Examining the Taxonomic Identity of the Green Alga *Oophila Amblystomatis* (Chlamydomonadales), the Biogeography of its Symbiont *Ambystoma Maculatum* (Amphibia), and the Toxic Response of the Green Alga to Two Agricultural Herbicides

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

The yellow spotted salamander (*Ambystoma maculatum*) shares a unique endosymbiotic relationship with the unicellular green alga *Oophila amblystomatis.* Though a number of studies have isolated and identified *O. amblystomatis,* the alga's taxonomic identity is yet to be resolved. In this study the nuclear SSU rRNA gene was used to identify two well supported *Oophila* clades that included sequences from past studies in addition to isolates from the current study, and showed that *O. amblystomatis* does not group monophyletically with its own members, and groups paraphyletically with other species of green algae. Past studies have also assessed the potential for indirect effects on embryo development via herbicidal exposure to the endosymbiotic algae, but few have taken into account the possibility of correct species identification, strain, or locale sensitivity. In this study, the response of *O. amblystomatis* to the exposure of two herbicides, atrazine and 2,4-dichlorophenoxyacetic (96 h exposure acute toxicity tests), followed by recovery (96 h recovery in untreated media) acid were characterized. Lowest growth inhibition no-observed effect concentrations of 70 µg/L and 30 mg/L of atrazine and 2,4-D, respectively, followed by full recovery at these concentrations, indicate that these herbicides do not pose a risk of growth inhibition to egg-inhabiting algae. This study proposes a revision of the current taxonomy of *O. amblystomatis*, and demonstrates the need for species identification and thorough phylogenetic reconstruction in toxicity testing.

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List of Figures

List of Tables

1. General Introduction

Ambystoma maculatum (Shaw), the yellow spotted salamander, shares a unique mutualistic relationship with a unicellular green alga commonly described as *Oophila amblystomatis* (Chlamydomonadales) Lambert ex Wille (Orr 1888, Wille 1905). Kerney (2011) suggested that the algal cells enter the salamander eggs after they are laid and provide the developing embryos with oxygen. This additional provision of oxygen has been shown to increase the viability and hatching success of the embryos (Gilbert & Perry 1944). More recently, it has been proposed that the embryos also incorporate fixed carbon produced by the algae, which further aids in their development (Graham et al. 2013). The algae in turn benefit from compounds produced in the nitrogenous waste of the embryos, such as ammonia, which the algae convert to proteins (Goff & Stein 1978, Kerney 2011). The gelatinous matrix encompassing the eggs may also serve as protection for the algae from grazing (Kerney 2011).

The habitat of *A. maculatum* ranges from Nova Scotia in eastern Canada to Florida in the southern United States and reaches westward towards Ontario and Texas (Phillips 1994). Two scenarios have been proposed to explain the biogeographic distribution of *A. maculatum* and other North American fauna, post-glacial advance (Zamudio and Savage 2003). Numerous studies have hypothesized that species could have either evolved in eastern North America then spread west after glaciation, or diversity within refugial populations could have already existed before glaciation, meaning that glaciation periods may not have served as the sole driver of genetic variation in North America (Pflieger 1970, Mayden 1985, 1987). Recent evidence postulated by Zamudio and Savage (2003) has suggested that genetic divergence

within *A. maculatum* populations points towards post-glacial dispersal and radiation of these organisms from at least two southern refugia in the southern Appalachian Mountains (Zamudio and Savage 2003).

Spotted salamanders inhabit forested areas, and migrate to ephemeral freshwater ponds to mate and lay their eggs in the spring (Newcomb et al. 2003). In farmed regions, these temporary ponds may be associated with agricultural runoff that could be contaminated with pesticides (Mann et al. 2009). Amphibians and their eggs are particularly sensitive to contaminants due to their highly permeable integument, which allows for gas exchange and osmoregulation (Hopkins 2007). Hence, *A. maculatum* embryos and their mutualistic algae are both at risk of being exposed to contaminants including agricultural pesticides, which may accumulate in their habitats and alter aquatic conditions (Rodríguez-Gil et al. 2014, Hopkins 2007).

There is specific concern that herbicides could impair the growth and viability of the algal symbionts within *A. maculatum* eggs, whereby reducing hatching success and development of the embryos, as reported for atrazine by Olivier and Moon (2009). Atrazine is one of the most widely used herbicides in North America (Solomon et al. 1996) and in Ontario, it is the second most applied pesticide for corn crops (after glyphosate). It is primarily used to control annual broadleaf and grass weeds (McGee et al. 2010, Solomon et al. 1996).

Another widely used broadleaf agrochemical in North America is 2,4 dichlorophenoxyacetic acid (2,4-D) (Boivin et al. 2005). This herbicide is auxin simulator, and operates by disrupting normal plant cell division (Relyea et al. 2005, Fairchild et al. 1997). Though the direct effects of this herbicide on *A. maculatum*

embryos have not yet been extensively documented, there is potential for the algal symbionts to be inhibited by the herbicidal properties of 2,4-D (Wong 2000). Herbicides such as atrazine and 2,4-D pose a potential threat for amphibian populations in agricultural areas (Mann et al. 2009). As such, there is increasing concern for the risks that these chemicals may pose to amphibian health (Mann et al. 2009).

1.1 Symbiotic Relationships between the Chlorophyta and Animals

From aquatic to subaerial and terrestrial habitats, algae have colonized environments that would seemingly be uninhabitable for many other forms of life (Seckbach, 2007; Wolfang, 2000). Aquatic algae have a wide ranging tolerance of pH, temperature, dissolved oxygen, and carbon dioxide concentrations, and thus can be observed in freshwater bodies to salt lakes, hot springs and even deserts (Barsanti et al. 2008). These organisms have also colonized extremely cold environments such as glaciers, and lowlight environments such as the marine benthos, at depths of 200 m (Barsanti et al. 2008). In addition to their capabilities to adapt to a wide range of conditions, algae are also notable for their many different associations with other organisms such as epiphytism, parasitism, and various symbioses (Barsanti et al. 2008). Algae observed growing on plants or other algae are known as epiphytes, while epizoic algae are those that grow on animals (Bourrelly 1968). An example of the latter is *Trichophilus welckeri*, a species of green algae that inhabits the coarse and water absorbent fur of *Bradypus* spp., the three-toed sloth (Suutari et al. 2010). Though this alga has been described as the predominant algal species to inhabit *Bradypus* spp., phylogenetic analyses have revealed a diverse algal community (at least 20 species

across multiple genera) that has adapted to live with *Bradypus* and other sloth species (Suutari et al. 2010, Pauli et al. 2014, Voirin 2015). Differing hair structure, ecology, and divergence of sloth genera approximately 20 million years ago have been proposed as possible mechanisms for algal diversity and co-evolution in this symbiotic relationship (Suutari et al. 2010, Delsuc et al. 2004).

Algae have also been observed having endosymbiotic associations (where algal cells are absorbed by host) with other organisms (Kerney 2011). Perhaps the most well known example of algal-animal endosymbiosis is that between the sea slug *Elysia chlorotica* and its algal food source *Vaucheria litorea* (Ochrophyta), a yellow-green marine alga (Rumpho et al. 2008). The kleptoplastic (plastid stealing) sea slug obtains the alga's plastids via ingestion (herbivorous feeding) and stores the organelles in its epithelium where they continue to photosynthesize, providing the sea slug with an energy source (Rumpho et al. 2008). Alga cells are ingested during the larval stages of *E. chlorotica*, and again after metaphorphosis when larvae transform into adults (Schwartz et a. 2014). Thus, *E. chlorotica* has demonstrated the ability to renew its plastid endosymbionts (Schwartz et a. 2014).

In a similar case of endosymbiosis, the transient invasion of *Oophila amblystomatis* cells into the egg fluid of amphibian eggs is a well-known relationship seen in North American (Kerney 2011). Though other amphibians such as ranid frogs are also known to harbour *O. amblystomatis* within their eggs, this symbiotic relationship has been mostly observed and studied in *Ambystoma maculatum*, from where the alga derives its name (Wille 1909, Kerney 2011; Pinder and Friet 1994). The mutualism between *O. amblystomatis* and *A. maculatum* was first noted by Gilbert

(1942, 1944) who, through a series of experiments, noted that salamander eggs inhabited by the algal cells had lower mortality, faster hatching time and a greater rate of development than those without algal cells (Hutchinson and Hammen 1958). Increased proliferation of the algae in the presence of the salamander embryos was also observed, relative to algal growth in eggs where embryos were removed (Gilbert 1942). These embryo exclusion experiments have yet to be reproduced however, and experiments examining benefits to the algal symbionts are few compared to those examining benefits to *A. maculatum* (Kerney 2011). There is thus a need for further evidence to establish this symbiotic relationship as case of mutualism in favour of the endosymbiotic algae (Kerney 2011).

1.2 Taxonomy of the "*Oophila"* Clade

Recently, there has been increasing interest in the phylogeny and identity of *Oophila amblystomatis*. Named informally in 1905 by Lambert, who collected algal samples from *A. maculatum* eggs, the alga has been accepted as a "Chlamydomonad green alga" but lacks enough phylogenetic evidence for its current taxonomic position (Collins et al. 1905, Wille 1909, Kim et al. 2014). Recent phylogenetic reconstruction of algae sampled from *A. maculatum* eggs has suggested that the algal symbionts form a strongly supported clade which includes free living chlamydomonad taxa as well as sequences previously identified as *O. amblystomatis* (Kim et al. 2014, Rodríguez-Gil, et al. 2014, Baxter et al. 2015, Bishop and Miller 2014). Increasing evidence of the taxonomic position of *O. amblystomatis* amongst other green algal species indicates that the algae present in *A. maculatum* eggs is paraphyletic and groups with other

members within the *Chlamydomonas* genus (Baxter et al. 2015). Past studies have focused on the biogeography of this egg inhabiting algae, although sampling of the organisms has not extended to the complete range of *A. maculatum* (Phillips 1994, Zamudio and Savage 2003, Zamudio and Wieczorek 2007). To date, research focused on the phylogeny of *O. amblystomatis* has included samples from parts of eastern, western and central Canada, and some parts of the southern and eastern US (Kerney et al. 2011, Kim et al. 2014, Graham et al. 2013, Rodríguez-Gil et al. 2014), but the data have not been consolidated. Furthermore, most of the available sequence data for *O. amblystomatis* has been sampled from the North American east coast; thus it is evident that central North American regions are not, comparatively, sufficiently represented in terms of sample size. The investigation by Rodríguez-Gil et al. (2014), which included a sequence taken from Nova Scotia (Kerney et al. 2011), Pennsylvania (Graham et al. 2013) and their own samples collected from southern Ontario, highlighted this need for increased sampling as their phylogenetic analyses revealed a possibility for biogeographic variability amongst the algae. More recently a study performed by Kim et al. (2014), which analyzed the majority of sequences from Nova Scotia along with a few other sequences across Canada and the United States, concluded that their samples formed one distinct "*Oophila"* clade, without an indication of possible variation, though they did not include sequence data from Rodríguez-Gil et al. (2014) or other available GenBank sequences in their analyses. Thus, there has been some evidence to indicate that some algal symbionts of *A. maculatum* may occupy a separate clade outside of *O. amblystomatis*, contrary to what has been previously accepted (Rodríguez-Gil et al. 2014).

1.3 History and Taxonomy of the Chlamydomonads

The genus *Chlamydomonas* Ehrenberg (1883) has historically been accepted as including all green algal species in which cells were biflagellated and housed one pyrenoid containing chloroplast with a cell wall (Ettl 1976; Pröschold et al. 2001). For this reason, the genus was considered by some taxonomists to be an artificial designation, as the described characteristics are not exclusive, or synapomorphic, to all Chlamydomonad species (Ettl 1976; Pröschold et al. 2001). Furthermore, many of the species within this genus were first identified using morphological classification, and did not take into account possible life cycle and population level variation within the Chlamydomonads (Pröschold et al. 2001). Thus, the genetic identity of many previously classified Chlamydomonad species have yet to be molecularly confirmed (Pröschold et al. 2001).

One characteristic that has been used to delineate species within *Chlamydomona*s is the number and position of pyrenoids within the chloroplast. A lack of pyrenoids within the cell has led some phycologists to transfer some *Chlamydomonas* species into a separate genus, *Chloromonas* Gobi emend. Wille (1903), however this designation has not been unanimously accepted (Harris 2009; Pröschold et al. 2001). For example, Harris (2009) has maintained that the presence of pyrenoids can be affected by certain environmental conditions and hence, they can be absent in some parts of the life cycle. Futhermore, there is molecular evidence that demonstrates that some species in *Chloromonas* and *Chlamydomonas* group together, resulting in taxonomic amendments of these genera (Buchheim et al. 1997; Harris et al.,

2009, Morita et al. 1999; Pröschold et al. 2001). For example, Pröschold et al. (2001) used molecular evidence from nuclear encoded small subunit (SSU) rRNA gene sequences to regroup previously described pyrenoid-containing *Chlamydomonas* species with the *Chloromonas* genus. Another study examined cold-tolerant and snow inhabiting species of *Chlamydomonas* and *Chloromonas,* and revised the relationships among species in both genera using the nuclear SSU rRNA gene and *rbc*L genes (encodes the large subunit of the Ribulose 1,5-biphosphate carboxylase/oxygenase enzyme) and observed that pyrenoids have been gained and lost multiple times between these genera (Hoham et al. 2002). This supports the postulate that neither genus is monophyletic, despite being widely studied, and requires extensive taxonomic revision (Hoham et al. 2002).

Employing the use of nuclear SSU and ITS (Internal transcribed spacer) rRNA barcodes, Demchenko et al. (2012) also used chloroplast/pyrenoid structure as a defining characteristic to propose a revision of 15 previously described *Chlamydomonas* strains. The authors described their strains to have highly similar morphologies in organelles of the vegetative cells, including flagella and cup shaped chloroplasts (Demchenko et al. 2012). Despite some similarities in morphotypes however, comparison of mature and younger cells revealed differentiable organelle characters such as cup-shaped chloroplast versus lobed chloroplasts and ring-shaped versus horseshoe shaped pyrenoid. The extensive morphological characterizations of these previously *Chlamydomonas* sp. strains were confirmed with the phylogenetic reconstruction, and the authors show a newly characterized monophyletic lineage (within the Chlorophyceae) which they propose to rename *Microglena* (Demchenko et

al. 2012). Thus, it is evident that the polyphyletic *Chlamydomonas* genus is continually being re-examined and its species re-classified with novel and dynamic molecular tools (Pröschold et al. 2001, Harris et al. 2009, Demchenko et al. 2012).

1.4 Effects of Pleistocene Climate Changes on North American Taxa

Within the past few decades sea bed, lake bottom, and ice sheet cores have provided scientists with an abundance of data that have helped deepen our understanding of paleo-climates (Hewitt 2000). Evidence from these core analyses show that temperature oscillations during the Pleistocene epoch (1-2 million years before present) lead to Earth's cooling climate, and fluctuations with increasing amplitudes became more prevalent (Hewitt 2000, Zeisset and BeeBee 2008). These temperature oscillations led to a series of glaciation events that caused both extinctions and post glacial range expansion of many taxa around the world (Zamudio and Savage 2003). Fluctuations in temperature were felt differently globally and were influenced by ocean currents, latitude, and landform characteristics (Hewitt 2000). The Last Glacial Maximum (LGM) is regarded as the most recent time period when ice sheets had reached their maximum global coverage, around 19 000 to 20 000 years ago (Mix et al. 2001, Clark et al. 2009). Species responded accordingly to ice-covered Earth and were largely compressed to warmer areas towards the equator as ice sheets expanded (Hewitt 2000). Thus, many populations that currently inhabit previously glaciated areas can trace their lineages back to a limited number of southern refugia from where they began their colonization (Zamudio and Savage 2003). In North America and Europe, evidence from pollen cores has indicated that taxa currently inhabiting boreal and

temperate regions took refuge south of the ice and permafrost during glaciation (Hewitt 2000). It has been proposed that post-glacial expansion of many of these taxa was fairly rapid and that northernmost populations of southern refugia would have expanded into large swathes of habitable area (Hewitt 2000). Long distance dispersers would have dominated this “leading edge expansion”, establishing colonies and dispersing before other populations arrived (Hampe and Petit 2005, Hewitt 2000). A continuation of this colonizing process would ultimately lead to successive founder events that would result in homozygosity and a loss of alleles (Ibrahim et al. 1996). Studies of fossils records in areas of post-glacial colonization show this proposed reduction in genetic diversity, and there have been a number of North American phylogeographic studies which show decreasing genetic diversity in populations north of southern refugia (Hewitt;1996, 2003, Soltis et al. 1997, Conroy et al. 2000). Mountain uplift has also been thought to be a major vicariance event that influenced the biogeography of these taxa (Zeisset and Beebee 2008).

Within amphibians, however, there are instances where secondary contact of vicariant lineages post-glacial colonization has led to an increase in genetic diversity (Zeisset and Beebe 2008). For example, Austin et al. (2002) investigated the phylogeographic distribution of the amphibian *Pseudacris crucifer* (spring peeper): a small woodland frog that inhabits eastern North America. Through a series of mitochondrial haplotype studies, the authors inferred that recolonization of *P. crucifer* began from two isolated southern refugia in the southern Appalachians which then converged in Ontario post-glaciation (Austin et al. 2002). Studies have also revealed that post glacial expansion of both aquatic and terrestrial species in North America

reflect the allopatric differentiation caused by glacial movement (Zamudio and Savage 2003, Slots et al. 2006). Thus, geomorphological features such as mountain formation have, at different evolutionary scales, influenced both species diversification and intraspecific variation (Zamudio and Savage 2003)

To establish whether the phylogenetic patterns of *A. maculatum* today follow the hypothesized patterns of post glacial biogeography of North American taxa described above, Zamudio and Savage (2003) employed the use of two mitochondrial gene regions to examine the intraspecific phylogeny of *A. maculatum* throughout its range in eastern North America. Their extensive sampling from 82 locations was able to reveal the presence of two monophyletic clades which they hypothesize arose from two isolated refugia populations in the southern Appalachian region (Zamudio and Savage 2003). The aim of this study was to expand the sample area of Zamudio and Savage (2003), in hopes of gaining further insight into the biogeographic variation of *A. maculatum* within its range in southern Ontario. Thus, the mitochondrial gene regions ND4 and control region (including D-Loop) described by Zamudio and Savage (2003) were also employed in this study. Figure 1 shows Zamudio and Savage (2003)'s proposed clade hypothesis.

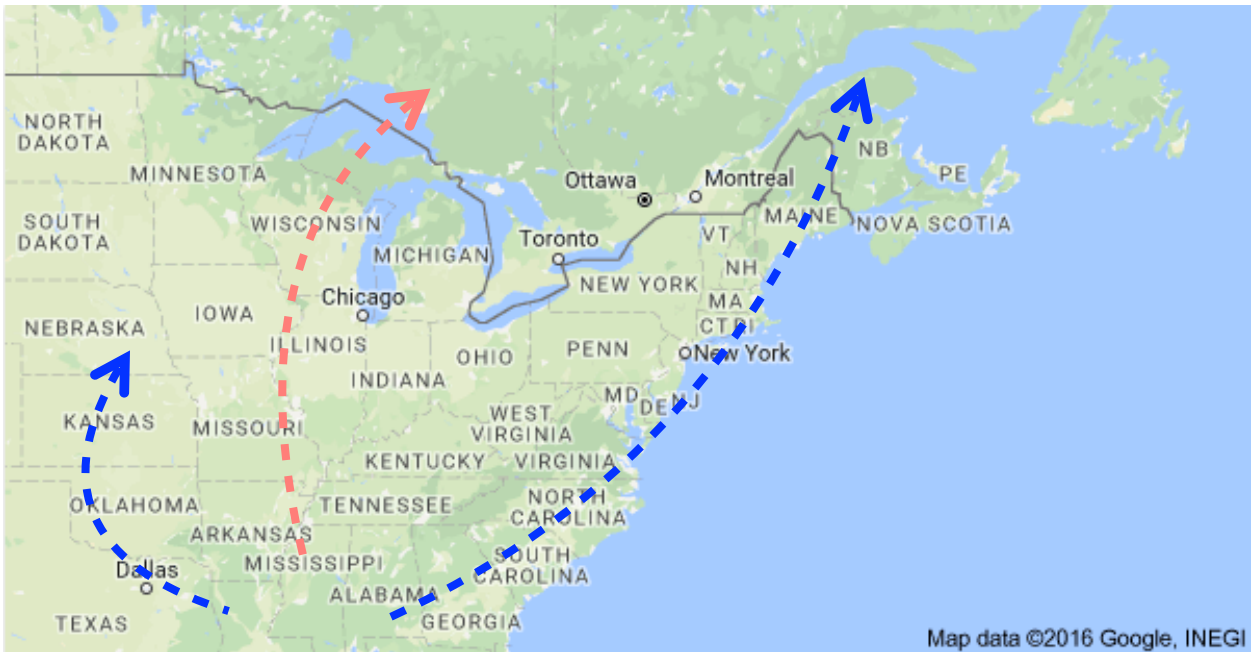

Figure 1. Map of eastern North America depicting *A. maculatum* lineage distribution proposed by Zamudio and Savage (2003). The blue arrows represent sample localities falling within the author's proposed "coastal clade hypothesis". The red arrow delineates samples falling into the "interior clade" (which consists of two strongly supported lineages).

1.5 Biogeography of Freshwater Algae in North America

Due to their great ability to disperse and form desiccant resistant cells in unfavourable conditions, fresh water algae are considered to be geographically widespread (Bodeker et al. 2010). One species of filamentous freshwater algae, *Cladophora glomerata* (Chlorophyta) for example, is found globally in a wide range of habitats including streams, estuaries, and even polluted lakes (Whitton 1970, Dodds and Gudder 1992). Within the coccoid flagellates, geographic distribution is equally expansive, with some genera such as *Chloromonas,* and *Chlamydomonas*, also inhabiting snow and ice (Wehr 2015). Of the estimated 100 freshwater green flagellated genera, 50 have been identified across North America (Wehr 2015).

Some species of algae are not as dispersive and require specific environmental conditions that meet their ecological demands in order to thrive (Coesel 1996, Baedeker et al. 2010). Within the cosmopolitan cyanobacteria for instance, there are some specialized cryptic species that have a very narrow range of habitat (Hoffmann 1996, Joyner et al. 2008). Intuitively, the more limited the distribution of a species is, the less opportunities it may have for dispersal, via faunal vectors such as birds for example (Kristiansen 1996, Bodeker et al. 2010). For this reason, less ubiquitous algal species may provide more insights into paleo-biogeography of these organisms as historic geographic distributions are not masked by vector distribution (Boedeker et al. 2010).

1.6 The Effects of Herbicides on *Oophila amblystomatis*

1.6.1 Atrazine

Research on the effects of the herbicide atrazine on *A. maculatum* eggs has shown both direct and indirect effects on growth and hatching success of the embryos (Olivier and Moon 2010), though recent and more expansive work was not able to replicate these initial findings (Baxter et al., 2015). The ability of atrazine to inhibit growth of *O. amblystomatis* within the salamander eggs is the theorized cause of these indirect detrimental effects. The herbicide's mode of action begins by binding to plastoquinone B located in the D1 subunit of PSII, and thus disrupting the flow of chloroplast electrons from plastoquinone A to B (Mullet and Arntzen, 1981; Steinback et al., 1981). This results in reduced ATP, and NADPH production, CO_2 fixation, and an ultimate reduced rate of photosynthesis (Mullet and Arntzen, 1981; Steinback et al., 1981; Zhu et al. 2009). Indirectly, the blockage of electron transport in PSII also causes the toxic accumulation of free radicals, such as reactive oxygen species, in PSII (Bowyer et al., 1991; Rutherford and Krieger-Liszkay, 2001; Zhu et al. 2009). These free radicals are subsequently responsible for protein and pigment damage, and ultimate plant death (Zhu et al. 2009).

With a low vapour pressure and Henry's law constant, atrazine's volatilization from both water and soil is negligible (Solomon et al. 1996). Furthermore, the movement of the chemical from soil to water systems is favoured, as it has a moderate solubility and a small partition coefficient (Solomon et al. 1996). The persistent of atrazine in the water column is further facilitated by its chemical structure, which contains an s-triazine ring, hindering microbial degradation (Solomon et al. 1996). Chemical degradation of

atrazine via hydrolysis is thus the most important form of degradation of the compound. In waters with pH levels ranging from 6.0 to 7.0 and the addition of 5mg/L fluvial acid (naturally occurring levels), half lives of 398 and 742 days, respectively, have been observed (Solomon et al.1996).

The effects of atrazine have been previously tested on algal samples isolated from *A. maculatum* eggs, as well as *A. maculatum* embryos themselves (Baxter et al. 2014, Olivier & Moon 2009). However, differences in experimental design and herbicide test concentrations have resulted in some contradictory findings amongst these studies with regard to the sensitivity of the salamanders and their symbionts. Most recently, a comprehensive weight-of-evidence review by Van Der Kraak et al. (2014) maintained that *A. maculatum* and their symbionts are relatively insensitive to the concentrations of atrazine found in the environment. In a study investigating atrazine concentrations in southern Ontario surface waters, Byer et al. (2011) tested 158 locations within the region and reported peak atrazine concentrations at 3.9 µg/L. Furthermore, in rural and agricultural areas, they observed these peak concentrations of the herbicide occurring during the spring and early summer season when pesticides may be washed from soils during high rainfall periods (Byer et al. 2011).

1.6.2 2,4-Dichlorophenoxyacetic Acid

The second herbicide that will be tested in this study is 2,4-D, a phenoxy compound which is also a widely applied agrochemical used to control broad-leaved weeds (Boivin et al. 2005, Mangat et al. 1999).Through runoff and leaching, it has the potential to contaminate aquatic systems, and thus raises concern for biota in

freshwater systems that may be susceptible to its toxic effects (Mangat et al. 1999). With a relatively low molecular mass, high solubility, and low Henry's constant, 2,4-D is limited in its ability to volatilize (Boivin et al. 2005). However, though it is retained in water, studies have shown that the herbicide is readily mineralized by microfauna, thus limiting its long term availability to the water column (Boivin et al. 2005). The compound is rapidly degraded into residues that are bound to various soil types, and it has been shown that 50% of an application dose of the herbicide can mineralize within 10 days of contact with clay or loamy soils (Boivin et al. 2005). Despite its rapid rate of degradation 2, 4-D is a compound of concern because of the detrimental effects it can have on the growth and metabolism of aquatic plants (Wong 2000). In the environment, 2,4-D concentrations in urban and rural Canada and the U.S have been reported within the 0.1 - 1.0 µg/L range (Glozier et al. 2012, US Geological survey 2006).

Being an auxin simulator, 2,4-D has been observed to promote growth in low concentrations while significantly inhibiting growth at higher concentrations— thus its use in the control of unwanted plants and weeds (Fairchild et al. 1997, Wong 2000). Although the effects of this herbicide on *O. amblystomatis* have never been investigated, Wong (2000) examined the effects of 2,4-D on *Scenedesmus quadricauda*. After examining the growth rate (at two day intervals until the stationary phase), photosynthetic rate (at 10 min. intervals for 60min), and chlorophyll-a content (at log and stationary phases of growth) of the alga at varying concentrations, their findings suggest that low concentrations (2-20 mg/L) of 2,4-D will promote growth of the algae, and only higher concentrations (200 mg/L) exceeding environmental levels

significantly inhibit algal growth (Wong et al. 2000). The effects of 2,4-D on *A. maculatum* embryos have not yet been investigated to our knowledge.

2.0 Objectives

1. To confirm the taxonomic identity of the green algal species that is symbiotic with the yellow spotted salamander, *A. maculatum*, and to determine if there are more than one species of green algae involved in this relationship. This will include increasing the current sample area through more of the *A. maculatum* range within eastern North America, and analysis of the nuclear SSU RNA gene to determine placement of these collections with existing available data.

Hypothesis

Phylogenetic analyses of the nuclear SSU RNA gene will depict multiple species within the *Oophila* clade that are involved in the symbiotic relationship with the yellow spotted salamander.

2. To investigate the biogeography and patterns of coevolution of both *A. maculatum* and its algal symbionts using analyses of the nuclear SSU RNA gene described in Objective 1 (*Oophila* sequences) and the mitochondrial ND4 and control region genes (*A. maculatum*). Current genetic distributions and discontinuities in amphibian and algal populations will confirm previously hypothesized theories of post-glacial expansion of lineages in various North American taxa.

Hypotheses

a. The evolutionary pattern depicted in the phylogenies of both *Oophila* species and *A. maculatum* will be similar when the gene phylogenies are compared due to co-evolution.

b. The biogeographic patterns in the gene phylogenies will be defined by Pleistocene climatic changes.

3. To isolate and culture algal samples obtained from *A. maculatum* eggs across south Ontario, and to test their sensitivity (response and recovery) to two agricultural herbicides: atrazine and 2,4-D. Toxic effects on these algae have been demonstrated to have indirect growth effects on *A. maculatum* embryos, and these data will be useful for the purposes of ecological risk assessment as it relates to these unique symbiotic relationships. Concentrations examined will include environmentally realistic exposures.

Hypotheses

a. Adverse effects of atrazine on algae cultured from *A. maculatum* egg masses will not be observed at environmental concentration levels.

b. Adverse effects of 2,4-D on cultured algae will only be observed at concentration levels exceeding environmental exposure levels.

3.0 Methods

3.1 Sampling and Algal Culturing

Ambystoma maculatum egg collections were sampled from four locations across southern Ontario (Table 1, Figure 2). An additional collection was attempted at Backus Woods, however there were no egg masses present and it is possible the embryos had already hatched at this southern most location (Table 1). Appropriate permission from conservation authorities and/or owners of private properties, and the Ministry of Natural Resources and Forestry was retrieved (No. 1079477). The salamander embryos were observed to be at stages 25-37, based on Harrison and Wilens (1969) classification of development, and varied per location and time collected. However, the development of the embryos was noticeably more advanced at southerly locations (Table 1). In addition, embryos collected from Sudden Tract were the largest (most developed) while those from Bruce Peninsula were the smallest (least developed), with samples from Kingston and Niagara falling in between (presumably characterized by latitudinal geography). Environmental conditions upon sample collection and dates of collection are summarized in Table 2. One egg mass was collected from each location, and these were transported back to the University of Waterloo where they were stored at the Wet-lab aquatic animal facility. Water temperature was maintained at 10 °C with constant flowing well water, and light conditions were maintained at a 12 h cycle of light and dark to mimic day and night. A select number of eggs (8-10) from each sample location were detached from jelly masses and preserved in 97% ethanol (v/v) for future sequencing. Appropriate ethics approval was obtained from the University of Waterloo Animal Care Committee (Issue Number A-15-04).

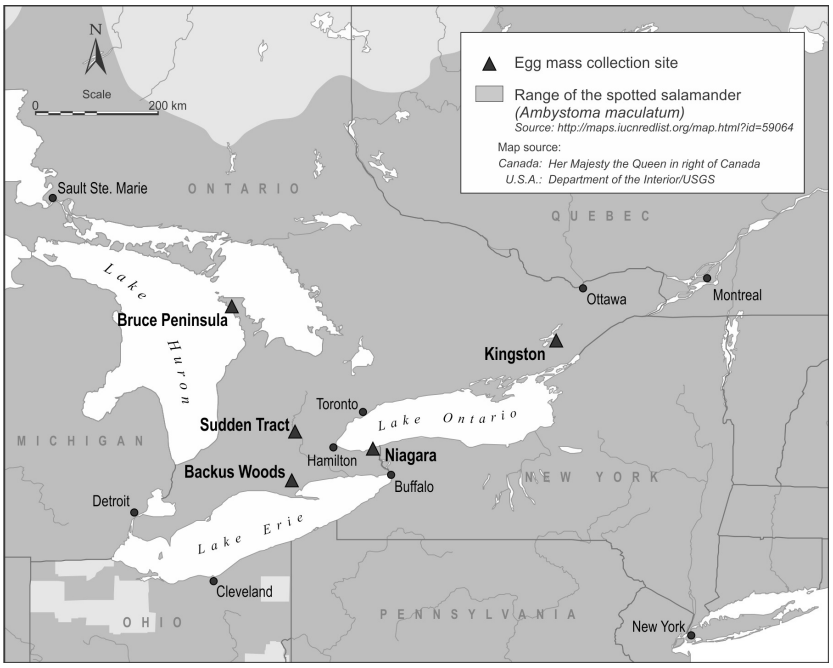

Figure 2. Southern Ontario sample locations (Google Maps, 2016a). Triangles denote the four sample locations from which *A. maculatum* eggs were collected, and Backus Woods where there were no egg masses present.

Table 1. Egg Mass Collection Sites

Sample Location	Coordinates	Property Owner
Sudden Tract - Cambridge, ON	43°18'30.8"N 80°22'05.1"W	Region of Waterloo
Niagara College Campus Niagara, ON	43°08'55.7"N 79°09'44.8"W	Niagara College
326 Lindsay Rd. #20 - North Bruce Peninsula	45°03'16.5"N 81°27'02.0"W	Private Property
Indian Lake - Elgin, ON	44°35'17.4"N 76°20'18.3"W	Queens University Biological Station
*Backus Woods - Walsingham, ON	42°40'31.1"N 80°28'52.7"W	Nature Conservancy of Canada

*No egg masses present

Table 2. Sampling Environmental Conditions

Sample Location	Collection Date	PAR* (µmol m^{-2} s^{-}) Above/Below Water***	Temp. (°C) Air/Water	Water Depth (cm)	pH	DO (mg/L)	Conductivity (uS)
Sudden Tract	May 5, 2015	1123/600	23/20	21.5	5.5	8.5	70
Niagara College Campus	May 8, 2015	1027/910	27/16.6	27.0	8.6	7.4	NA**
North Bruce Peninsula	May 8, 2015	Private Property	22/NA**	25.0	NA**		NA**
Indian Lake	May 9, 2015	1013/675	28.5/27.9	23.0	7.7	5.7	481.4

*PAR - Photosynthetically Active Radiation
**NA - Data unavailable or unobtainable due to instrument malfunction
***Below Water - reading taken at position of egg mass under water

For algal cell culturing, salamander eggs were first detached from jelly mass and rinsed with a 1x phosphate buffered saline solution (Rodríguez-Gil et al. 2015). Egg contents including algal cells were then extracted with a syringe and placed in modified NH_4^+ Bristol's media (Rodríguez-Gil et al. 2015). These cultures were maintained on bench top in autoclaved 50 mL Erlenmeyer flasks, at room temperature, with autoclaved media refreshed every two weeks until cell isolation was performed (initial isolations within three months). Single-cell isolation techniques were conducted as described by Anderson (2005) and Rodríguez-Gil et al. (2014) without deviation. Cells were isolated and deposited in Nunc 4—well dishes in order to create unialgal cultures. Once wells were visibly green, contents were transferred to 50 mL and then to 250 mL erlenmeyer flasks. Of the four locations sampled, the algal sample from Niagara was not successfully isolated for *Oophila* sp., and therefore not included in subsequent analyses. For toxicity tests, subcultures were prepared using United States Environmental Protection Agency (USEPA) protocols (USEPA 1996). Based on these guidelines, cultures were maintained (same conditions as test conditions) in NH4+ modified Bristol's media as per Rodríguez-Gil et al. (2014), with temperature controlled at 18 ± 1 °C and a 24 h white fluorescent light source at 200 ± 15 µmol m^{-2} s^{-1}. Cultures were placed on a shaker table set to continuous shaking at 90 rpm.

3.2 DNA Extraction and Polymerase Chain Reaction (PCR) Amplification

Genomic DNA of *A. maculatum* was isolated using a DNeasy Blood & Tissue Kit (Qiagen, Toronto) following manufacturers protocols. Two mitochondrial gene regions were amplified using the ND4-tRNA and control region markers (Zamudio and Savage

2003, McKnight and Shaffer 1997). The ND4 region was amplified using the primers ND4 (Forward 5' CAC CTA TGA CTA CCA AAA GCT CAT GTA GAA GC 3') and LEU (Reverse 5' CAT TAC TTT TAC TTG GAT TTG CAC CA 3') (Arevalo et al. 1994). The control region was amplified with the primers THR (Forward 5' AAA CAT CGA TCT TGT AAG TC 3') and DL1 (Reverse 5' AAT ATT GAT AAT TCA AGC TCC G 3') (McKnight and Shaffer 1997). Amplifications were carried out in 50 µL solutions containing 5 µL template, 5 µL Thermopol buffer (10x, containing MgCl2), 1 µL of each primer (10 µM), 1 µL dNTPs (10 µM), and 0.5 µl Taq Polymerase. A Multi Gene II (Labnet International) thermocycler was used to perform amplification under the following parameters: initial denaturation 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 48-55 °C for 30 s, and extension at 72 °C for 1 min 20 s, with a final extension at 72 °C for 5 min.

A DNeasy Plant Minikit (Qiagen, Toronto) was used to extract DNA from the algal culture obtained from the egg masses after rupturing cells using liquid nitrogen and a mortar and pestle. The nuclear SSU rRNA gene was amplified using the primers SSU1 (Forward 5' TGG TTG ATC CTG CCA GTA G 3') and SSU2 (Reverse 5' TGA TCC TTC CGC AGG TTC AC 3') (Shoup and Lewis 2003). Amplification of the nuclear SSU rRNA gene was carried out in 50 µl solutions containing 5 µl template, 5µl Thermopol buffer (10x, containing MgCl), 1 µl of each primer (10 µM), 1 µl dNTPs (10 µM), and 0.5 µl Taq Polymerase. A Multi Gene II (Labnet International) thermocycler was used to perform amplification under the following parameters: initial denaturation 95 °C for 3 min, 34 cycles of denaturation at 95 °C for 30 s, annealing at 51 °C for 30 s, and

extension at 72 °C for 1 min 20 s, with a final elongation at 72 °C for 5 min. Amplified PCR products of all gene regions were viewed on a 1% agarose gel.

3.3 Sequencing and Phylogenetic Analyses

Successfully amplified PCR products were cleaned using a QiaQuick PCR Purification Kit (Qiagen, Toronto) and sent for Sanger sequencing to the Robarts Research Institute in London, Ontario. Sequences were subsequently aligned using SeaView Multi-platform Graphical User Interface (Gouy and Gascue 2010). Multiple sequence alignments were created for all markers, with *Ambystoma gracile* (AY691773) and *Amystoma opacum* (EF649952) used as outgroups for both mitochondrial ND4 and control region alignments. The outgroup selected for the nuclear SSU rRNA gene alignment was borrowed from Kim et al. (2009) and included *Pyramimonas vacuolata* (AB999994), *Micromonas commoda* (KU244632), and *Chara drouetii* (U18495) (Hoham et al. 2002, Wodniok et al. 2011). Maximum likelihood (PhyML) trees using the GTR (Generalised time reversal) model were created with 1000 iterations and bootstrap thresholds of 70% or more are indicated on trees.

3.4 Toxicity Testing

To assess the concentration-response of each alga to atrazine, standard 96 h toxicity tests were performed at herbicide concentrations of 0 µg/L, 3 µg/L, 10 µg/L, 30 µg/L, 100 µg/L and 300 µg/L (n=3) based on the range finding test of Baxter et al. (2014). These concentrations were chosen by the authors to achieve at least 50% growth inhibition and photosystem II (PSII) effective quantum yield (Baxter et al. 2014).

Test solutions were prepared by dissolving technical grade atrazine (96% purity, supplied by Syngenta Crop Protection, Product No. G30027H) in NH_4^+ modified Bristol's media as per Rodríguez-Gil et al. (2014).

In order to investigate the toxic effects of 2,4-D to algal cultures, a range finding test from 0 to 200 mg/L was first conducted. This range was chosen based on the study of Wong (2000) which investigated the effects of 2,4-D on the green algal species, *Scenedesmus quadricada*. Toxicity tests were then performed at herbicide concentrations of 0 mg/L, 3 mg/L, 10 mg/L, 30 mg/L, and 100 mg/L. Test solutions were prepared by dissolving technical grade 2,4-D (98.4% purity, supplied by Dow Agrosciences, Lot no. UB07161101) in modified Bristol's media without the use of solvents.

Toxicity tests were performed by inoculating test flasks containing 100 mL of modified Bristol's media plus atrazine with a 4-6 day old algal culture in log phase, to achieve a 10000 cells/mL cell density. Three replicates were used for each concentration, and six replicates were used for control (untreated media). Flasks were then positioned in haphazard order on a shaker table set to 90 rpm in a growth chamber (Percival Scientific). Conditions in the chamber were set at 18 (±1) °C and 24 h cool white fluorescent light at 200 (±10) µmol m^2/s for 96 hours. These parameters were in conjunction with USEPA guidelines (USEPA 1996) for standard acute algal toxicity tests and were set to mimic those defined by in Baxter et al. (2014).

The recovery phase of the test was initiated by first collecting algae from the exposure phase for each concentration (replicates pooled) via centrifugation (4500 rpm, at 14°C for 30 min). The algae were subsequently washed twice with sterile media and

then reinoculated in fresh flasks containing untreated media to achieve a cell density of 10000 cells/mL. Three replicates for each atrazine concentration (and six for control) were prepared for the recovery phase. Flasks were placed randomly on a shaker table in a growth chamber for 96 hours, under the same conditions as the exposure phase mentioned above.

3.5 Measurement of Endpoints

Growth rate (as function of cell density), chlorophyll absorbance and effective quantum yield of Photosystem II (PSII) were measured after the exposure and recovery phase of all tests. PS II yield was only measured for the mode of action of atrazine. Cell counts were taken using a Fuchs-Rosenthal hemacytometer, with two sub samples counted (and averaged) for each replicate. Growth rate was calculated using Equation 1, where N = cell number, the time interval is 96 h, and k is the growth rate constant.

$$ln(N_2/N_1)=k(t_2-t_1) \quad (1)$$

A number of absorbance wavelengths have been proposed for monitoring chlorophyl content (Geiss et al. 2000). Environment Canada suggests readings at 430nm wavelength, and USEPA guidelines recommend 750 nm (Environment Canada 1992, USEPA 1996). For the purposes of the current study, readings were taken at 430 and 680 nm, although values at 430 nm were utilized in later analyses to maintain conformity with the methods of (Baxter et al. 2014). The average absorbance reading of three subsamples was used for each replicate.

Effective quantum yield of PSII was measured using a WATER-PAM (pulse-amplitude-modulation) chlorophyll fluorometer (Heinz Walz GmbH). Aliquots of each "light adapted" sample were subject to the "saturation pulse method" which involves measurement of fluorescence yield (F), followed by a pulse of saturating light to determine maximum fluorescence (Fm') (Klughammer and Schreiber 2008). Effective quantum yield (Y) is the resulting difference of fluorescence yield from maximum fluorescence divided by maximum fluorescence as seen in Equation 2 (Klughammer and Schreiber 2008).

$$Y=(Fm'-F)/Fm' \quad (2)$$

It was ensured that the current fluorescent yield, Ft, value for each sample was initially at 300 - 400 mV as per the manufacture's recommendations. The Pm gain setting was adjusted, or the sample diluted with de-ionised water to reach the appropriate Ft range. Five readings, 30 s apart, were taken for each replicate. The average of the last three readings were used for later analyses (Baxter et al. 2014).

3.6 Confirmation of Atrazine Test Concentrations

Atrazine and 2,4-D prepared stock concentrations for each exposure level (at 0h, before test initiation) were measure in duplicate via enzyme-linked immunosorbent assay (ELISA) according to manufacturer's protocol (Atrazine Microtitre Kit - Abraxis, Warminster). Recovery samples, taken from each recovery flask (before test initiation, at 0 h of recovery phase) were also analyzed in the same manner (see Appendix,

Tables A5, A6). Samples were frozen and stored in the dark until ELISA analysis. Samples were measured in duplicate and the average of values used in data analyses. Atrazine exposure and recovery values were within 20% of nominal concentrations, however 2,4-D exposure and recovery samples were not successfully quantified.

3.7 Statistical Analysis

One-way analysis of variance (ANOVA) followed by the post hoc Dunnett's test ($\alpha = 0.05$) were employed to determine no-observed-effect-concentrations (NOECs) and lowest-observed-effect-concentrations (LOECs) of atrazine and 2,4-D exposures. For recovery phase analysis, ANOVA followed by Dunnett's test were used to determine recovery concentrations that differed significantly from controls. Where distributions did not meet ANOVA assumptions, the nonparametric Kruskal-Wallis H test was employed to determine whether or not there was significance between controls and exposure level ($\alpha = 0.05$). These analyses were conducted in IBM SPSS Statistics software (IBM corp., New York).

The response of *O. amblystomatis* strains to atrazine and 2,4-D exposure was also modelled for endpoints (growth rate, chlorophyll absorbance, PSII yield) in all exposure tests by non-linear regression using Equation 3, where a = the upper limit and b = the slope of the linear section of the curve (Baxter et al. 2014).

$$y = a/1(1 + e^{(b[\log(x) - \log(EC50)])}) \quad (3)$$

Regression models and calculations were conducted in RStudio interface for R statistics software (Vienna) using the DRC package and script for ED.dcr, “Estimating effective doses” (Ritz and Streibig, 2005).

4.0 Results

4.1 Phylogenetic Reconstruction

For nuclear SSU rRNA algal genes, sequences obtained after amplification were approximately 1700 base pairs (bp) in length, after primer annealing regions at the 5' and 3' ends were trimmed. Thus, phylogenetic analysis included GenBank sequences that were 900-1750 bp in length for the alignment. Partial sequences (e.g 200 bp) were excluded from analysis. An intron (248 bp) was observed in the Bruce Peninsula sequence, but this was trimmed before inclusion in the alignment. The nuclear SSU rRNA gene alignment also included *Oophila* sp*.* sequences obtained from Genbank in addition to representative Chlorophycean algal sequences that were included in the analyses by Kim et al. (2009). The maximum likelihood tree shows these *Oophila* sp. sequences grouping into two different, but well supported clades (Figure 3). The first "*Oophila"* Clade B (light green) includes sequences isolated/obtained by Kim et al. (2009), which originate from New Jersey, Tennessee, Nova Scotia, British Columbia, and Ontario (see Figure 4 for labelled map of North America), and has 100% bootstrap support. This clade also includes two sequences (denoted by arrows) retrieved from two southern Ontario locations (Sudden Tract and Lost Ray Lake) from Baxter al. (2014). Sudden Tract was revisited and included in the current study, however this sequence (2015) groups within *Oophila* Clade A (dark green). "*Oophila*" Clade B also includes three sequences from *Chlamydomonas* (AY220572, AF517097, AB701502) that are paraphyletic with "*Oophila"* sp. sequences. Strong bootstrap values (90 to100%) support the grouping of these sequences within "*Oophila*" Clade B.

The second group, *Oophila* Clade A (Figure 3), includes sequences from the current study as well as unpublished *Oophila* sp. sequences obtained from GenBank, and holds 86% bootstrap support. At least one of these sequences was sampled from the type locality of *Oophila amblystomatis* in Middlesex Fells, Massachusetts (personal communication with Craig Schneider in possession of Lambert's type species - Phycological Society of America Annual Meeting 2016; Wille 1909). It is notable that one *Oophila* sequence (KJ635663) falls outside of both described groups, but groups with two *Chlamydomonas* sequences (U57694, KF879589) with 84% bootstrap support. In addition to the various *Chlamydomonas* species, Clade B also shows *Oophila* species grouping with members of other green algal genera, *Chlorococcum* (AB490286, AB490288, U70586) and *Characium* (M63001), with strong bootstrap support of 85 - 90%.

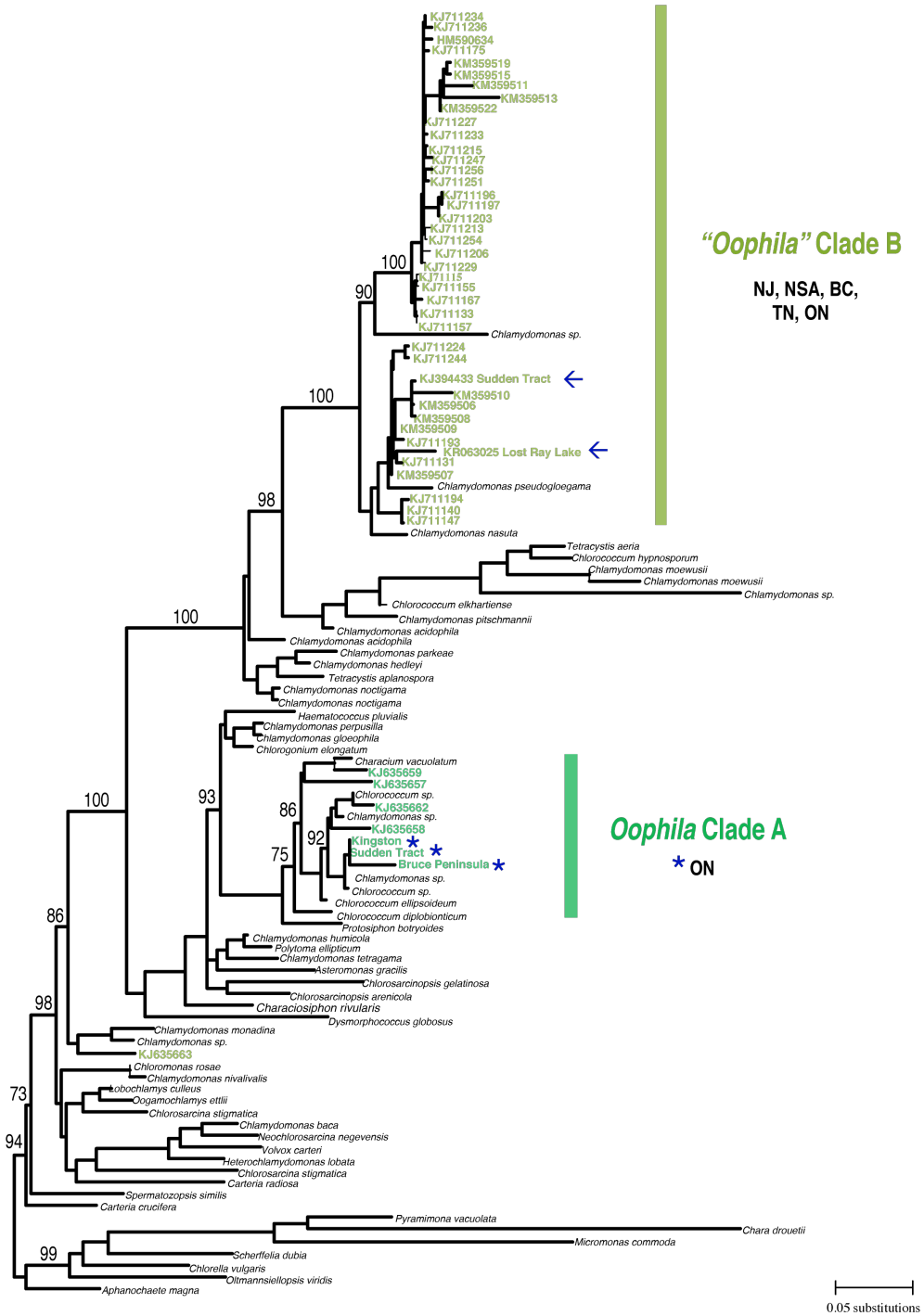

Figure 3. Maximum likelihood tree of *O. amblystomatis* nuclear SSU rRNA gene sequences includes samples from the current study (asterisk) grouping with type locality (Clade A in dark green), samples retrieved by Baxter et al. (2014) (arrows, belonging to Clade B in light green), and representative green algal sequences from Kim et al. (2014). State/province abbreviations are included where sample information was available. Bootstrap values greater than 65% (1000 replicates) are indicated on major lineages. PhyML ln(L) = -20057.0 1778 sites GTR 4 rate classes

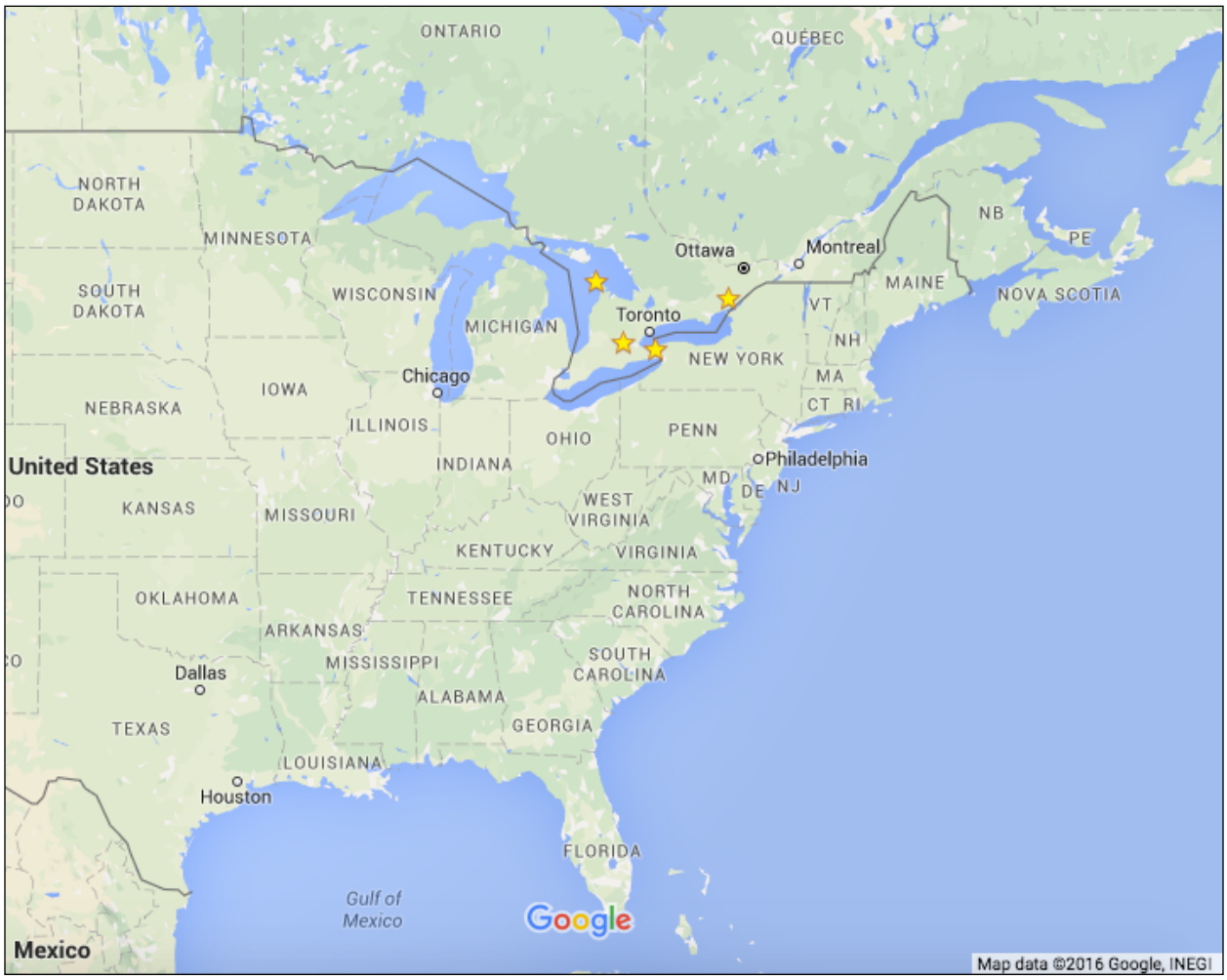

Figure 4. Map of North America (Google Maps, 2016b) includes U.S. and Canadian provinces within *A. maculatum* range. Stars denote sample locations within southern Ontario. State and province abbreviations from Figure 3 match labels above.

Phylogeographic analysis of *A. maculatum* included the construction of two maximum likelihood trees using the mitochondrial ND4 and control region markers (Figures 5, and 6). Sequences of the ND4 region obtained for the current study had final lengths of ~ 800bp in length, and sequences of the control region consisted of edited sequences ~ 900bp, after 5' and 3' ends were trimmed. Additional sequences of both regions for the alignments were obtained from Zamudio and Savage (2003) and collated with sequences from the current study. Our ND4 mtDNA samples (asterisk) grouped within the expected Eastern Clade (blue) shown in Figure 5, and there is strong bootstrap support for this grouping (92%). Though this clade includes members from eastern provinces and states such as Ontario, and Maine, there is also evidence of these haplotypes within Interior Clade B (92% bootstrap support).

Sequences of the control region taken from the five southern Ontario sample locations (asterisk) group together within Eastern Clade A with 94% bootstrap support (light blue, Figure 6). These Ontario individuals group with members from Nova Scotia, Maine, Maryland, Massachusetts, Wisconsin, Virginia, Connecticut, Pennsylvania, and New York with strong bootstrap support of 95%. Since sequences obtained from the current study grouped within the expected Eastern Clade A (or Coastal Clade as proposed by Zamudio and Savage (2009), it was deemed unnecessary to carry out any further analysis into determining the biogeographic distribution patterns of these samples within the relatively small sample region of southern Ontario.

The approximate geographic distribution of the above mentioned Interior and Eastern Clades are depicted in Figure 7. This map shows all four proposed *A.*

maculatum lineages in relation to the Appalachian Mountain discontinuity as proposed by Zamudio and Savage (2003) and others (Austin et al. 2002, Soltis et al. 2006)

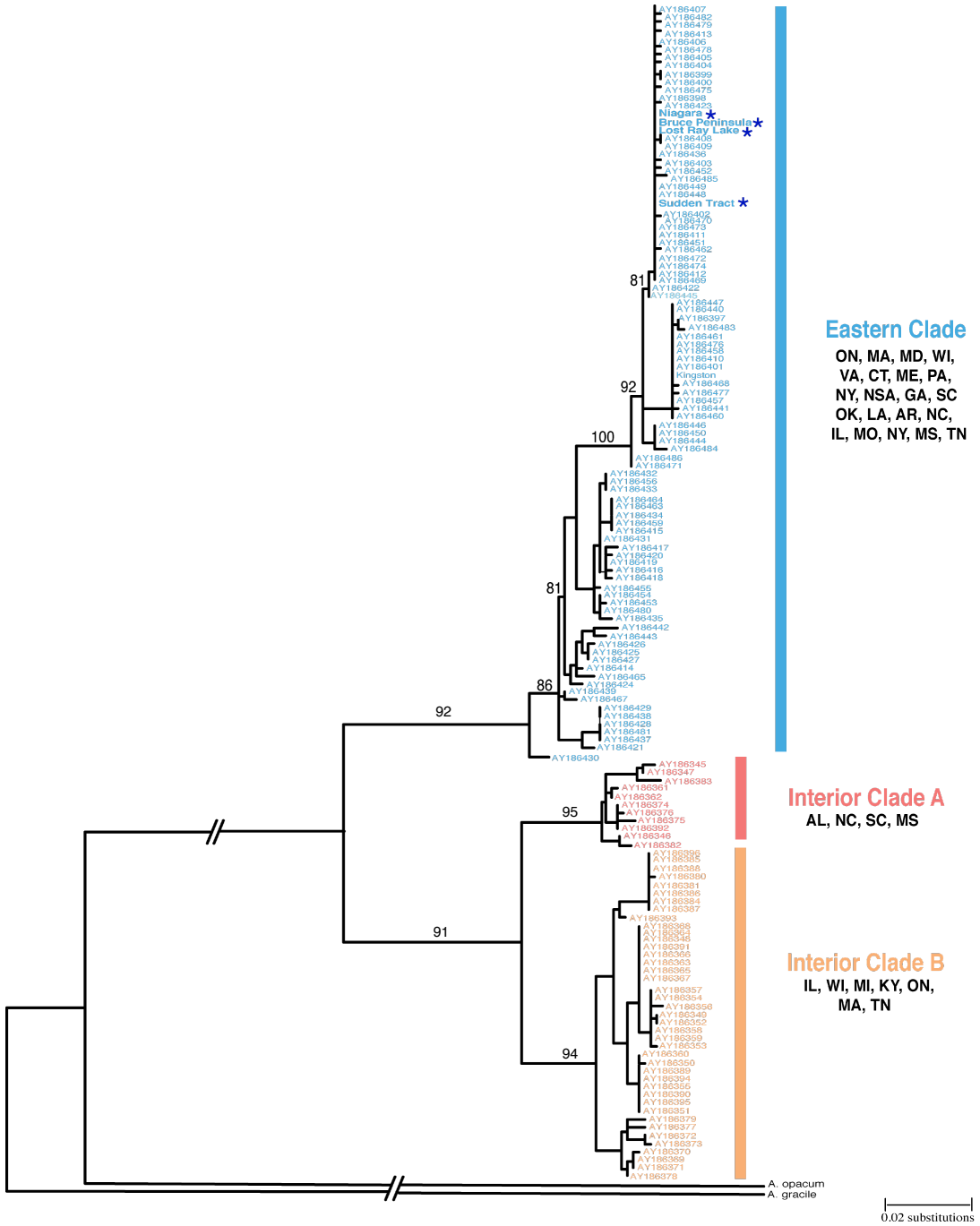

Figure 5. Maximum likelihood tree of *A. maculatum* mtDNA ND4 sequences includes data from Zamudio and Savage (2003) and samples from the current study (asterisk) grouping in the Eastern Clade (blue). State/province abbreviations under each clade represent the localities from which samples were retrieved. Bootstrap values greater than 65% (1000 replicates) are indicated on major lineages. PhYL ln(L) = -3094.3 802 sites GTR.

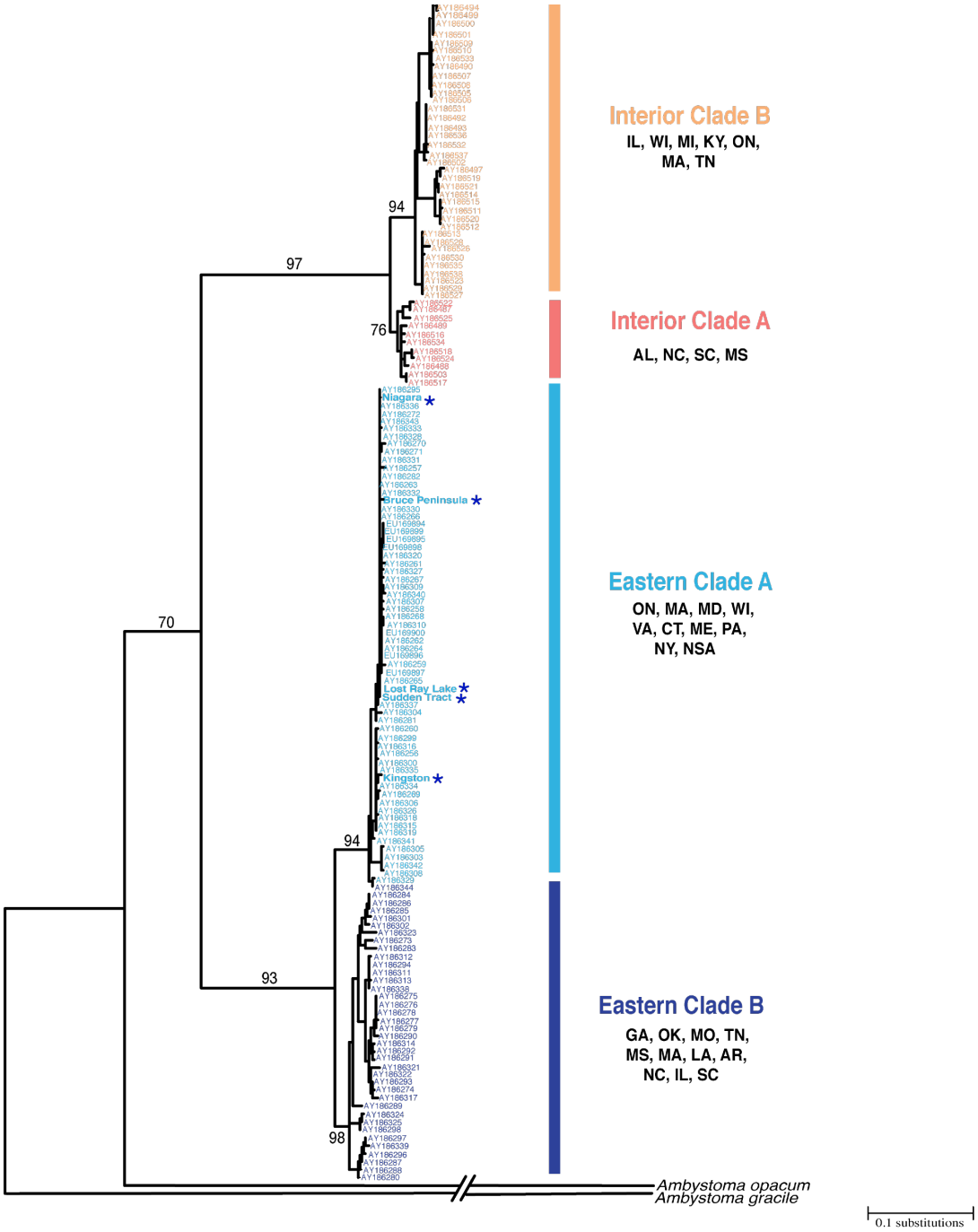

Figure 6. Maximum likelihood tree of *A. maculatum* mtDNA control region sequences includes data from Zamudio and Savage (2003) and samples from the current study (asterisk) grouping in Eastern Clade B (dark blue). State/province abbreviations under each clade represent the localities from which samples were retrieved. Bootstrap values greater than 65% (1000 replicates) are indicated on major lineages. PhyML ln(L) = -5049.7 sites GTR

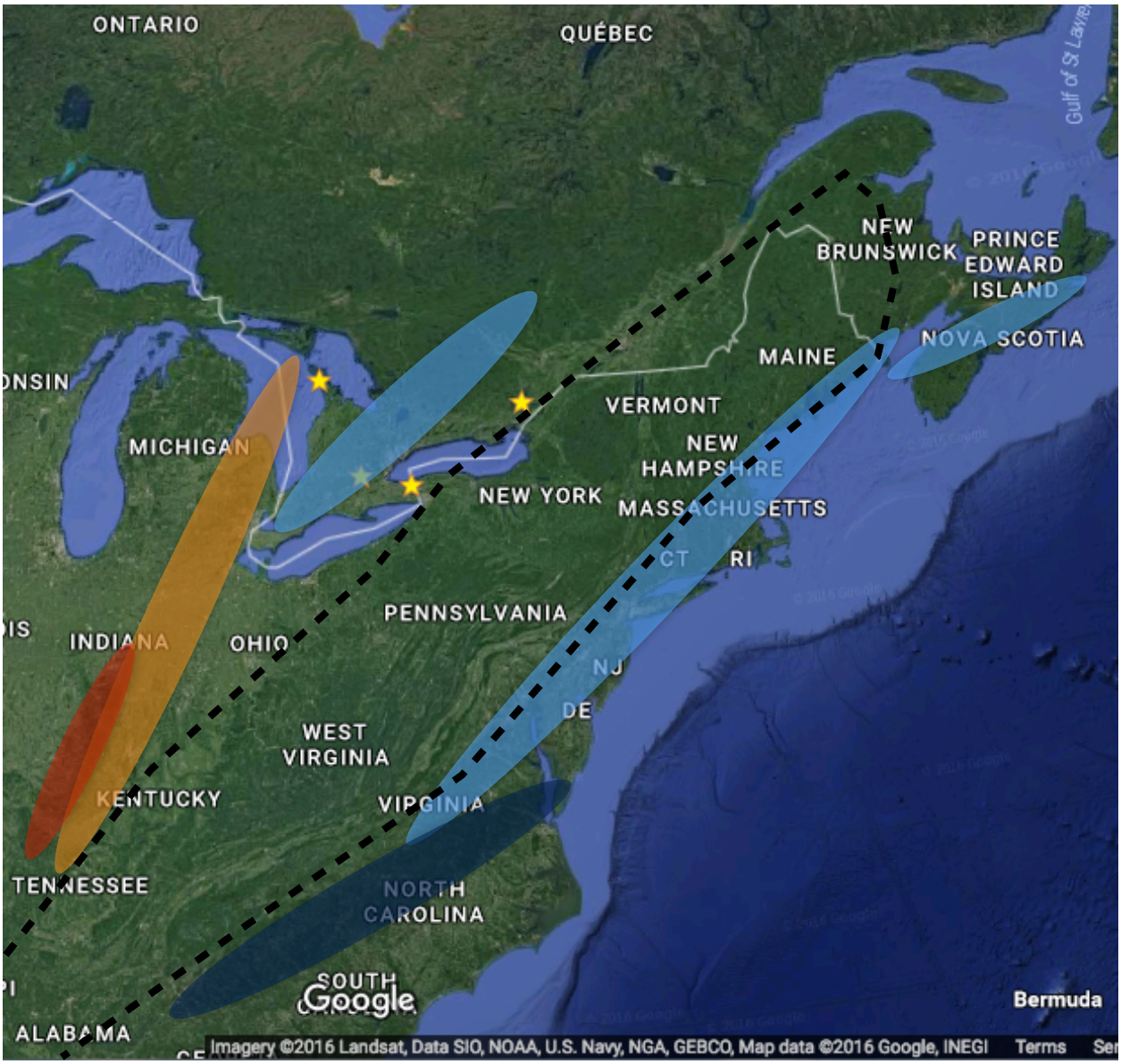

Figure 7. Map depicting proposed *A. maculatum* lineage distribution by Zamudio and Savage (2003) (Google Maps, 2016c) includes sequences collected from the current study (locations starred) which group within Eastern Clade A highlighted in light blue. Also represented in the map are Eastern Clade B in dark blue, and Interior Clade A in red, and Interior Clade B in orange. Colours coincide with phylogenetic trees in previous two figures. The Appalachian Mountain discontinuity is depicted by the dotted region.

4.2 Algal Toxicity Tests

4.2.1 Atrazine

Exposure effective concentration values for atrazine as it relates to growth rate, chlorophyll absorbance, and PSII yield are listed in Table 3. The goal of 50% inhibition was not reached for all endpoints, with the exception of Bruce Peninsula growth rate, and PSII yield, and Sudden Tract PSII yield. Relatively, chlorophyll absorbance was the most sensitive response, though it displays large SE. The least variable endpoint is PSII yield, with the lowest SE values compared to growth rate and chlorophyll absorbance. Of the extrapolated PSII values, Bruce Peninsula had the lowest 96 h EC50 at 292 µg/L, and Kingston the greatest at 803 µg/L. This trend was also observed in the other two endpoints with Bruce Peninsula having a chlorophyll absorbance 96 h EC50 of 218 µg/L, Sudden Tract an EC50 of 273 µg/L, and Kingston an EC50 of 352 µg/L. Bruce Peninsula, Sudden Tract and Kingston 96 h EC50s for growth rate were 425 µg/L, 508 µg/L, and 526 µg/L respectively. Again, with such high SE values, these data values may only show relative trends between endpoints.

Analysis of variance revealed PSII NOECs had the lowest values, and were substantially different from chlorophyll absorbance and growth rate endpoints (Table 4). Bruce Peninsula had the lowest PSII 96 h NOEC at 3.3 µg/L, Sudden Tract had a 96 h NOEC of 9 µg/L and Kingston a 96 h NOEC of 10 µg/L. Chlorophyll absorbance was the least sensitive endpoint with Kingston having the greatest value of 405 µg/L, and Sudden Tract with 360 µg/L. Kingston also had the greatest growth rate NOEC of the three locations with a value of 140 µg/L, Sudden Tract and Bruce Peninsula followed with values of 80 µg/L and 70 µg/L respectively. An observed power analysis reveals

that chlorophyll absorbance is underpowered for Kingston, and has less power the other endpoints for Sudden Tract, and Bruce Peninsula as well.

For the recovery phase of all three strains, full recovery was observed at all endpoints and previously atrazine exposed test cultures were equal to or more productive than controls (Figures 7, 8, and 9). Chlorophyll absorbance was visibly the most variable endpoint in both exposure and recovery phases for all three strains.

Table 3. Atrazine effective concentration estimates with standard error of measured endpoints in Kingston, Sudden Tract and Bruce Peninsula *O. amblystomatis*

Sample	EC	Growth rate		Chlorophyll absorbance		Photosystem II yield	
		Estimate (µg/L)	SE	Estimate (µg/L)	SE	Estimate (µg/L)	SE
Kingston	EC10	352*	94	145*	162	29*	9
	EC25	450*	93	226*	163	155*	23
	EC50	576*	382	352*	155	803*	138
Sudden Tract	EC10	276*	204	78*	110	17	3
	EC25	374*	41	146*	129	86	8
	EC50	508*	482	273*	142	442	56
Bruce Peninsula	EC10	130	26	118*	268	14	2
	EC25	235	15	160*	231	64	5
	EC50	425	55	218*	138	292	27

* 50% inhibition was not reached at endpoint, and EC50 values were extrapolated from regression model

Table 4. Atrazine exposure NOECs, LOECs, and percent differences from the control group mean of measured endpoints in Kingston, Sudden Tract and Bruce Peninsula *O. amblystomatis*

Sample	Endpoint	NOEC		LOEC		Observed Power
		Value (µg/L)	% difference	Value (µg/L)	% difference	
Kingston	Growth rate	140	2	405	17	1.0
	Chlorophyll absorbance	405	56	> 405	NA	0.47
	Photosystem II Yield	10	1	27	9	1.0
Sudden Tract	Growth rate	80	0	360	23	1
	Chlorophyll absorbance	360	55	> 360	NA	0.54
	Photosystem II Yield	9	4	21	11	1.0
Bruce Peninsula	Growth rate	70	3	270	31	1.0
	Chlorophyll absorbance	270	65	> 270	NA	0.64
	Photosystem II Yield	3.3	1	10	7.75	1.0

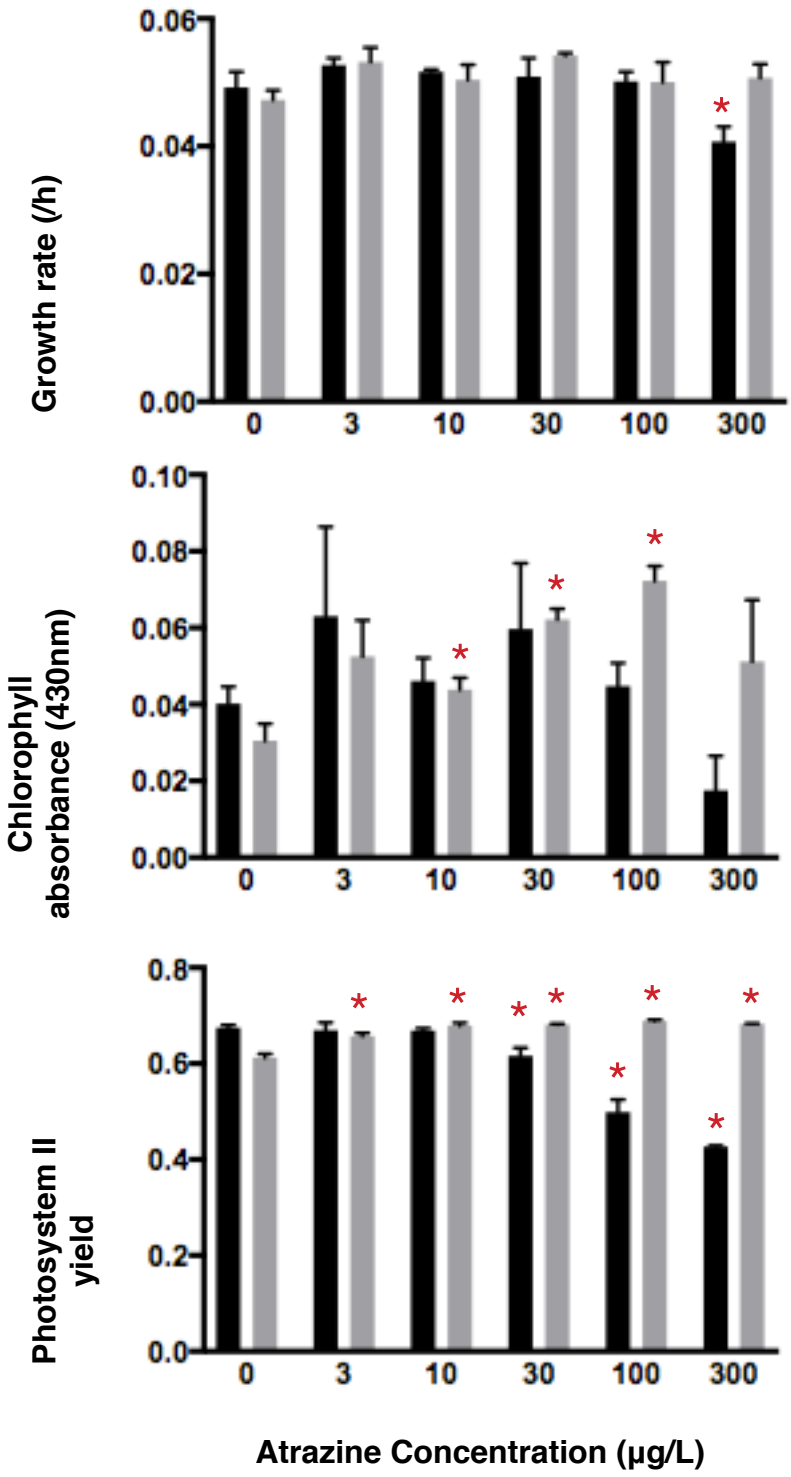

Figure 8. Kingston *O. amblystomatis* atrazine exposure and recovery. Endpoints monitored after 96 h exposure of atrazine to algae (dark bars) followed by 96 h recovery (light bars). Nominal concentrations are shown with standard error bars. Significant differences from control (Dunnett's test, Kruskal-Wallis H test α = 0.05) represented by asterisk.

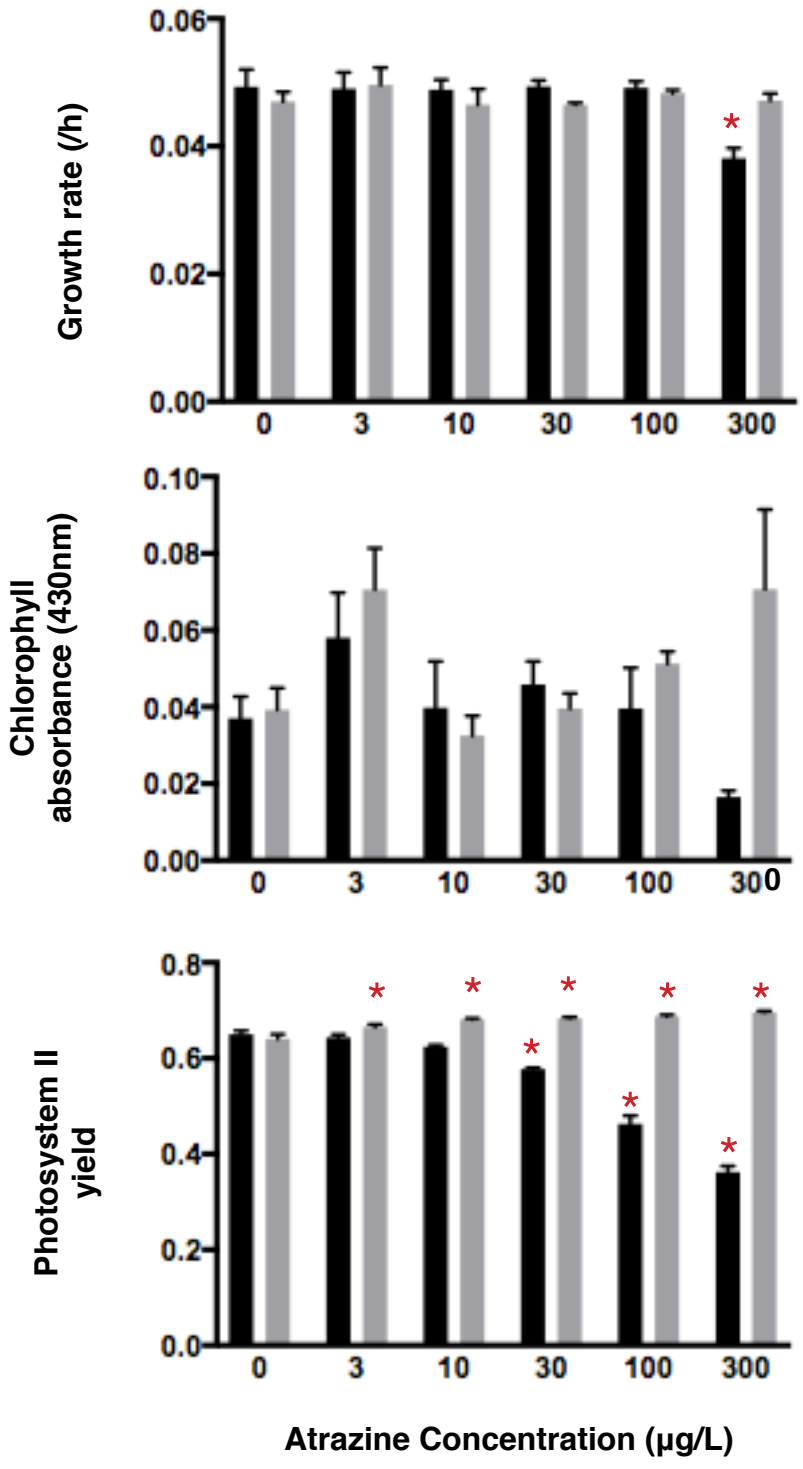

Figure 9. Sudden Tract *O. amblystomatis* atrazine exposure and recovery. Endpoints monitored after 96 h exposure of atrazine to algae (dark bars) followed by 96 h recovery (light bars). Nominal concentrations are shown with standard error bars. Significant differences from control (Dunnett's test, Kruskal-Wallis H test α = 0.05) represented by asterisk.

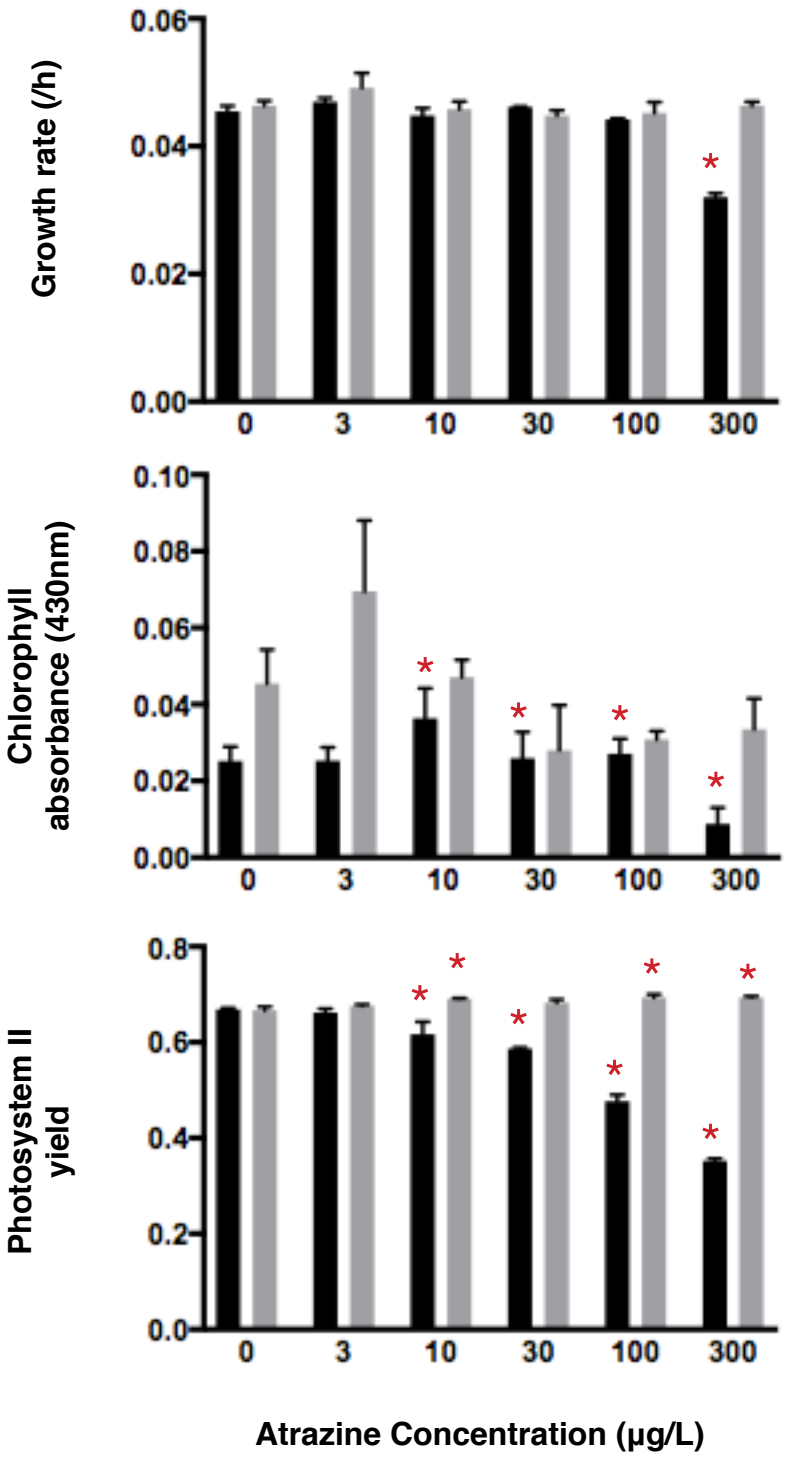

Figure 10. Bruce Peninsula *O. amblystomatis* atrazine exposure and recovery. Endpoints monitored after 96 h exposure of atrazine to algae (dark bars) followed by 96 h recovery (light bars). Nominal concentrations are shown with standard error bars. Significant differences from control (Dunnett's test, Kruskal-Wallis H test α = 0.05) represented by asterisk.

4.2.2 2,4-Dichlorophenoxy acetic acid

Nominal concentrations were used in the concentration-response regressions and ANOVA analyses for 2,4-D as analytical confirmation of exposure concentrations was not successful with ELISA. Growth rate and chlorophyll absorbance for Bruce Peninsula were similar, with 96 h EC50 values of 69 mg/L and 67 mg/L, respectively (Table 5). However SE values for chlorophyll absorbance were very large for Bruce Peninsula, as well as Kingston. Sudden Tract had 96 h EC50 values of 92 mg/L and 8 mg/L for growth rate and chlorophyll absorbance, respectively. The latter value is noticeably the lowest value of all three samples and endpoints, and reflects high variability within the replicates for that test. Kingston had a 96 h EC50 of 77 mg/L for growth rate, and 50 mg/L for chlorophyll absorbance.

Nominal 96 h NOECs for 2,4-D were 30mg/L for all endpoints measured for the three sample locations, except chlorophyll absorbance of Sudden Tract (Table 6). This does mirror the skewed 96 h EC50 values, which suggests that the variance between replicates likely interfered with the regression and ANOVA analyses. This is evident in the chlorophyll absorbance power, as it is lower for growth rate, similar to that of the previous atrazine tests.

Full recovery (equal to or greater than control productivity) was observed in all strains exposed to 2,4-D except for chlorophyll absorbance for Sudden Tract, however exposure readings for this endpoint were also unexpectedly low.

Table 5. 2,4-D effective concentration estimates based on nominal concentrations with standard error of measured endpoints in Kingston, Sudden Tract and Bruce Peninsula *O. amblystomatis*

Sample	EC	Growth rate		Chlorophyll absorbance	
		Estimate (mg/L)	SE	Estimate (mg/L)	SE
Kingston	EC10	42	8	26	15
	EC25	57	7	36	17
	EC50	77	5	50	23
Sudden Tract	EC10	73	81	0	0
	EC25	82	56	1	1
	EC50	92	24	8	5
Bruce Peninsula	EC10	41	13	41	55
	EC25	53	12	52	52
	EC50	69	10	67	43

Table 6. 2,4-D exposure nominal NOECs, LOECs, and percent differences from the control group mean of measured endpoints in Kingston, Sudden Tract and Bruce Peninsula *O.* amblystomatis

Sample	Endpoint	NOEC		LOEC		Observed Power
		Value (mg/L)	% difference	Value (mg/L)	% difference	
Kingston	Growth rate	30	3	100	72	1.0
	Chlorophyll absorbance	30	1	100	88	0.79
Sudden Tract	Growth rate	30	+2	100	67	1.0
	Chlorophyll absorbance	10	37	30	63	0.84
Bruce Peninsula	Growth rate	30	0	100	83	1.0
	Chlorophyll absorbance	30	+16	100	83	0.98

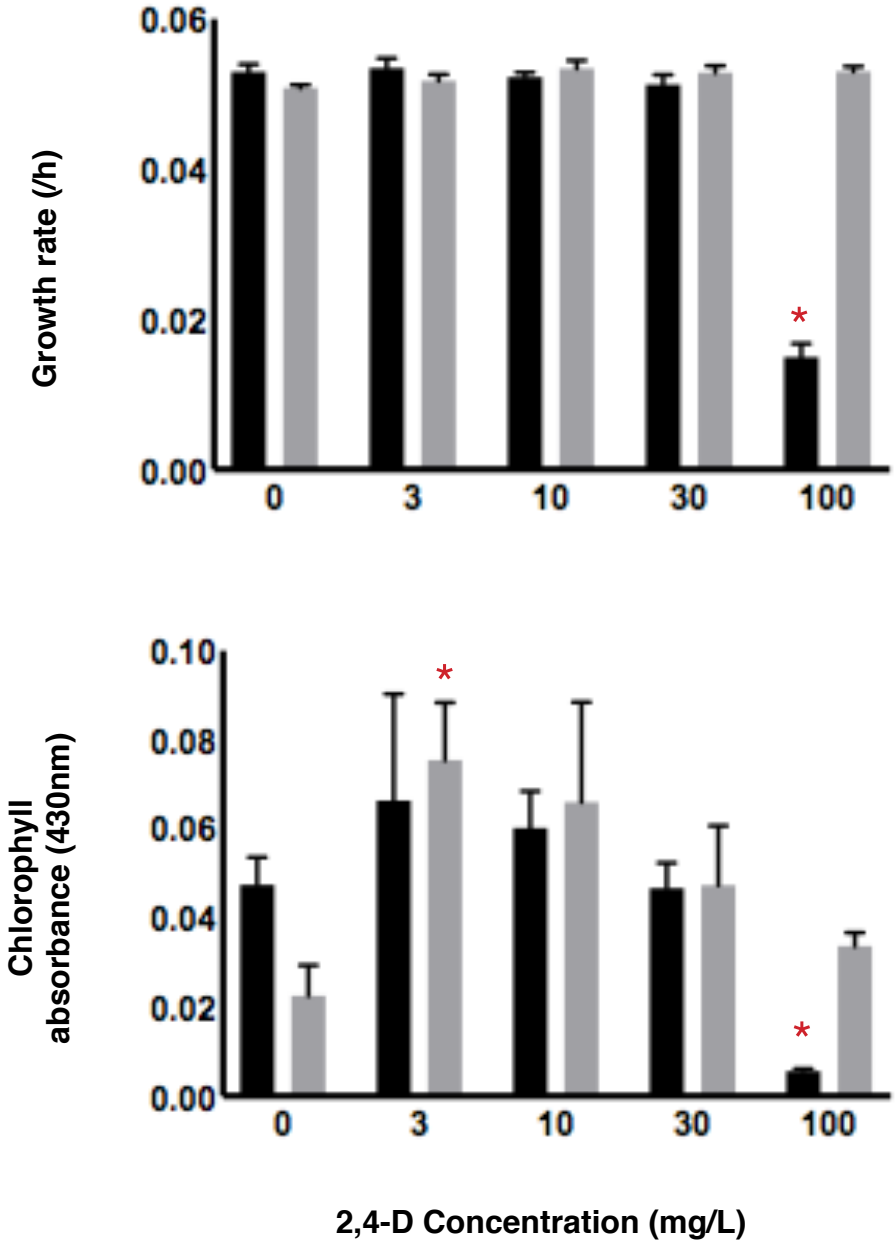

Figure 11. Kingston *O. amblystomatis* 2,4-D exposure and recovery. Endpoints monitored after 96 h exposure of 2,4-D to algae (dark bars) followed by 96 h recovery (light bars). Nominal concentrations are shown with standard error bars. Significant differences from control (Dunnett's test, Kruskal-Wallis H test α = 0.05) represented by asterisk.

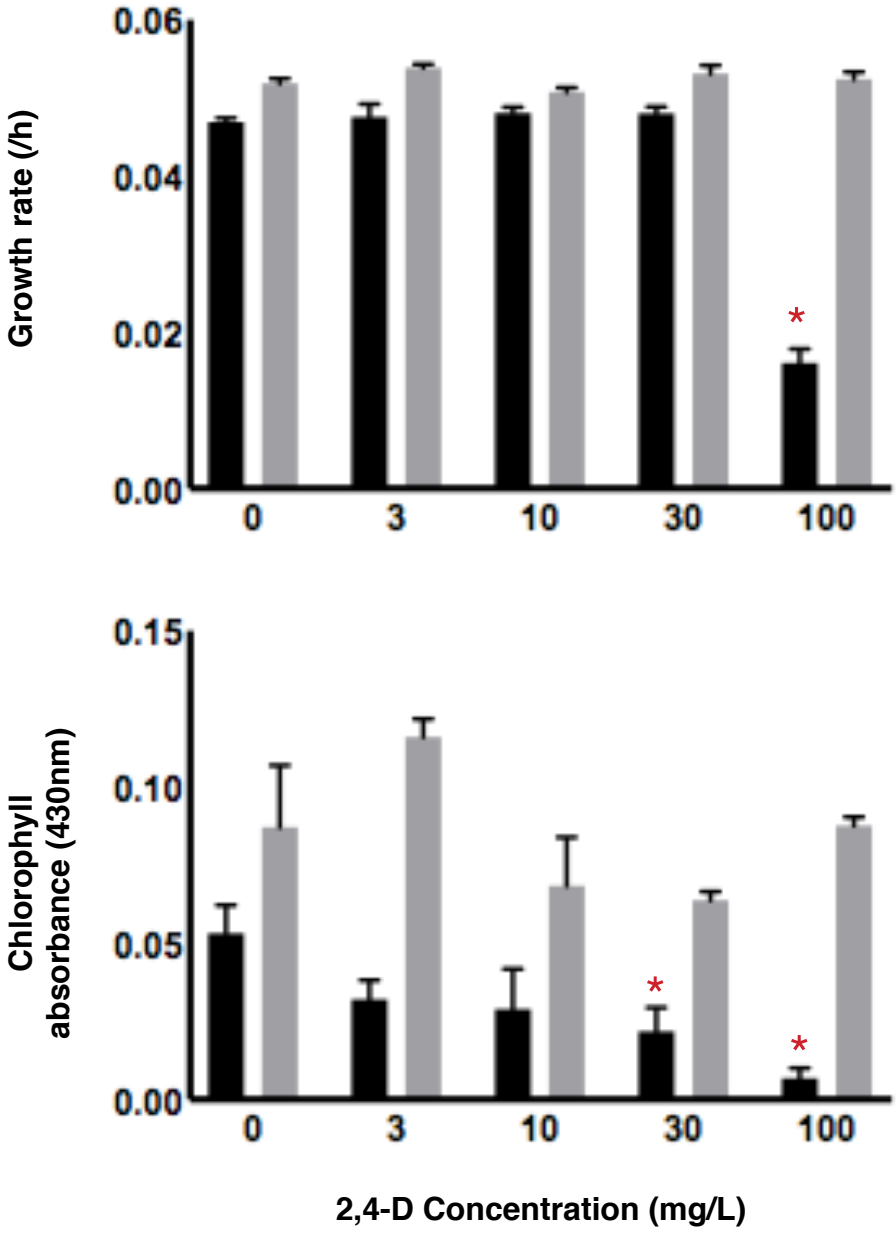

Figure 12. Sudden Tract *O. amblystomatis* 2,4-D exposure and recovery. Endpoints monitored after 96 h exposure of 2,4-D to algae (dark bars) followed by 96 h recovery (light bars). Nominal concentrations are shown with standard error bars. Significant differences from control (Dunnett's test, Kruskal-Wallis H test $\alpha = 0.05$) represented by asterisk.

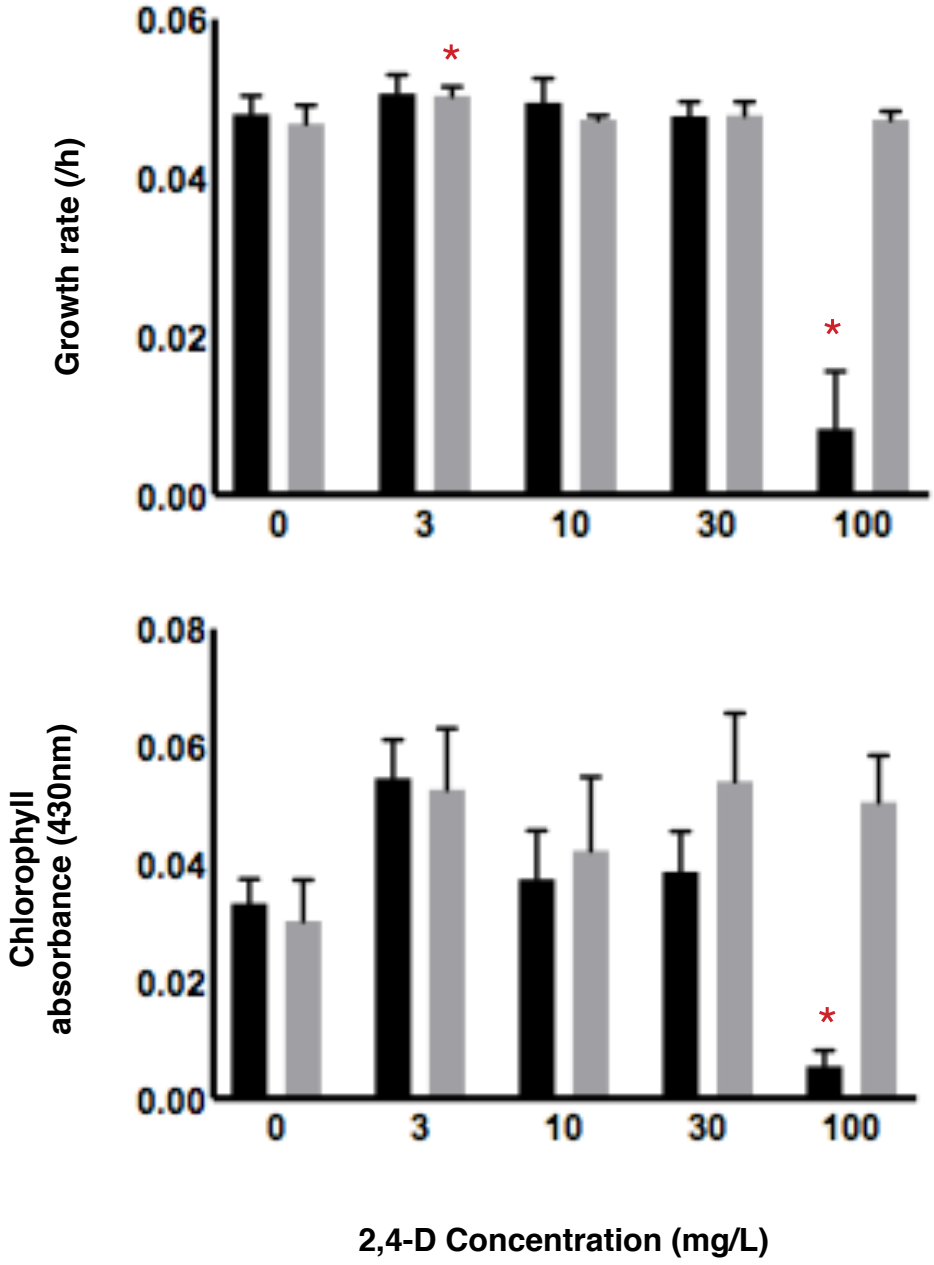

Figure 13. Bruce Peninsula *O. amblystomatis* 2,4-D exposure and recovery. Endpoints monitored after 96 h exposure of 2,4-D to algae (dark bars) followed by 96 h recovery (light bars). Nominal concentrations are shown with standard error bars. Significant differences from control (Dunnett's test, Kruskal-Wallis H test $\alpha = 0.05$) represented by asterisk.

5.0 Discussion

Standard toxicity tests should produce data that can be harmonized and compared with other studies, and be able to provide a relative context through which environmental risks may be assessed. In an effort to first determine the identity of the egg inhabiting algae *O. amblystomatis,* the phylogenetic analysis from this study was compared with the results of Kim et al. (2014) and others, who demonstrate that *Oophila* sp. is not a monophyletic species. Though the data from this study are partially congruent with previous results, the inclusion of other available *Oophila* sp. sequences from GenBank as well as samples from the current study in our phylogenetic reconstruction suggests that the species requires substantial taxonomic revision. The diverse nature of *Oophila* sp. as currently described is significant from a risk assessment standpoint, as toxic sensitivity is generally regarded as a species specific response (Blanck 1984, Ellesat et al. 2007). Thus, toxicity tests that examine species that are in fact genetically dissimilar would produce confounding results (Leung et al. 2016). In their study, Leung et al. (2016) highlight this complexity in conducting toxicity tests with cryptic species such as *Hyalella azteca* Saussure*,* an amphipod crustacean. Only two clades of this species are cultured and employed in toxicity laboratories across North America, though the species complex includes 85 genetically diverse lineages in the wild (Leung et al. 2016). The authors exposed two clades of *H. azteca* to Nickel and Copper in a 14 d test, and reported one clade being significantly more tolerant to the metals than the other (endpoint measured being mortality) (Leung et al. 2016). Thus, the problematic nature of conducting toxicity tests on a cryptic species complex that is

otherwise accepted as a “standard toxicity test species” provides some context for the nature of the current study.

In this study, the sensitivity of the egg inhabiting algae *O. amblystomatis* to herbicides atrazine and 2,4-D was also investigated. The results from the atrazine exposure and recovery tests in this investigation do differ from those in the study of Baxter et al. (2014), and the genetic dissimilarity between *O. amblystomatis* strains from each study may reflect these differences in toxic sensitivity. Our results also include data from the sample site used in Baxter et al. (2014) that was re-visited in the current study.

5.1 Phylogenetic reconstruction of symbionts

To gain a better understanding of the phylogeny of collected *Oophila* sp. samples, the nuclear SSU rRNA gene was sequenced and included in maximum likelihood tree with other related Chlorophycea species. The results depict *Oophila* sp. members grouping in two strongly supported clades, neither of which are monophyletic. “*Oophila*” Clade A, which contains sequences largely from Nova Scotia, includes three Chlamydomonad species mirroring the results of Kim et al. (2014), Lin and Bishop (2015) and Bishop and Miller (2014). Though the authors of these studies show *Chlamydomonas* members grouping within the “*Oophila*” clade, they exclude five *Oophila* sp. sequences available in GenBank (Lewis and Landberg, meeting abstract, 2014). Four of these sequences, along with the southern Ontario sequences from the current study, group together to form *Oophila* Clade A in the nuclear SSU rRNA tree. It has recently been learned that at least one of the four unpublished GenBank sequences

was sampled from the type locality of *Oophila amblystomatis* F.D.Lambert ex N.Wille*,* namely, from Middlesex Fells, Massachusetts (personal communication with Craig Schneider, in possession of Lambert's type species - Phycological Society of America Annual Meeting 2016; Wille 1909). Thus, it is postulated that samples from the current study are more likely to be *Oophila amblystomatis*, as they group strongly with the type locality. Within *Oophila* clade A members of other green coccoid or ovoid genera including *Chlamydomonas* (AY220599, AY220094), *Characium* (M63001), and *Chlorococcum* (U70586, AB490288, AB490286, U70587) are also observed*.* Some of these sequences are unpublished and U70586 has been described in GenBank only as a "Chlamydomonad flagellate". With such a lack of verified characterization within the other species of *Oophila* Clade A, it is hypothesized that these other algae may be subject to mis-identification, and may in fact be *O. amblystomatis* members. Alternatively, it is possible that members within "*Oophila*" Clade B may indeed be *O. amblystomatis*, with the three chlamydomonad species, mentioned previously, possibly misidentified. This number of "*Oophila* sp." sequences (over 40) grouping in a clade so distantly from sequences collected from the type locality may not be the most parsimonious explanation. It is more likely (and parsimoniously sound) that members of "*Oophila*" Clade B have indeed been mislabelled.

Within the Chlamydomonadales (Chlorophyceae, Chlorophyta), taxonomic revision is not uncommon; Nakada et al. (2008) constructed an extensive phylogeny of the nuclear 18S rRNA gene region including over 400 sequences within the Chlamydomonadales (previously Volvocales). With the objective of reclassifying traditional taxonomic descriptions based on morphological characters, the authors

delineated 21 strongly supported clades within the Chlamydomonadales (Nakada et al. 2008). One of their proposed clades (not a taxonomic group), Stephanosphaerinia, displays a similar grouping of *Chlamydomonas* spp. and *Chlorococcum ellipsoideum*, as observed in the present study (Nakada et al. 2008). The species within this described lineage are not monophyletic, but characterize the close genetic relationships between the above mentioned polyphyletic genera (Nakada et al. 2008). The clade is named after the representative genus, *Stephanosphaera* proposed by the authors (Nakada et al. 2008). The Stephanosphaerinia clade has been referenced in more recent literature and the grouping has been seemingly recognized in various taxonomic studies within the Chlorophyta (Hollzinger et al. 2014, Lemieux et al. 2015). Another study examined diversity within one *Chlamydomonas* species, *C. reinhardtii*, Jang and Ehrenreich (2012) employed a genome-wide assessment of interspecific variation within this model species. The authors isolated almost all available isolates of the species in North America and provide insight into the diversity of the species in addition to identifying geographical subpopulations of *C. reinhardtii (*Jang and Ehrenreich 2012). The authors assert the importance of their results as *C. reinhardtii* is a well studied species used as a model system for not only biological investigations, but also research in emerging algal applications such as biofuel (Jang and Ehrenreich 2012). Thus, it is evident that the characterization of genetic diversity within species, that are tested and utilized in different applications, is paramount to gain a complete understanding of the species' sensitivity and applicability.

This study proposes that "*Oophila*" Clade B as described by previous authors likely does not contain *O. amblystomatis* members, we do not discredit the role that the

isolated algae from “Oophila” Clade B may have in the symbiotic relationship with their amphibian symbionts. Since these sequences were in fact isolated from *A. maculatum* eggs, we suggest that the relationship between the symbionts may not be restricted to just *O. amblystomatis*. Rather, the egg inhabiting algae may be opportunistic, with species adapting to environmental conditions which can vary in temperature, salinity, light, dissolved oxygen etc. Variations amongst these parameters within sample sites of this study were observed within one breeding season alone. Thus, variations in environmental conditions year to year would, undoubtedly, allow for even more competition between green algal species, as certain species would proliferate under conditions they are best suited to. Though Bishop and Miller (2014) maintain that their characterized “*Oophila* sp.” is the predominant alga within *A. maculatum* eggs, our phylogeny shows that isolates of egg inhabiting algae form distant clades. This could suggest that species within each clade are able to proliferate under separate sets of environmental conditions. In initial microscopic observations of *A. maculatum* egg contents for this study, a number of green algal species including *Scenedesmus* sp. and *Chlorella* sp. were noted. Furthermore, “*Oophila*” Clade B contains a sequence from a sample location that was re-visited in the current study, and which now groups in *Oophila* Clade A. Thus, though *O. amblystomatis* was isolated and sequenced, the possibility that there may be more Chlorophyta species that would contribute photosynthate products to *A. maculatum* embryos, and thus take part in this symbiotic relationship cannot be precluded.

The second objective of this study was to examine two mtDNA gene regions of our *A. maculatum* samples, and determine whether sequences fell within previously

described patterns of post glacial distribution of taxa since the last glacial maximum. Furthermore, we wanted to compare the phylogeography of *A. maculatum* with any potential phylogeographic patterns observed within the algal symbionts. The results show that both ND4 and control region mtDNA sequences of out salamander samples are falling within the expected Eastern Clade A as described by (Zamudio and Savage 2003). Since both our gene regions displayed congruent phylogenies of our southern Ontario samples, it was not deemed necessary to carry out any further network analysis or concatenate the phylogenies to get further resolution. The Eastern and Interior clade hypothesis proposed by Zamudio et al (2003) is a distribution pattern also described by Soltis et al. (2006) who sampled a vast range of taxa including amphibians, reptiles, fish, and algae, across North America. Their mtDNA restriction site analysis show the Appalachian mountain discontinuity as an east versus west pattern that delineates the genetic distribution of salamanders (*A. maculatum*, *A. tigrinum*) and turtles (*Sternotherus odouratus, S. minor*) among other species, in eastern North America (Soltis et al. 2006). It has been proposed that post glacial distribution of taxa after the LGM may have emerged from two refugial populations on either side of the city of Apalachicola, Florida (Soltis et al. 2006, Church et al. 2003).

This east-west divide of genetic diversity on either side of the Appalachians is not apparent in the nuclear SSU rRNA *"Oophila"* sp. phylogeny. Since both *Oophila* Clades A and B include members from Ontario, and thus members from at least one same geographical region, it is unclear that any phylogeographic patterns exist amongst the *Oophila* species. Furthermore, members of Clade A include not only East Coast sequences from Nova Scotia and New Jersey, but also sequences from the West Coast

in British Columbia. This vast distance between sample locations further suggests a lack of any clear phlyogeographic patterns.

In order to delineate the biogeographic patterns within the red agal genus *Hildenbrandia* (Rhodophyta), Sherwood and Sheath (1999) employed chloroplast and nuclear SSU rRNA gene markers to resolve the relationships of the widespread marine, and more isolated freshwater samples of the genus (Sherwood and Sheath 1999). Within the freshwater species, the authors observed paraphyly with marine samples and suggested that multiple invasions by different populations were responsible for this genetic diversity (Sherwood and Sheath 1999). It is possible that vectors such as marine waterfowl are responsible for the establishment of freshwater *Hildenbrandia* populations from coastal marine environments (Sherwood and Sheath 1999). Also within the red algae, the biogeography of the Boldiaceae (Rodophyta) family was investigated by Rintoul et al. (1999) using combined analyses of nuclear SSU rRNA, *rbc*L, and other genes. In their results, the authors describe such high levels of species divergence within this family that sequence alignment was not possible (Rintoul et al. 1999). Despite these differences however, the authors propose a number of historical events that could explain this high interspecific variation within Boldiaceae (Rintoul et al. 1999). For example, algal populations around the Great Lakes could only have been established after the retreat of the Laurentide Ice Sheet (Rintoul et al. 1999). More southerly populations, such as those sampled from Virgina however, may have been unaffected by the Last Glacial Maximum (approx. 20000 years ago), and thus may have been established much longer (Rintoul et al. 1999). This could account for the north-south genetic divide amongst these Boldaceae species (Rintoul et al. 1999). To explain

the high degree of divergence amongst Boldaceae species within Ontario, the authors also propose the possibility of vector mediated transport such as that of migrating waterfowl (Rintoul et al. 1999).

It is evident that *Oophila* sp., from its wide geographic distribution across North America, has an excellent ability to disperse, and can occupy a wide range of habitats (from east coast, to central, and west coast North America). This high affinity for dispersal can potentially mask any paleo-phylogeographic patterns that can be attempted to uncover using genetic data (Boedeker et al. 2010). Thus, with such widely dispersed populations, and apparent ability to thrive in a range of environments, there is not enough evidence to suggest any strict phylogeographic patterns of the algal symbionts of *A. maculatum* due to coevolution.

5.2 Response of *O. amblystomatis* to herbicides

Past studies on the effects of atrazine on fresh water algae have indicated that inhibition of growth, largely, is detected at 10 to 20 µg/L (Lockert et al. 2006, Huber 1993). DeNoyelles et al. (1982) however, examined the indirect effects of atrazine on three fish species via the reduction of their phytoplankton food source, and reported inhibitory effects (in chlorophyll absorbance, biomass, and species distribution) on phytoplankton growth at 1 to 5 µg/L of treated atrazine. These authors employed longer term (136 d) mesochosm studies which involved exposing whole phytoplankton communities to atrazine, and monthly monitoring of the mentioned endpoints (DeNoyelles et al. 1982). For the current study, 96 h acute toxicity tests (including exposure and recovery) were conducted with unialgal cultures isolated from three

sample locations. The 96 h NOEC values for PSII Sudden Tract and Kingston support the results of Lockert et al. (2006) who reported that any measurable effects of atrazine were only apparent at > 20 µg/L. Lockert et al. (2006) examined the effects of atrazine on a wide range of taxa including green algae, cyanobacteria, and diatoms representative of North American freshwater streams. The authors measured chlorophyll absorbance and growth rate as endpoints, and obtained readings for 5 days of atrazine exposure (Lockert et al. 2006). *O. amblystomatis* cultures from the current study showed less susceptibility to atrazine however, in chlorophyll absorbance and growth rate data than the green algal species tested by Lockert et al. (2006). In comparison with the atrazine exposure tests by Baxter et al. (2014), the 96 h EC50s for all three endpoints in this study were much higher. The methodology and test protocol (including range values) of Baxter et al. (2014) was followed, so it was initially surprising that the algal cultures showed generally less sensitivity. After confirming the taxonomic position of the *O. amblystomatis* isolates (Figure 3, "*Oophila"* Clade A) in comparison with the sequence from Baxter et al. (2014) however, it can be surmised that the genetic differences in these two strains may contribute to their toxicological sensitivity to atrazine. Also worth re-iterating is that the sample location from which Baxter et al. (2014) had obtained their *A. maculatum* eggs (Sudden Tract, Township of North Dumfries Ontario), was re-sampled in the current study. As mentioned previously, the sequence from Sudden Tract did not group within the clade including sequences from Baxter et al. (2014) and other authors (Bishop and Miller 2014, Kim et al. 2014).

After the exposure phase, full recovery was observed for all three sample cultures, and endpoints measured were equal to or more than the recovery controls.

This mirrors the observations of Baxter at el. (2014) who, through similar 96 h atrazine exposure followed by 96 hour recovery tests, noted full recovery for all endpoints except chlorophyll absorbance at 300 µg/L. The recovery data from this study are also in line with the results of Brain et al. (2012) who exposed three algal species, including the green alga *Pseudokirchneriella subcapitata* (Chlorophyta) to pulse exposures of atrazine. Growth inhibition of *P. subcapitata* was tested at nominal atrazine concentrations ranging from 5- 250 µg/L, and after a two day pulse exposure followed by a two day recovery, the authors observed no significant differences between recovery test concentrations and control (Brain et al. 2012). Furthermore, the concentration test range of Brain et al. (2012) was maintained to encompass the maximum worst-case pulse exposure of atrazine, that may potentially be measured in North American fresh water. With seasonal pulses taken into account, the highest recorded atrazine concentrations in the White River, Indiana (located in the U.S. Corn Belt) for example have peaked at approximately 15 µg/L (Gilliom, 2006). In Ontario, atrazine concentrations in surface waters have been reported at < 0.1 to 3.9 µg/L , with seasonal peaks of herbicide concentrations in the spring/early summer (Byer et al. 2011). Thus, with the results of exposure and full recovery observed at the maximum test concentration, 405 µg/L in the current study, there is sufficient evidence to indicate that phytoplankton productivity, specifically that of *O. amblystomatis* and other chlamydomonad-like taxa, would likely be unaffected at environmentally realistic atrazine exposures in North America.

The herbicide 2,4-Dichlorophenoxyacetic acid is an auxin simulator, and as such it promotes plant growth at low doses, while at high doses, accelerates plant growth

which results in plant deformities and eventually, plant death (Song, 2013). The physiological abnormalities as a result of 2,4-D exposure described in vascular plants was not observed in the cells of our *O. amblystomatis* cultures. However, the herbicide did have an observable hormetic effect at low doses, approximately 3 to 10 mg/L, which was observed in two of the three strains (Kingston, Bruce Peninsula). Hormetic effects were not statistically significant from controls, but were observable in both growth rate and chlorophyll absorbance endpoints (see exposure/recovery graphs Figures 10, 11, and 12.

Though quantification of 2,4-D test concentrations in this study was not obtained, response results at nominal concentrations coincide relatively with existing data on the inhibitory effects of this herbicide (Song 2013, Wong 2000). In a 96 h 2,4-D toxicity test on *Selenastrum capricornutum* (Chlorophyta), Fairchild et al. (1996) observed the cell biomass NOEC to be 24 mg/L and the EC50 ranging from 37 to 46 mg/L of 2,4-D. The results from the current study suggested *O. amblystomatis* to be less sensitive with NOEC values of 30 mg/L for all endpoints and strains except for Sudden Tract chlorophyll absorbance. The 96 h EC50 values were also higher than those reported by Fairchild et al. (1996), but were within the same or order of magnitude. Though Fairchild et al. (1996) did not discuss the stimulatory effects of 2,4-D at low concentrations, Wong (2000) did observe promotion of growth in their growth rate and chlorophyll-a content endpoints of their test alga *Scenedesmus quadricauda* (Chlorophyta). Wong (2000) reported promotion of algal growth at 0.02 to 0.2 mg/L of 2,4-D, complete inhibition at 200 mg/L, and significant chlorophyll-a reduction (compared to control) at 20 mg/L. Though some stimulatory effects at > 2 mg/L were observed, and significant growth

reduction at 30 to 100 mg/L of 2,4-D, the initial range finding test showed complete inhibition at 200 mg/L of the herbicide. This result coincides with that of Wong (2000), though the rest of the data may suggest our *O. amblystomatis* strains to be less sensitive than *Scenedesmus quadricauda* and *Selenastrum capricornutum*.

There is a paucity of data on the recovery response of freshwater algae exposed to 2,4-D. Similar to that of the atrazine recovery phase tests however, full recovery (greater or equal to control) for 2,4-D exposed algae was observed. It is unlikely that the concentrations at which significant inhibition of growth detected in the current study would be found in the environment (Gilliom, 2006). According to the National Water-Quality Assessment Program led by the U.S Geological Survey, although 2,4-D is one of the most highly detected compounds in herbicide mixtures within agricultural and urban environments, its detection at 1.0 µg/L is at the lowest limit of their detection capabilities (Gilliom, 2006). In Canada, the highest 2,4-D concentrations have been reported at approximately 0.95 µg/L (Glozier 2012). Thus, in conjunction with the chemical properties of 2,4-D that allow it to be readily degraded and mineralized by soil and organic particles in the water column (Boivin et al. 2005), it is proposed that 2,4-D is unlikely to be a risk to freshwater chlamydomonad algae at environmentally relevant concentrations (Gilliom, 2006).

6.0 Conclusions

The taxonomic identity of *Oophila amblystomatis* is in need of revision, and in this study we identified that this group is currently not monophyletic, and includes sequences that group in two separate clades, and with members of other green algal species. The genetic diversity of algae isolated from *A. maculatum* eggs leads us to suggest that the symbiotic relationship between salamander and alga(e) may not be as exclusive as was until now accepted. Rather, the egg inhabiting algae may be opportunistic, and certain species may predominate under certain environmental conditions. It is possible that the algae participating in this relationship may comprise of a whole community of symbionts. The diversity observed within *Oophila* sp. itself does not coincide with its widespread distribution, in contrast with the biogeographic patters observed in *A. maculatum* lineages. This suggests that the green alga has likely been subject to vector transport, and is able to adapt and thrive in a wide range of habitats.

In order to thoroughly assess the indirect effects that herbicides, such as atrazine and 2,4-D, may have on *A. maculatum* embryos via their symbiotic algae, the next step into characterizing these potential risks may involve identifying and testing all the agal species that participate in this symbiotic relationship. Our study and others have shown that green algae is differentially sensitive to atrazine (Lockert et al. 2006, Baxter et al. 2014) and 2,4-D (Wong 2000, Fairchild et al. 1998). For atrazine, NOEC for growth rate in our study was observed at 70 µg/L or more, and for 2,4-D the nominal NOEC was 30mg/L or more. Though these responses exceed environmentally relevant exposure levels, there is potential for inhibitory effects to be observed at lower concentrations for more sensitive green algal species. Future directions with this research include

assessing the species of egg inhabiting algae that are the most sensitive to herbicides such as atrazine and 2,4-D, and establishing these as a standard for future toxicity tests. The results from this study for example, indicate that the *O. amblystomatis* strains isolated and cultured in this investigation are less sensitive than the strain tested by Baxter et al. (2014). From an environmental context, it is intuitive that the more sensitive a species is, the higher it is at risk, and this should be taken into consideration when conducting future toxicity tests with these unique egg inhabiting algae.

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Appendix / Supplemental Data

Table A1. Oophila sp. Genbank Compilation

Accession Number	
KJ635663	KJ711227
KJ635662	KJ711224
KJ635659	KJ711215
KJ635658	KJ711213
KM359522	KJ711206
KM359519	KJ711203
KM359515	KJ711197
KM359513	KJ711196
KM359511	KJ711194
KM359510	KJ711193
KM359509	KJ711175
KM359508	KJ711167
KM359507	KJ711157
KM359506	KJ711155
KJ711131	KJ711151
KJ711256	KJ711147
KJ711254	KJ711145
KJ711251	KJ711140
KJ711247	KJ711133
KJ711244	KJ635670
KJ711236	
KJ711234	
KJ711233	
KJ711229	

Table A2. Green Algal Sequences Taken from Kim et al. (2014)

Species, GenBank Accession Number
Chlamdomonas nasuta AB701502
Chlamydomonas pseudogloegama AF517097
Tetracystis aeria U41175
Chlorococcum hypnosporum U41173
Chlamydomonas moewusii U41174
Chlamydomonas moewusii EU925396
Chlamydomonas sp HM754412
Chlorococcum elkhartiense AJ628976
Chlamydomonas pitschmannii AJ628982
Chlamydomonas acidophila AJ628977
Chlamydomonas acidophila AJ852427
Chlamydomonas parkeae AB058373
Chlamydomonas hedleyi AJ781312
Tetracystis aplanospora JN903992
Chlamydomonas noctigama AJ781311
Chlamydomonas noctigama AF008242
Haematococcus pluvialis AF159369
Chlamydomonas perpusilla AB753036
Chlamydomonas gloeophila KJ635670
Chlorogonium elongatum U70589
Characium vacuolatum M63001
KJ635659 (Louise)
KJ635657 (Louise)
Chlorococcum sp. AB490286
KJ635662 (Louise)
Chlamydomonas sp. AY220599
KJ635658 (Louise)

Species, GenBank Accession Number
Chlamydomonas sp. AY220094
Chlorococcum sp. AB490288
Chlorococcum ellipsoideum U70586
Chlorococcum diplobionticum U70587
Protosiphon botryoides U41177
Chlamydomonas humicola U13984
Polytoma ellipticum U22933
Chlamydomonas tetragama AB007370
Asteromonas gracilis M95614
Chlorosarcinopsis gelatinosa AB218707
Chlorosarcinopsis arenicola AB218701
Characiosiphon rivularis AF395437
Dysmorphococcus globosus X91629
Chlamydomonas monadina U57694
Chlamydomonas sp. KF879589
KJ635663 (Louise Lewis)
Chloromonas rosae U70796
Chlamydomonas nivalivalis U57696
Lobochlamys culleus AJ410461
Oogamochlamys ettlii AJ410469
Chlorosarcina stigmatica AB218709
Chlamydomonas baca U70781
Neochlorosarcina negevensis AB218715
Volvox carteri X53904
Heterochlamydomonas lobata AF367858
Chlorosarcina stigmatica AB218711
Carteria radiosa D86500
Spermatozopsis similis X65557

Species, GenBank Accession Number
Carteria crucifera D86501
Pyramimona vacuolata AB999994
Chara drouetii U18495
Micromonas commoda KU244632
Scherffelia dubia X68484
Chlorella vulgaris X13688
Oltmannsiellopsis viridis D86495
Aphanochaete magna AF182816

Table A3. ND4 Region *A. maculatum* sequences Taken from Zamudio and Savage (2003)

Genbank Accession Number		
AY186486	AY186459	AY186434
AY186485	AY186458	AY186433
AY186484	AY186457	AY186432
AY186484	AY186456	AY186431
AY186483	AY186455	AY186430
AY186482	AY186454	AY186429
AY186481	AY186453	AY186428
AY186480	AY186454	AY186427
AY186479	AY186453	AY186426
AY186478	AY186452	AY186425
AY186477	AY186451	AY186424
AY186476	AY186450	AY186423
AY186475	AY186449	AY186422
AY186474	AY186448	AY186421
AY186473	AY186447	AY186420
AY186472	AY186443	AY186419
AY186471	AY186444	AY186418
AY186470	AY186445	AY186417
AY186469	AY186446	AY186416
AY186468	AY186442	AY186415
AY186467	AY186441	AY186414
AY186465	AY186440	AY186413
AY186464	AY186439	AY186412
AY186463	AY186438	AY186411
AY186462	AY186437	AY186410
AY186461	AY186436	AY186409

Table A3. ND4 Region *A. maculatum* sequences Taken from Zamudio and Savage (2003)

Genbank Accession Number		
AY186460	AY186435	AY186407
AY186408	AY186383	AY186358
AY186407	AY186382	AY186357
AY186406	AY186381	AY186356
AY186405	AY186380	AY186355
AY186404	AY186379	AY186354
AY186403	AY186378	AY186353
AY186402	AY186376	AY186352
AY186401	AY186375	AY186351
AY186400	AY186374	AY186350
AY186399	AY186373	AY186349
AY186398	AY186372	AY186348
AY186397	AY186371	AY186347
AY186396	AY186370	AY186346
AY186395	AY186369	AY186345
AY186394	AY186368	EF649952 (*A. opacum,* outgroup)
AY186393	AY186367	AY691773 (*A. gracile*, outgroup)
AY186392	AY186366	
AY186391	AY186365	
AY186390	AY186364	
AY1863989	AY186363	
AY186387	AY186362	
AY186386	AY186361	
AY186385	AY186360	
AY186384	AY186359	

Table A4. Control Region A. maculatum sequences Taken from Zamudio et al. (2003)

Genbank Accession Number		
AY186495 (IL)	AY186328 (?)	AY186532 (WI)
AY186496 (IL)	AY186270 (?)	AY186511 (IN)
AY186498 (IL)	AY186331(ON)	AY186520 (KY)
AY186491 (IL)	AY186332(ON)	AY186513 (IN)
AY186494 (IL)	AY186263 (ON)	AY186536 (WI)
AY186499 (IL)	AY186282 (WI)	AY186528 (WI)
AY186500((IL)	AY186266 (ON)	AY186526 (WI)
AY186501 (IL)	AY186330 (?)	AY186530 (WI)
AY186509 (MI)	AY186261 (?)	AY186523 (MI)
AY186510 (KY)	EU169894 (?)	AY186522 (MI)
AY186533 (MI)	EU169895 (?)	AY186535 (TN)
AY186490 (ON)	EU169899 (?)	AY186529 (WI)
AY186507 (MI)	AY186262 (MA)	AY186487 (AL)
AY186508 (MI)	AY186320 (MA)	AY186489 (AL)
AY186505 (MI)	AY186340 (VA)	AY186525 (MS)
AY186506 (MI)	AY186307 (CT)	AY186516 (NC)
AY186519 (KY)	AY186268 (ME)	AY186524 (AL)
AY186521 (KY)	EU169896 (?)	AY186488 (AL)
AY186514 (IN)	EU169897 (?)	AY186517 (NC)
AY186537 (WI)	AY186267 (ME)	AY186518 (NC)
AY186497 (IL)	AY186310 (PA)	AY186503 (SC)
AY186502 (IL)	AY186258 (NY)	AY186504 (SC)
AY186512 (IN)	EU169900 (?)	AY186534 (NC)
AY186515 (IN)	AY186264 (MA)	AY186295 (IN)
AY186492 (IL)	AY186309 (PA)	AY186336 (ON)
AY186493 (MA)	AY186327 (?)	AY186271 (ON)

Table A4. Control Region A. maculatum sequences Taken from Zamudio et al. (2003)

Genbank Accession Number		
AY186304 (VA)	AY186337 (ON)	AY186297 (NC)
AY186281 (VA)	AY186323 (MS)	AY186296 (NC)
AY186316(NY)	AY186338 (MA)	AY186287 (NC)
AY186335 (ON)	AY186283 (?)	AY186317 (MO)
AY186260 (?)	AY186275 (LA)	AY186288 (NC)
AY186300 (NY)	AY186273 (?)	EF649894 A. opacum
AY186318 (MA)	AY186276 (LA)	AY186597 A. gracile
AY186299 (NY)	AY186313 (OK)	
AY186326 (NSA)	AY186278(LA)	
AY186269B (?	AY186312 (OK)	
AY186256 (MD)	AY186314 (MO)	
AY186319 (MA)	AY186290 (LA)	
AY186303 (VA)	AY186279 (LA)	
AY186315 (NY)	AY186302 (TN)	
AY186344 (MD)	AY186292 (AR)	
AY186334 (ON)	AY186321 (MO)	
AY186306 (CT)	AY186277 (LA)	
AY186329 (?)	AY186291 (AR)	
AY186305 (VA)	AY186293 (MO)	
AY186342 (VA)	AY186325 (NC)	
AY186341 (VA)	AY186324 (NC	
AY186284 (?)	AY186289 (OK)	
AY186286 (GA)	AY186298 (NC)	
AY186285 (?)	AY186274(IL)	
AY186311 (OK)	AY186339 (TN)	

Table A4. Control Region A. maculatum sequences Taken from Zamudio et al. (2003)

Genbank Accession Number	
AY186294 (MO)	AY186322(MO)
AY186301 (TN)	AY186280 (SC)

Table A5. ELISA analytical quantification of atrazine exposure test concentrations

Nominal	Kingston	Sudden Tract	Bruce Peninsula
3	4.5	4.2	3.3
10	10	9	10
30	27	21	21
100	140	80	70
300	405	360	270

Table A6. ELISA analytical quantification of atrazine recovery test concentrations

Nominal	Kingston	Sudden Tract	Bruce Peninsula
0	0.12	0	0
3	0.12	0.2	0
10	0.5	0.5	0
30	0.9	0.25	0
100	5	5	0
300	6	6	1.1