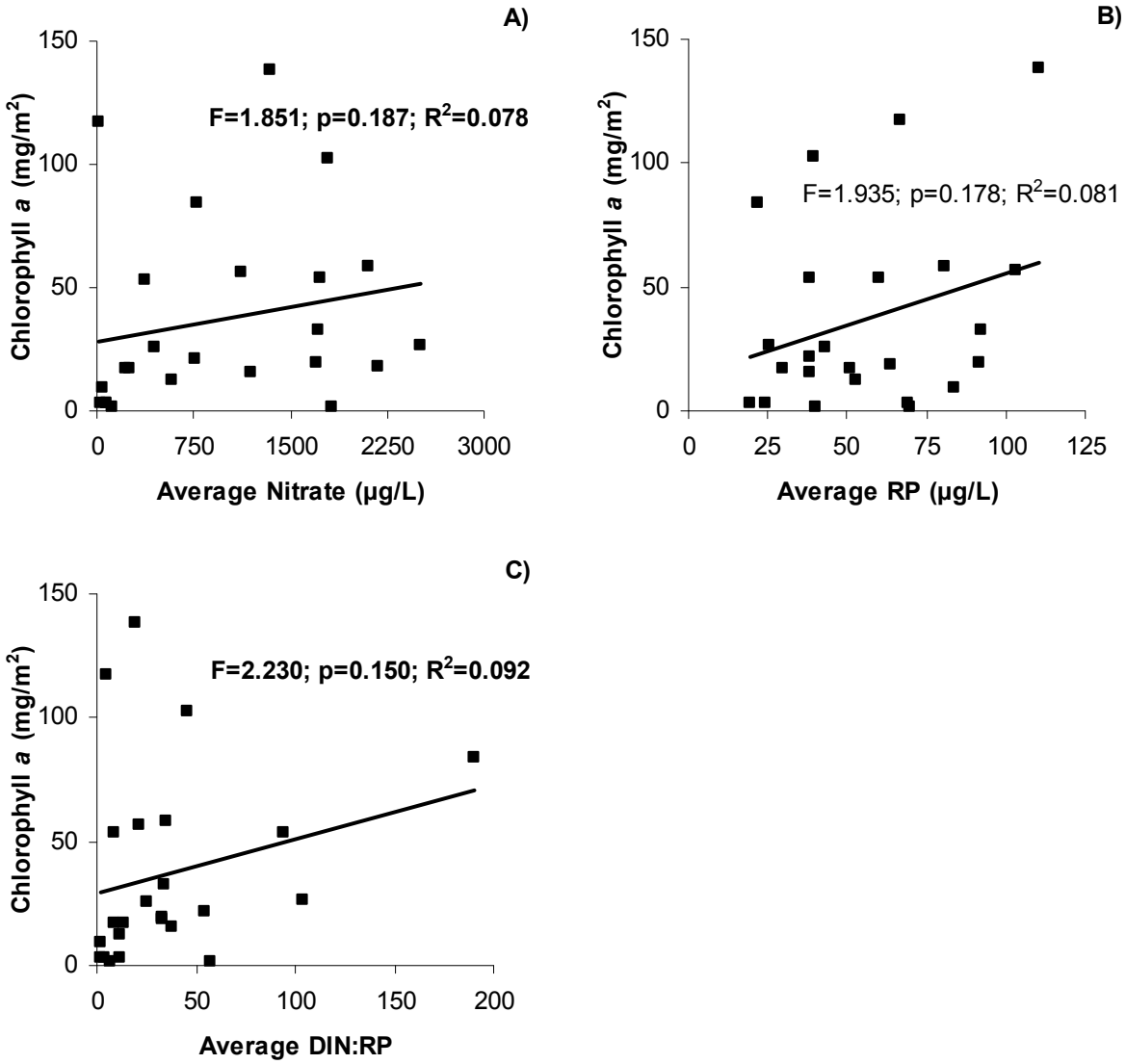

Fig. C.1 Relationship between periphyton biomass (mg/m^2 chlorophyll *a*) at 12 paired sites (24 in total) and A) nitrate, B) reactive phosphate (RP) and C) ratio of dissolved inorganic nitrogen to reactive phosphate (DIN:RP). Data were averaged from samples collected in May, June and July 2010. Regression statistics and trend lines are shown.