EVALUATING ENVIRONMENTAL DNA (eDNA) DETECTION OF INVASIVE WATER SOLDIER (STRATIOTES ALOIDES)

A Thesis Submitted to the Committee on Graduate Studies in Partial Fulfillment of the Requirements for the Degree of Master of Science in the Faculty of Arts and Science

TRENT UNIVERSITY
Peterborough, Ontario, Canada
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Environmental and Life Sciences M.Sc. Graduate Program
May 2017
Abstract

Evaluating Environmental DNA (eDNA) Detection of Invasive Water Soldier (*Stratiotes aloides*)

Allison Karen Marinich

In 2008, the first North American water soldier (*Stratiotes aloides*) population was discovered in the Trent River, Ontario. Water soldier is an invasive aquatic plant with sharp, serrated leaves that has the potential to spread rapidly through dispersed vegetative fragments. Although it is too late to prevent water soldier establishment in the Trent River, its local distribution remains limited. In this study, environmental DNA (eDNA) was explored as a potential tool for early detection of water soldier. Species-specific markers were designed from chloroplast DNA regions *matK* and *rbcL*, and a qPCR assay with *rbcL* primers yielded the most sensitive detection of water soldier eDNA. Positive detections were obtained from six of 40 sampling locations, of which five were collected in Seymour Lake, an area with large patches of water soldier. As water soldier plants were known to be present at these sites, high eDNA concentrations were expected. The sixth positive detection from Trent Lock 5 (50 km downstream of Lake Seymour) was unexpected as it was obtained at a site with no water soldier sightings. This is one of the first studies to demonstrate the effectiveness of eDNA detection from aquatic plants.

**Keywords**: invasive species, aquatic plant, water soldier (*Stratiotes aloides*), environmental DNA (eDNA)
Acknowledgements

I would first like to express sincere thanks to my thesis supervisor, Joanna Freeland, for her quick feedback, motivation and words of encouragement throughout this project. I thank Chris Wilson, my thesis co-supervisor for all his help and constructive comments. I am appreciative of Bill Crins for being a part of my graduate committee and sharing his attention to details.

I would like to acknowledge the individuals in the Fish Genetics Lab including Kristyne Wozney, for her time spent helping me develop qPCR primers and troubleshooting amplification. I would like to thank Jenn Bronnenhuber for teaching me how to filter my first eDNA sample. I am also very appreciative to Bill Sloan for helping me collect water samples during the fall of 2013.

I thank all of my fellow graduate students in the Freeland lab for being fantastic lab mates. I also thank the undergraduate students who completed filter extractions for my project.

I am thankful for all the friends I’ve made at Trent University. I thank April, my sister, who is my best friend and biggest supporter. I am also extremely grateful to Dan, my partner for his unlimited support and encouragement while writing this thesis.

Lastly, but most importantly, I wish to thank my parents, Randy and Karen, for providing me with unfailing guidance, unconditional love and support. This accomplishment would not have been possible without them.
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Introduction

An alien or non-native species is an organism that has been introduced, usually by humans, to a location outside of its previous natural range. Alien species are introduced through many means, either intentionally or accidentally. Intentional introductions of non-native plants have most commonly occurred for agricultural or ornamental purposes, with plant nurseries as one common source of invasive species (Martin and Coetzee, 2011; Hussner, 2012; Ciotir and Freeland, 2016). Accidental introductions may also occur through several systems. For example, alien species may enter through cargo transport, fire wood movement, accidental release from captivity, the opening of canals and waterways, ballast water from foreign ships, recreational boating and movement of equipment (Environment Canada, 2016a). Due to increasing travel, tourism and trade among countries in recent decades, the numbers of alien species around the world continue to rise (Sekar, 2012). Climate change may also play a role in the increasing number of worldwide alien species, as it increases the quantity and quality of suitable habitats for invaders (Bertelsmeier et al., 2013).

Not all organisms that arrive in new environments thrive and establish populations. The likelihood of an alien species succeeding in its new environment can be explained by a number of factors including escape from enemies, propagule pressure, life history traits, invasion meltdown, and adaptation to disturbed environments. The ‘escape from enemies’ hypothesis states that alien species arrive and thrive because they have no natural predators, competitors or pathogens in
their new environment (Crawley, 1986; Lawton and Brown, 1986; Blossey and Notzold, 1995). Propagule pressure, which is the quality, quantity and frequency of invading organisms (i.e., seeds, spores, statoblasts, eggs, etc.) (Groom et al., 2006), is another factor that can determine whether an alien species will be successful, as those that are introduced more frequently and in greater numbers may have a better chance of persisting and eventually establishing populations (Lockwood et al., 2005). Once the organisms have arrived, life history traits can help determine whether their populations will persist or perish. Researchers have found that age at first reproduction and growth rate may be predictors of whether a species will be successful in their new environment, for example, early maturity has been found to promote rapid population growth (Pimm, 1991). This hypothesis has been supported by studies on plants (Richardson and Rejmanek, 2004), reptiles and amphibians (van Wilgen and Richardson, 2012). However, early maturity and establishment success do not correlate in birds (Cassey et al., 2004; Blackburn et al., 2009; Sol et al., 2012) and mammals (Sol et al., 2008). Rapid growth rate has also been linked to establishment success in plants (Grotkopp et al., 2002; Burns, 2004; Garcia-Serrano et al., 2005; Grotkopp and Rejmanek, 2007; Graebner et al., 2012). In these studies, high specific leaf area (SLA) is a trait often linked with rapid growth rate and invasion success, because a high SLA allows the plant to capture more solar energy to promote growth (Grotkopp et al., 2002; Lake and Leishman, 2004; Hamilton et al., 2005; Leishman and Thomson, 2005). The ‘invasion meltdown theory’ states that the introduction of one invasive species may ease the establishment of another invasive species (Simberloff and Von Holle, 1999). For
example, when zebra mussels (*Dreissena polymorpha*) invaded the Great Lakes in the mid-1980s, they quickly consumed large quantities of phytoplankton which increased water clarity and allowed sunlight to penetrate deeper down the water column (Holland, 1993; MacIsaac, 1996). This increase in sunlight penetration facilitated the growth of invasive macrophytes including Eurasian watermilfoil (*Myriophyllum spicatum*) (MacIsaac, 1996). Finally, alien species may have an initial advantage in disturbed environments as there is reduced competition from native species less adapted to the disturbed environment (Marvier *et al*., 2004; Tilman, 2004).

**Invasive species**

Alien species are deemed “invasive” when they adversely impact the environment (Environment Canada, 2016b). Invasive species may hybridize with native species leading to a decrease in native species abundance and richness (Levin *et al*., 1996; Blackburn *et al*., 2004; Gaertner *et al*., 2009), alter ecosystem processes (Raizada *et al*., 2008) and change community structure (Hejda *et al*., 2009). By modifying habitats and outcompeting native organisms for resources, invasive species may lead to species extirpation or even extinction (Environment Canada, 2016c). In addition, extirpation and extinction of native species may lead to a reduction in biodiversity. For example, Japanese knotweed (*Fallopia japonica*), an aggressive perennial weed native to eastern Asia, was brought to North America in the 1800s as an ornamental plant and was also planted for erosion control (Anderson, 2012). Japanese knotweed is a highly invasive species that can displace
virtually all other types of vegetation such as native wildflowers by forming dense thickets that degrade wildlife habitats (Anderson, 2012). Like many invasive species, Japanese knotweed is extremely difficult to eradicate once populations are established (Hollingsworth and Bailey, 2000; Mack et al., 2000; Price et al., 2002).

Invasive species not only threaten biodiversity on land, but in aquatic systems as well. Freshwater habitats occupy less than one percent of the Earth’s surface, yet support about ten percent of all known species (Strayer and Dudgeon, 2010). Unfortunately, fresh waters are experiencing significant declines in biodiversity, partly as a result of invasive species, making freshwater ecosystems one of the most endangered habitats on Earth (Dudgeon et al., 2006; Thomsen et al., 2012b). The Laurentian Great Lakes in North America hold 21 percent of the world’s fresh water (Ghassemi, 2007). The Great Lakes are threatened by many aquatic invasive species (e.g., *Gymnocephalus cernuus* [Simon and Vondruska, 1991; Pratt et al., 1992] *Orconectes rusticus* [Lodge et al., 2000; Olden et al., 2006], *Bythotrephes longimanus* [Barbiero et al., 2004], *Phragmites australis* [Wilcox et al., 2003; Tulbure and Johnston, 2010; Bourgeau-Chavez et al., 2013], *Myriophyllum spicatum* [Trebitz and Taylor, 2007] and others) that degrade water quality, destroy habitat and prey on or compete with native species. With many connections to shipping pathways, invasive species that enter the Great Lakes can spread across the continent through linked river systems (Strayer, 1991; Vander Zanden et al., 2009). For example, the round goby (*Neogobius melanostomus*) is a small, bottom-dwelling fish native to the Black Sea and Caspian Sea in Eastern Europe. Round
gobies were brought over to North America in the ballast water of European ships and were first found in the St. Clair River in 1990 (Jude et al., 1992; Jude and DeBoe, 1996). Since then, round gobies have spread across the Great Lakes Basin and have also entered some inland waters (Raby et al., 2010). These bottom dwelling invaders are aggressive predators that prey on the eggs of native fish species and outcompete native fish for food and habitat (Dubs and Corkum, 1996; French and Jude, 2001; Balshine et al., 2005). Round goby can also spawn several times a season, allowing them to multiply and spread rapidly (Marsden et al., 1996; Yavno and Corkum, 2010).

In another example, zebra mussels (Dreissena polymorpha) are freshwater bivalves native to the Black Sea in Eurasia. Zebra mussels were introduced to North America in the late 1980s by ballast water from transoceanic ships (Hebert et al., 1991). Since their introduction to North America, zebra mussels have spread across the Great Lakes and beyond (Roberts, 1990; Ludyanskiy et al., 1993), forming dense colonies of sharp shells that attach to surfaces and clog water intake pipes (Roberts, 1990). The dense colonies of zebra mussels affect spawning habitats and can impact the survival of native fish eggs (Marsden and Chotkowski, 2001). Zebra mussels also filter water, which leads to growth of aquatic vegetation as sunlight penetrates the water column (Zhu et al., 2006).

In addition to invasive animal species, many invasive aquatic plants such as curlyleaf pondweed (Potamogeton crispus), Eurasian watermilfoil (Myriophyllum spicatum) and flowering rush (Butomus umbellatus) have made their way into the Great Lakes Basin (Trebitz and Taylor, 2007). Introduction of invasive aquatic
plants can occur via recreational boaters, the ballast water of transoceanic ships or by escape from aquatic water gardens or ponds (Johnson et al., 2001; Azan et al., 2014). The invasion and spread of invasive aquatic plants is facilitated by their ability to disperse via seeds, vegetative growth and fragments of established plants (Fleming and Dibble, 2015). Invasive aquatic plants can reduce species richness and abundance of native biota (Sax and Ganies, 2003; Winter et al., 2009), change habitat and ecosystem function (Hulme, 2007; Vilà et al., 2009), and have a negative impact on ecosystem services and human well-being (Schlaepfer et al., 2011).

**Invasive water soldier**

Growing concern surrounds the possibility that water soldier (*Stratiotes aloides*), an invasive aquatic perennial plant native to Europe and northwest Asia (Cook and Urm-König, 1983; Czerepanov, 1995), may enter the Great Lakes. Water soldier grows in freshwater systems including ditches, lakes and rivers in depths of up to 5 metres (Cook and Urm-König, 1983). Water soldier can be either submerged or emergent, allowing its simple and fibrillose roots growing up to 180 cm in length to be free-floating or loosely embedded in the soil (Toma, 2006). Water soldier typically rises to the water surface in the spring, when newly formed leaves containing gas bubbles allow it to float to the surface. In late fall, older leaves die back and become waterlogged, sinking the plant back down the water column (Forbes, 2000). However, not all populations include an emergent stage, with some remaining submerged throughout the summer (Erixon, 1979; Renman, 1989;
Nielsen and Borum, 2008). This difference in plant buoyancy is explained by site conditions including carbon dioxide and radiation levels (Harpenslager et al., 2015).

Anatomical and morphological differences exist between the submerged and emergent forms of water soldier. Emergent leaves are thick, stiff and dark green growing near the surface, while submerged leaves are thin, flaccid, lighter green (often reddish-purple in Ontario) growing near the bottom of the river or lake in deeper water (Snyder et al., 2016). The submerged leaves of water soldier are generally longer than the emergent leaves (Cook and Urmi-König, 1983), with the submerged leaves growing up to 60 cm long, while the emergent leaves grow to less than 40 cm long (Toma, 2006). Both submerged and emergent forms of water soldier’s leaves are sessile, linear, lanceolate or narrowly triangular and arranged in a rosette (Tomlinson, 1982; Snyder et al., 2016). As mentioned above, the leaves of water soldier are sharp and serrated with spines around the margins (Cook and Urmi-König, 1983; Toma, 2006). The spines on submerged water soldier leaves tend to be relatively weak compared to those of emergent plants (Snyder et al., 2016).

In its native range, water soldier is eaten by the European beaver (Castor fiber L.), muskrat (Ondatra zibethicus), and livestock including cattle and sheep (Veen et al., 2013). Grazing waterfowl have been shown to greatly reduce water soldier biomas in both lakes and wetlands (Van der Haterd and Heerdt, 2007; Veen et al., 2013). In addition, the Spilosoma lucticipeda L. caterpillar has been observed
feeding on water soldier causing damage to leaves (Smolder and Van der Velde, 1996). However, this species is not an aquatic insect and would die when the plants sink down the water column in the fall. Finally, species of Coleoptera, Diptera and Lepidoptera reportedly feed on water soldier (Cook and Urmi-Konig, 1983; Scheffer et al., 1984).

A number of aquatic species have been reported using water soldier as shelter. For example, a species of dragonfly (Aeshna viridis) use water soldier as shelter for protection from predators such as fish (Prejs et al., 1997; Rantala et al., 2004). Leaves of water soldier may also serve as shelter for macroinvertebrate crustaceans Cyclopoida (Strzalek and Koperski, 2009) and Isopoda (Obolewski et al., 2013). Furthermore, water soldier is reported to provide a daytime shelter for water fleas (Cerioidaphnia) (Strzalek and Koperski, 2009).

**Water soldier reproduction**

Water soldier is dioecious, often forming unisexual populations (Cook and Urmi-Konig, 1983; Toma, 2006). The plant produces either staminate or pistillate white flowers with three petals that are each 1 to 3.5 cm in length. In its native range, the flowering period of water soldier is between May and August (Markgraf, 1981). Since many populations are unisexual, fruit and seed production is sporadic. However, female plants in mixed-gender populations can produce a fruit, which is a berry-like capsule, leathery and brownish-green to green with 12-24 seeds (Cook and Urmi-Konig, 1983; Smolders et al., 1995). In North America, only female plants
have been observed and seed production has not been detected (Snyder et al., 2016). There are no reports of water soldier hybridizing with any other plant species in either its native or invasive range.

The production of two types of vegetative propagules, offsets and turions, provide an opportunity for water soldier to disperse and rapidly dominate a given area in the absence of sexual reproduction. Mature plants produce up to 12 offsets in the early summer of each year, which are smaller juvenile plants that bud off the adult through deteriorated lateral shoots beginning in August (Erixon, 1979; Kornatowski, 1979). Turions, which are small overwintering buds rich in nutrients, normally begin to grow on adult plants from mid-summer onwards. These buds sink to the substrate and remain dormant during winter or unfavourable conditions but sprout and develop into adult plants the following spring (Kornatowski, 1979). In Europe, an average of 3.1 and 14.5 turions were found on adult plants in Poland and Finland, respectively (Toma, 2012). A higher number of offsets and turions are generally found in the emergent compared to the submerged form of water soldier (Renman, 1989; Toma, 2006).

**Water soldier in North America**

The first known wild occurrence of water soldier in North America was documented in the Trent River, Ontario in 2008 (Snyder et al., 2016). The Trent River is part of the Trent-Severn Waterway, a historical waterway connecting Lake Ontario and Lake Huron through a series of natural waterways connected by
navigational locks and canals (Angus, 1998). Towards its eastern terminus, the waterway drains into the Bay of Quinte in Lake Ontario. The initial water soldier population was discovered in the section of the river that runs through Trent Hills, Ontario behind Hardy Island, County of Northumberland (Figure 1). Water soldier could potentially enter the Great Lakes through the Bay of Quinte if plants from the established population disperse 40-60 km downstream to Lake Ontario (Snyder et al., 2016). To date, four water soldier populations in addition to the one in the Trent River have been discovered in south-central Ontario, making a total of five known wild populations in North America (Snyder et al., 2016). These are in the Black River, near Sutton, Regional Municipality of York (M. Shapiera, OMNR, pers. comm.); in a pond in Blackstock, Township of Scugog, Regional Municipality of Durham; in an artificial pond near Bayfield, Huron County; and in a watering pond for cattle in the Township of Seymour, County of Northumberland (Snyder et al., 2016).

The largest and oldest water soldier population in Ontario (and North America) is in the Trent River. Surveys conducted in 2011 reported water soldier along a 11 km stretch in the Trent River, covering an area of approximately 32 ha (Snyder et al., 2016). Patches of plants varied in size with patches of a few plants to several hundreds. Surveys of the Trent River were again conducted in 2014, and water soldier was detected along a 16 km segment of the same river with some patches of plants numbering in the thousands (Snyder et al., 2016). In addition,
Figure 1. Trent-Severn Waterway and adjoining water bodies. The yellow star marks the area of initial water soldier invasion behind Hardy Island in Seymour Lake, Trent Hills, Ontario, Canada. The inset map shows the location of the study system in southern Ontario.
flowering North American water soldier plants were first spotted in June 2014 towards the eastern region of the population in the Trent River, Ontario (L. Wensink, OFAH, pers. comm). The Black River population, near Sutton, Regional Municipality of Durham was discovered in 2015 with an established population of several hundred plants (Snyder et al., 2016). Both the Blackstock and Bayfield ponds mentioned above hold dense monocultures of water soldier with plants numbering in the hundreds.

The exact mode of water soldier introduction to the wild in North America is unknown. However, it is likely that water soldier plants or seeds escaped cultivation as an ornamental plant, as they can be purchased from a number of nurseries in Ontario and are often planted in private water gardens or ponds. Like many invasive species, the introduction of water soldier has harmful effects on the surrounding community. Water soldier has negative ecological effects on native species as plants can form dense floating mats of vegetation that crowd out native species (Renman, 1989; Minchin and Boelens, 2012). The serrated leaves of this invasive plant are sharp and endanger swimmers, fishermen and aquatic recreational activities (Toma, 2006; Obolewski and Strzelczek, 2009).

Little is known regarding herbivory of water soldier in North America, although field observations conclude that some herbivory occurs (Synder et al., 2016). Furthermore, molluscs including Amnicola linosa, Martonia sp. and Gyraulus circumstriatus have been observed on water soldier in the Trent River, however,
these species prey on periphyton and do not consume water soldier (Synder et al., 2016).

**Control of invasive water soldier**

Water soldier populations can be controlled by either chemical or mechanical removal. Experimental research conducted at the University of Florida treated water soldier plants from the Trent River with diquat, a chemical herbicide, at concentrations ranging from 0 to 370 μg L⁻¹ with exposure times varying from 2 to 24 hours (Netherland, 2012). A minimum of 16 hours exposure time was required at the highest diquat concentration for total mortality of plants without regeneration. In 2009 and 2010, diquat was applied to water soldier in the Trent River. Treatment was most effective in shallow waters with low flow rates. On the contrary, treatment was least effective in deep waters with higher flow rates, where total mortality of adult plants was not achieved (Anonymous, cited in Snyder et al., 2016). In 2015, diquat was once more applied to water soldier populations in the Trent River, but this time using applicators mounted on fan boats to minimize turbidity during the process (R. McGowan, OFAH, pers. comm.). It is not yet known whether this method is more effective as results have not been evaluated. Manual removal of water soldier by hand-pulling was found to be inefficient in the Trent River. This control strategy was only moderately successful as plants were difficult to detect due to dense communities of other plants and low visibility caused by water turbidity (Anonymous, cited in Snyder et al., 2016). In October, 2015,
hundreds of plants were pulled from the Black River population, approximately 0.5 kilometres upstream from a dam in Sutton, Ontario. In November, 2015, the remaining water soldier plants were treated with chemical herbicide (diquat). The Black River was surveyed in June 2016 for signs of water soldier regrowth. Only two plants were discovered and pulled from the river. In July 2016, however, a new patch of plants was discovered upstream from the previous year’s patch. Manual removal of this patch began just two days after its discovery and an herbicide treatment was carried out in October 2016 (M. Shapiera, OMNRF, pers. comm.).

**Environmental DNA (eDNA)**

The Ontario provincial government and some of its partners are working to control or eradicate existing water soldier populations in Ontario. A main component of this plan consists of early detection and tracking of newly established populations. Early detection is key in the control and eradication of invasive species, in order to eliminate invaders before they become widely established (Vander Zanden et al., 2009). Environmental DNA (eDNA) screening has recently emerged as a tool for detecting aquatic species in early stages of invasion or establishment (Darling and Mahon, 2011; Jerde et al., 2011). eDNA ends up in the aqueous environment when it is sloughed off or excreted by dead or living organisms. This eDNA can be filtered and extracted from environmental samples such as water, and molecular markers can be used to determine whether some of the eDNA originated from a particular target species of interest. The presence of this DNA in the environment may indicate that the target species is (or was
recently) present in the sampled habitat. This method has been used to detect endangered native species (Thomsen et al., 2012b; Boothroyd et al., 2016) and has also been used for surveillance and early detection of aquatic invasive species (Ficetola et al., 2008; Jerde et al., 2011; Mahon et al., 2013; Takahara et al., 2013). eDNA detection has mostly been conducted on animals (Ficetola et al., 2008; Takahara et al., 2012; Deiner and Altermatt, 2014; Díaz-Ferguson et al., 2014; Rees et al., 2014; Turner et al., 2014; Pilliod et al., 2014; Wilson et al., 2014; Amberg et al., 2015; Boothroyd et al., 2015; Hunter et al., 2015; Dougherty et al., 2016; Eichmiller et al., 2016; Tucker et al., 2016), but a small number of studies have begun targeting invasive plant eDNA (Scriver et al., 2015; Fujiwara et al., 2016; Matsuhashi et al., 2016; Newton et al., 2016).

**eDNA studies**

The high sensitivity of eDNA surveillance allows researchers to detect species at low densities or abundances without actually having to capture or directly encounter the target species (Thompson, 2004; Thomsen et al., 2012b; Janosik and Johnston, 2015; Laramie et al., 2015; Sigsgaard et al., 2015). As a result, less time is spent searching for target species in the field compared to traditional monitoring methods, which may reduce project costs (Ficetola et al., 2008; Dejean et al., 2011, Sigsgaard et al., 2015).

Although eDNA detection can be highly sensitive, less time consuming and more inexpensive than traditional monitoring methods, limitations to the method
exist. Detection of eDNA does not provide biomass of the eDNA source nor the distance which eDNA may have traveled downstream, however, eDNA studies have investigated these variables (Takahara et al., 2012; Thomsen et al., 2012a; Pilliod et al., 2013; Deiner and Altermatt, 2014; Eichmiller et al., 2014; Janet et al., 2014; Kelly et al., 2014; Lamarie et al., 2015). Other factors that affect eDNA results include the rate of eDNA degradation (Thomsen et al., 2012a,b; Goldberg et al., 2013; Barnes et al., 2014), the optimal number of samples and volume of water to collect (Thomsen et al., 2012b; Mächler et al., 2015), the different sources from which eDNA can be derived (Ficetola et al., 2008; Jerde et al., 2011; Thomsen et al., 2012b; Merkes et al., 2014), the optimal method for eDNA filtering and extraction (Eichmiller et al., 2016; Minamoto et al., 2016) and polymerase chain reaction (PCR) inhibition (Gibson et al., 2012; Hunter et al., 2015; McKee et al., 2015; Renshaw et al., 2015). Studies on these factors either yield conflicting results, vary between species and habitats being sampled or are limited in number. Consequently, there is no universal method to determine these factors for all eDNA studies.

**Biomass quantification**

eDNA studies have explored the relationship between biomass and eDNA concentration of a target species. These studies have mostly been conducted by sampling controlled environments with known quantities of target biomass, and other variables (flow rate, sampling distance, etc.) held constant. For example, Takahara et al. (2012) sampled water from aquaria holding 1, 5 and 10 common carp (*Cyprinus carpio*), and found that eDNA concentration increased with fish
abundance. Furthermore, a lake study of common carp suggested the rate of detection and eDNA concentration positively correlated with fish abundance (Eichmiller et al., 2014). Similar correlations have also been shown with amphibian species in streams (Pilliod et al., 2013), ponds (Thomsen et al., 2012a), and mesocosms (Kelly et al., 2014). However, limited field-based surveys have suggested that eDNA abundance positively correlates with the target species’ density and biomass (Takahara et al., 2012, 2013).

**Downstream displacement of eDNA**

Several studies have also investigated the distance that eDNA can travel through aquatic habitats. These studies are done by collecting water samples in regular intervals away from an eDNA source. For example, Deiner and Altermatt (2014) collected water samples from eleven sites in regular intervals downstream from a population of *Daphnia longispina*. The eDNA of these macroorganisms was detected up to 12 kilometres downstream from their population. Although eDNA is capable of downstream transport, Lamarie et al. (2015) were unable to find a consistent relationship between distance traveled and eDNA concentration while quantifying eDNA of Chinook salmon (*Oncorhynchus tshawytscha*). This lack of a relationship is likely partially explained by the varying flow rates in each stream, as Janet et al. (2014) found that eDNA travels further with higher flow rates. Furthermore, Klymus et al. (2015) showed that dilution also occurs with higher flow rates as more eDNA gets flushed away.
Rate of eDNA degradation

In addition to examining the distance eDNA travels through aquatic habitats, recent studies have examined the amount of time that eDNA can remain detectable in habitats. These studies are conducted by removing target organisms from controlled environments and monitoring the persistence of target eDNA over time. Most of these studies have been conducted in a laboratory setting. For example, eDNA became undetectable beyond 0.9 day and 6.7 days for each of two fish species in aquaria (Thomsen et al., 2012a). In a similar aquarium experiment, the probability of common carp eDNA detection was <5% after four days of removing the fish (Barnes et al., 2014). eDNA remained detectable for up to 21 days after removing New Zealand mudsnails (*Potamopyrgus antipodarum*) from aquaria (Goldberg et al., 2013). In addition to indoor aquarium experiments, other studies have quantified the persistence of eDNA in outdoor environments. For example, Thomsen et al. (2012b) reported that eDNA of two amphibians persisted 7-14 days in an outdoor mesocosm following removal of the organisms.

Number and volume of eDNA samples

Studies have evaluated the number and volume of water samples to collect for eDNA extraction. The number of samples taken per site varies across studies; however, collecting three samples per site is a relatively common number of replicates (Ficetola et al., 2008; Dejean et al., 2011; Takahara et al., 2012; Thomsen et al., 2012b; Hunter et al., 2015). Thomsen et al. (2012b) examined the probability
of detecting the target species with up to three samples collected from each site, and found that detection probabilities were significantly higher when based on three versus only one or two samples per site. In addition, Mächler et al. (2015) examined the effect of sampled water volume (0.25L to 2L) on the detection rates of three macroinvertebrate species. Although all three species were detected in all volumes of water, only one species showed a positive relationship between increased detection rate and increased sampled water volume. Consequently, Mächler et al. (2015) suggested the optimal sample volume may depend on the target species and habitats being sampled.

**eDNA tissue sources**

Limited eDNA research has described the various eDNA tissue sources, and eDNA assays are unable to differentiate one tissue source from another. For example, sources of eDNA include feces (Thomsen et al., 2012b), bodily fluids such as slimy coatings on fish (Jerde et al., 2011) and amphibians (Ficetola et al., 2008). In addition, Merkes et al. (2014) revealed that dead carcasses and predator feces may even act as an eDNA source. More recently, eDNA of aquatic macrophytes has been detected using PCR assays (Scriver et al., 2015; Fujiwara et al., 2016; Matsuhashi et al., 2016; Newton et al., 2016). However, the specific part of the plant (i.e., leaf, stem, seeds, flowers, etc.) from which the eDNA was sloughed cannot be determined with eDNA detection.
Filtration and extraction methods

Studies have compared methods of extraction and filtration for detection and quantification of eDNA. Eichmiller et al. (2016) compared six commercially available DNA extractions kits for their ability to detect common carp eDNA from water. Of the six DNA extraction kits tested, MoBio PowerSoil DNA Isolation Kit was recommended for aquatic eDNA studies as it had the least amount of variation in extraction efficiency and showed no signs of inhibition. In another study, Minamoto et al. (2016) compared a variety of extraction, isolation and preservation steps using field water samples. They proposed that the chosen method for eDNA analysis should be based on context. For example, ethanol precipitation was most advantageous when a high concentration of target DNA was expected. However, when target DNA was expectedly rare, filtration followed by DNA extraction using phenol and ethanol were most successful. They also suggested that filter type should be chosen based on the characteristics of the water to be analyzed. For example, 1.5 μm pore-sized, glassfiber (GF) filters were most effective when sediment was present in water samples. However, 0.7-μm GF filters were fragile and clogged easily by sediment but yielded higher eDNA copy numbers if sediment was limited. Furthermore, Eichmiller et al. (2016) examined various types of filters and found that Glassfibre filters performed better than similar pore sized polycarbonate (PC) filters and 1.5 μm pore-sized GF filters were recommended for eDNA detection.
**Polymerase chain reaction (PCR) inhibition**

In addition to filtration and extraction methods, eDNA studies have reported techniques to reduce PCR inhibition in environmental DNA samples. This is an important procedure to address as PCR inhibition can prevent eDNA detection (Gibson *et al.*, 2012). Many studies have reported spiking samples with internal positive controls (IPCs) to gauge the presence of inhibitors (Hunter *et al.*, 2015; McKee *et al.*, 2015; Sigsgaard *et al.*, 2015; Spear *et al.*, 2015; Turner *et al.*, 2015; Boothroyd *et al.*, 2016). However, limited research has addressed methods to overcome PCR inhibition. For example, post-extraction purification (phenol-chloroform, silica-based columns) (McKee *et al.*, 2015; Renshaw *et al.*, 2015) and diluting DNA samples has been shown to reduce inhibition (McKee *et al.*, 2015). In addition, using quantitative PCR (qPCR) versus conventional PCR may alleviate amplification failure, as qPCR is less sensitive to inhibitors (Gibson *et al.*, 2012).

eDNA detection can be highly sensitive (Thompson, 2004; Thomsen *et al.*, 2012b; Janosik and Johnston, 2015; Laramie *et al.*, 2015; Sigsgaard *et al.*, 2015), less time consuming and more inexpensive than traditional monitoring methods (Ficetola *et al.*, 2008; Dejean *et al.*, 2011, Sigsgaard *et al.*, 2015). Despite some potential limitations and uncertainties, eDNA is a promising new tool that could greatly improve the detection of invasive aquatic species.
Species-specific markers

eDNA research either involves targeting one or two focal species through eDNA assays, or multiple species (metabarcoding). This paper will focus on the former, which requires the development of species-specific markers that will only amplify DNA of the target organism. Animal eDNA studies usually utilize markers which amplify DNA from mitochondrial DNA (mtDNA) barcoding regions cytochrome b, 12sr RNA and COI (e.g. Deiner and Altermatt, 2014; Piaggio et al., 2014). For plants, chloroplast DNA (cpDNA) segments are recommended for barcoding because of the high copy number of chloroplast DNA within cells (Thomas, 2013; Ali et al., 2014). The Consortium for the Barcode of Life (CBOL) proposed that chloroplast DNA regions rbcL and matK be used for plant barcoding (Little, 2009). A study conducted by Ghahramzadeh et al. (2013) suggested the trnH-psbA noncoding spacer be used for barcoding aquatic plants, as it was the best performing barcode in a study which used markers to differentiate among closely related aquatic plant species. Barcoding markers are not necessarily the most appropriate for developing species-specific eDNA primers, for example matK was found to be more useful than trnH-psbA or rbcL for designing species-specific markers for aquatic plants (Scriver et al., 2015). To date, few studies have detected aquatic plant eDNA in the field. One study used the region of an intergenic spacer between trnL and trnF in chloroplast DNA (Fujiwara et al., 2016). This same study successfully detected eDNA of Brazilian waterweed (Egeria densa) in five ponds, confirming that the detection of aquatic plants by eDNA analysis is possible. Another study used the internal transcribed spacer (ITS) region of ribosomal DNA to
detect Eurasian watermilfoil (*Myriophyllum spicatum*) (Newton *et al.*, 2016). The ITS locus region was used to design primers as it contains many polymorphisms that distinguish watermilfoil species. This study detected Eurasian watermilfoil in two water bodies (Half Moon Lake, Michigan, USA and Jefferson Slough, Montana, USA) where the plant was known to occur. A third study developed a primer/probe set for *Hydrilla verticillata* using *matK* sequences and successfully detected eDNA in five of 21 ponds in Japan (Matsuhashi *et al.*, 2016).

In this project, the feasibility of inferring the presence or absence of invasive aquatic water soldier from eDNA was investigated. The goals of this study were to (1) determine whether species-specific markers could be developed for water soldier from existing databases, (2) determine whether an invasive aquatic plant species can be detected in the wild using eDNA surveillance, (3) compare methods of eDNA assays to determine the most sensitive method of water soldier eDNA detection, and (4) map positive water soldier detections from the Trent-Severn Waterway. This study is useful to environmental managers dealing with the threat of water soldier and other invasive plants. Sensitive detection of water soldier eDNA may unveil sites at the early invasion stage (i.e., before plants have been observed), facilitating their eradication before they develop into patches that are not as easily controlled. If detection of water soldier DNA from an environmental sample is successful, then these methods could be adapted for other invasive aquatic plants and be an important tool for early detection of aquatic invasive plants species, as well as assisting with informing control or eradication efforts.
Methods

A) eDNA marker development and validation

(i) Primer design

GenBank (www.ncbi.nlm.nih.gov/genbank) was searched for *rbcL*, *matK* and *trnH-psbA* chloroplast gene region sequences belonging to water soldier and a range of potentially sympatric plant species that occur in or near the Trent River: coontail (*Ceratophyllum demersum*), curly-leaf pondweed (*Potamogeton crispus*), flatstem pondweed (*Potamogeton zosteriformis*), Canadian waterweed (*Elodea canadensis*), tape grass (*Vallisneria americana*), Richardson’s pondweed (*Potamogeton richardsonii*), Eurasian watermilfoil (*Myriophyllum spicatum*), common bladderwort (*Utricularia vulgaris*), variegated pond-lily (*Nuphar variegata*), water marigold (*Megalodonta beckii*), nodding waternymph (*Najas flexilis*), water stargrass (*Heteranthera dubia*), large-leaf pondweed (*Potamogeton amplifolius*), lesser pondweed (*Potamogeton pusillus*), star duckweed (*Lemma trisulca*), fragrant water-lily (*Nymphaea odorata*), variable-leaf watermilfoil (*Myriophyllum heterophyllum*), Siberian watermilfoil (*Myriophyllum sibiricum*), longbeak buttercup (*Ranunculus longirostris*), and Robbin’s pondweed (*Potamogeton robbinsii*). As water soldier has no congeneric species, sequence comparisons were therefore made between potentially sympatric species from seven different plant families, thereby increasing the likelihood of finding species-specific mutations in regions suitable primer design.
Sequences for \textit{rbcL}, \textit{matK} and \textit{trnH-psbA} gene regions belonging to \textit{M. beckii}, \textit{H. dubia} and \textit{R. longirostris} were unavailable in GenBank and therefore not included in the primer design. Of the remaining seventeen plant species, \textit{matK} sequences were unavailable for two plant species, \textit{rbcL} sequences were unavailable for one plant species, and \textit{trnH-psbA} sequences were unavailable for eight of the plant species (Appendix A).

Since relatively few \textit{trnH-psbA} sequences were available, primer design focused on the \textit{rbcL} and \textit{matK} gene regions. Sequences for \textit{rbcL} and \textit{matK} were separately aligned using ClustalX and compared in BioEdit to identify nucleotide sequences that occurred only in water soldier. Each primer was designed manually, contained 18-28 base pairs and contained a minimum of two nucleotide mismatches with all potentially sympatric plant species that were included in the alignment. In addition, primers were designed with non-complementary 3’ ends, a maximum of two cytosine (C) or guanine (G) bases at the 3’ end, a GC-content of approximately 50%, melting temperatures between 55-80°C and a maximum melting temperature difference of 2°C between the forward and reverse primers of primer pairs (Table 1).
**Table 1.** Gene region, primer name, primer sequence, product size (number of base pairs), melting temperature ($T_m$) and annealing temperature ($T_a$) of six primer pairs developed to detect water soldier from environmental DNA.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Sequence (Forward; Reverse)</th>
<th>Product size (#bp) *</th>
<th>$T_m$ (F)</th>
<th>$T_m$ (R)</th>
<th>$T_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>rbcL</strong></td>
<td>Sal_rbcL1</td>
<td>F- TAACTCCGCAACCTGGAGTTC; R- CACGTACCTGCAGTAGCATTC</td>
<td>574</td>
<td>64°C</td>
<td>64°C</td>
<td>59°C</td>
</tr>
<tr>
<td></td>
<td>Sal_rbcL2</td>
<td>F- CGTTACTGGGGAGGAAGATC; R- TCACACGTACCTGCAGTAGC</td>
<td>437</td>
<td>62°C</td>
<td>62°C</td>
<td>53°C</td>
</tr>
<tr>
<td></td>
<td>Sal_rbcL3</td>
<td>F- TCTTCCACTGTCATGGAC; R- CACACGTACCTGCAGTAGC</td>
<td>542</td>
<td>60°C</td>
<td>60°C</td>
<td>54°C</td>
</tr>
<tr>
<td><strong>matK</strong></td>
<td>Sal_matK1</td>
<td>F- ATTCTACAATCCCGTATAAAGATG; R- GGTTCTCTTTTGAAGCGAGGATTG</td>
<td>359</td>
<td>66°C</td>
<td>68°C</td>
<td>59°C</td>
</tr>
<tr>
<td></td>
<td>Sal_matK2</td>
<td>F- TTCTCCGTAGACATTCCCTCTATTTAC; R- GCTTTGACAATGATCCAATTGAGG</td>
<td>407</td>
<td>74°C</td>
<td>72°C</td>
<td>69°C</td>
</tr>
<tr>
<td></td>
<td>Sal_matK3</td>
<td>F- CAAATTCTACAATCCGTATAA; R- CCTTGGTATCTAACATAATGC</td>
<td>316</td>
<td>58°C</td>
<td>58°C</td>
<td>53°C</td>
</tr>
</tbody>
</table>

* Not including primers
(ii) Testing and validation

Plant samples collection

In the summer of 2013, a water soldier plant was collected from the Trent River, Ontario. In addition, thirteen potentially sympatric plant species in southern Ontario were collected from elsewhere in the Trent-Severn Waterway (Lakefield, ON or Peterborough, ON), or from commercial retailers: fragrant water lily (*Nymphaea odorata*), coontail (*Ceratophyllum demersum*), Canadian waterweed (*Elodea canadensis*), tape grass (*Vallisneria americana*), curly-leaf pondweed (*Potamogeton crispus*), large-leaf pondweed (*Potamogeton amplifolius*), broadleaf cattail (*Typha latifolia*), narrowleaf cattail (*Typha angustifolia*), common reed (*Phragmites australis*), broad-leaf arrowhead (*Sagittaria latifolia*), Carolina fanwort (*Cabomba caroliniana*), frogbit (*Hydrocharis morsus-ranae*), and water chestnut (*Trapa natans*).

Extraction

Frozen plant tissue from each sampled plant (approximately 50 mg per plant) was powdered in a pestle and mortar after adding liquid nitrogen. DNA was extracted from the ground plant material using the fresh/frozen protocol for the E.Z.N.A. plant DNA kit (Omega Biotek). DNA extractions were eluted in 200 μL of ddH₂O and visualized on a 1.5% agarose gel.
**Water soldier testing**

Amplification of water soldier DNA using the designed primer pairs (Table 1) was optimized using a temperature gradient for primer annealing during PCR. This was achieved by setting a 10°C gradient and setting the annealing temperature at 5°C below the calculated melting temperature \[T_m = 4(G/C) + 2(A/T)\]. All thermal gradient PCRs were carried out using an Eppendorf Mastercycler. Amplifications were done in 20 μL reaction mixtures which included 1x Taq reaction buffer (UBI Life Sciences), 3 mM MgCl₂, 0.2 mM dNTPs, 0.6 μM forward and reverse primer, 2U Taq (UBI Life Sciences) and ~2 ng water soldier DNA. The thermal cycling parameters were 96°C for 5 minutes, followed by 34 cycles of 96°C for 30 seconds, the marker-specific annealing temperature (\(T_a\); Table 1) for 30 seconds and 72°C for 1 minute. All six primer pairs successfully amplified water soldier DNA, and the temperature which resulted in the strongest, brightest bands without secondary bands or smears on a 1.5% agarose gel was selected as the optimal annealing temperature.

**Sympatric plant species testing**

Since all of the designed primers amplified water soldier DNA, they were further tested on DNA extractions from the thirteen sampled potentially sympatric plant species (see above), to determine non-target amplification. Two rbcL (Sal_rbcL1, and Sal_rbcL3) and one matK (Sal_matK2) primer pairs amplified DNA from all thirteen of the non-target sympatric plant species, whereas one rbcL
(Sal_rbcL2) and two matK (Sal_matK1 and Sal_matK3) primer pairs did not amplify non-target sympatric plant species DNA. Consequently, the latter three primer pairs (Sal_rbcL2, Sal_matK1, Sal_matK3) were retained for further assessment via qPCR.

**Development of qPCR standards**

The number of DNA copies/5 μL of DNA was determined via absolute quantification using the standard curve method. This required the development of DNA standards to quantify the number of DNA copies in environmental samples. Primer pairs Sal_matK1, Sal_matK3 and Sal_rbcL2 were used to amplify extracted water soldier DNA using conventional PCR as previously described. The PCR product was quantified using a Picogreen plate (BMG FluoStar Galaxy 96-well plate system). The volume of extracted water soldier DNA containing $10^{10}$ DNA copies/reaction was quantified, based on the Picogreen reading and the molecular weights for the different rbcL and matK PCR products. A 10-fold serial dilution was performed on the volume of amplicons containing $10^{10}$ copies down to $10^0$ DNA copies. This was done by first adding 10μL of $10^{10}$ copies/reaction to 90 μL of low TE (TE Buffer, 1X Solution pH 8.0, low EDTA) and thoroughly mixing to achieve $10^9$ DNA copies/reaction. Then, this step was repeated until a concentration of $10^0$ copy/reaction was attained. Diluted samples ranging from $10^6$ to $10^0$ DNA copies were used as qPCR standards.

In qPCR assays, a positive sample is detected by an accumulation of fluorescent dye which bind to DNA. The cycle threshold (Ct) is the cycle number at
which the fluorescent signal crosses the fluorescent threshold, which is the level of fluorescence significantly above background fluorescence (Arya et al., 2014). A standard curve was developed by plotting the known DNA copy numbers against the cycle at which the signal passed the C\textsubscript{T}. Cycle numbers were then used to determine the number of DNA copies present in environmental samples. Two sets of DNA standards (i.e. two dilution series of positive control DNA) were ran with each qPCR assay.

**qPCR reactions**

Reaction cocktails consisted of 1 X Environmental Master Mix (Life Technologies), 0.2 μM of each primer, 5 μL of DNA, and deionized water in a total volume of 20 μL. Optimized cycling conditions consisted of an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and copy replication at 60°C for 1 minute.

**(iii) eDNA testing**

To detect water soldier eDNA using putatively species-specific primers (Sal_matK1, Sal_matK3 and Sal_rbcL2), positive controls of known water soldier eDNA were developed. Nine positive controls were obtained by placing approximately 10 grams of water soldier tissue into each of nine 1L Mason jars of deionized water and held at 4°C. After 24 hours, the water was filtered.
**Filtering**

Prior to filtering, lab equipment was soaked in a 10% bleach solution for five minutes and then triple rinsed in deionized water. Samples were filtered through GF/C Whatman 47 mm glass microfiber filters (1.2 μm pore size) using a filtering manifold attached to an EZ-Stream Pump (EMD Millipore). Filtering funnels were soaked in a 10% bleach solution for at least two minutes and then triple rinsed with deionized water between each sample. If filters became clogged due to sediment or debris in a sample, two filters were used to filter a single sample. Filters from the same 1L water sample were stored together. Funnel controls were made by filtering deionized water through funnels before and after filtering environmental samples. Filters were each stored in either a petri dish or a 1.5 mL centrifuge tube at -20°C or -80°C.

**Extractions**

To determine the optimal approach for water soldier eDNA extraction, two commercial DNA extraction kits, the MoBio Power Water extraction kit and the Qiagen DNeasy Blood and Tissue kit, were tested on positive eDNA controls. The Qiagen DNeasy Blood and Tissue kit protocol was modified by 1) adding 200 μL ATL buffer after proteinase K incubation, followed by vortexing (X. Guan, US Army Corps of Engineers, pers. comm.) and 2) eluting eDNA in two steps by adding 100 μL AE buffer twice (i.e. in two separate steps) instead of adding 200 μL Buffer AE to the spin column membrane at once. Three positive control filters were extracted using the Qiagen DNeasy Blood and tissue kit with the modified protocol and three
positive control filters were extracted using the MoBio Power Water extraction kit. DNA extractions were visualized on a 1.5% agarose gel. The MoBio Power Water extraction kit failed to extract eDNA from the positive control filters. However, the Qiagen DNeasy Blood and Tissue kit successfully extracted water soldier eDNA using the modified protocol. Three remaining positive control eDNA filters were subsequently extracted with the Qiagen DNeasy Blood and Tissue kit following the above-mentioned modifications. The presence of eDNA was verified on a 1.5% agarose gel.

**Extraction purification**

To determine which yields best results for PCR amplification of water soldier eDNA, three positive control Qiagen DNeasy Blood and Tissue kit eDNA extractions were purified and compared to three unpurified positive control Qiagen eDNA extractions. This was done to determine if post-extraction purification would remove PCR inhibitors that may inhibit water soldier eDNA detection. Each of three positive control eDNA extractions were purified using phenol/chloroform/isoamyl alcohol (PCI: 25:24:1; pH 6.7± 0.2) and chloroform washes followed by an ethanol precipitation (Sambrook and Russell, 2001). To begin, an equal volume of phenol/chloroform/isoamyl alcohol was added to each extraction. The tube was vortexed for approximately 10 seconds and then spun at 13000 g for 3 minutes. The aqueous layer was removed and pipetted into a new 1.5 mL centrifuge tube. This was repeated two more times, for a total of three phenol/chloroform/isoamyl alcohol washes. Next, an equal volume of chloroform was added to each tube. Each tube
was vortexed for approximately 10 seconds and then spun at 13000 g for 3 minutes. The aqueous layer was removed and pipetted into a new 1.5 mL centrifuge tube. This was repeated once more, for a total of two chloroform washes per extraction. For the ethanol precipitation, 1/10 the volume of sodium acetate (pH 5.2, 0.3 M) was added and mixed well. Then, two and half volumes of cold 100% ethanol (volume calculated after salt addition) was added and mixed well. Each tube was subsequently placed in the -20°C freezer. After 20 minutes, each tube was spun at maximum speed (21000g) for 15 minutes. The supernatant was decanted, 1 mL of cold 70% ethanol was added, mixed, spun briefly, and the supernatant was carefully decanted once more. Each tube was air dried and the pellet was suspended in 50 µL of deionized water.

Three purified and three unpurified positive control extractions were amplified using conventional PCR with putatively species-specific primers Sal_rbcL2, Sal_matK1 and Sal_matK3, using the PCR conditions described above. PCR product was visualized on a 1.5% agarose gel. Since best results (see results) were achieved with post-extraction phenol-chloroform washes, all subsequent eDNA extractions were purified in this manner.
B) Field application

i) Advanced preparation

Before sample collection, 1L Mason jars, transportation coolers and a collection snare pole were soaked in a 10% bleach solution for five minutes and then triple rinsed with deionized water. For each cooler, one 1L jar was randomly selected to act as a cooler control. This jar was filled with deionized water and then returned to the cooler.

ii) Sample collection

Collection of environmental water samples was done twice, in July and September of 2013. Field sampling protocols followed the University of Notre Dame Environmental DNA Monitoring and Surveillance Standard Operating Procedures (Mahon et al., 2010). During each sampling interval, all samples were collected over a five day period.

Four 1L water samples were collected at each of 40 sites upstream, downstream, and within the major area of water soldier infestation in Seymour Lake, Ontario (Figure 2). All four samples at each site were collected at the same location and approximately one foot below the surface of the water. Twenty of the 40 sampling sites were at locks in the Trent-Severn Waterway System. Fifteen of the lock sampling sites were located around Seymour Lake, the major area of water soldier infestation in the Trent-Severn System (Figure 2). Ten sites in Seymour Lake
and the Trent River spaced out by approximately 2 km were sampled and six sites in the Trent River upstream of Seymour Lake, also approximately 2 km apart, were sampled. The remaining four sites were in Rice Lake, located upstream from Seymour Lake (Figure 2). Sites were chosen prior to sample collection. Each site was saved as a waypoint into a Garmin Etrex 30 global positioning system (GPS). The same device was used to locate sampling sites during sample collection. The GPS coordinates of all 40 sample sites are in Appendix B.

Fresh nitrile gloves were worn during sample collection at each site. Lock samples were collected below each lock by leaning out from the side of the lock and filling the 1L mason jars with water. Trent River and Seymour Lake samples were collected in a downstream to upstream fashion, from a boat and using a snare pole to reach away from the gunwale. The snare pole was rinsed in a 10% bleach solution between sites. Once a sample was collected, it was placed in a cooler containing ice packs. At a randomly chosen site where sampling occurred, the cooler control jar was opened, resealed, submerged in water, and returned to the cooler.

**iii) Sample processing**

Collected samples were stored in the refrigerator (4°C) and filtered as described above within 24 hours to minimize DNA degradation. For samples, each 1L sample. Filters from July samples were stored in a petri dish at -20°C and
Figure 2. Sampling sites in Trent-Severn Waterway and adjoining water bodies from Nassau Mills Lock 22, Peterborough, ON downstream to Trenton Lock 1, Quinte West, ON. Sampling sites were heavily concentrated around Seymour Lake, the area with known water soldier patches. Black spheres represent sampling sites. Numbers indicate navigational locks.
filters from September samples were stored in a 1.5 mL centrifuge tube at -80°C.

Multiple filters from the same 1L water sample were stored together. Once field water samples were filtered, filters were extracted using the Qiagen DNeasy Blood and Tissue kit following the above mentioned modifications to the standard protocol. Each extraction was done on a single filter, and eDNA extractions were subsequently combined if they were from the same 1L water sample which required multiple filters. eDNA extractions were purified with PCl and chloroform washes and ethanol precipitation as described above. July eDNA samples were stored at -20°C for several months before being stored at -80°C and September samples were stored at -80°C. The presence of eDNA was verified on a 1.5% agarose gel.

Samples from Seymour Lake, the area with known water soldier populations, were assayed in triplicate for water soldier eDNA using conventional PCR and including a positive control which contained ~2 ng water soldier DNA and a negative control with no template DNA. Markers Sal_matK1, Sal_matK3 and Sal_rbcL2 were used following the previously described PCR protocol. The presence of amplified eDNA was verified on a 1.5% agarose gel, and successful amplifications were re-amplified and visualized in the same way to check for consistency of amplification.

Since conventional PCR was inconsistent in detecting eDNA (see Results), qPCR was used to assay all eDNA samples. To avoid contamination, pipettes, pipette
tips, ddH$_2$O, tubes and PCR plates were subjected to UV light for fifteen minutes prior to use. In addition, filtered pipette tips were utilized to prevent contaminated pipettes from contributing template to reactions. To determine which set of markers most efficiently amplified target DNA during qPCR, two sets of qPCR standards (i.e. serial dilutions of target DNA ranging from $10^6$-$10^0$ copy numbers as previously described) and two wells of no-template negative controls were assayed using primer pairs Sal_matK1, Sal_matK3 and Sal_rbcL2. Samples from which positive detections were expected (i.e., samples collected from Seymour Lake, area of known water soldier population) were assayed in triplicate (i.e., each sample was subjected to three qPCR assays), alongside two sets of qPCR standards and two wells of no-template negative controls using markers Sal_matK1 and Sal_rbcL2 following the cycling parameters described above. Subsequent eDNA assays of all field and control samples were based on Sal_rbcL2 primers, and all qPCR reactions were performed in triplicate.

The data for the raw qPCR values across all sites are provided in Appendix C.

**iv) Determining a detection threshold**

Determining whether a sample actually yields positive qPCR detection requires a detection threshold to clearly distinguish between positive and negative results (Darling and Mahon, 2011). This is necessary because the high sensitivity of qPCR can often pick up DNA copy numbers in negative samples that are a result of low levels of contamination or non-target DNA in environmental samples if the
similarity between target and non-target sequence is relatively high (Darling and Mahon, 2011). In this study, the minimum detection threshold was set just above the maximum copy number of DNA that was obtained from 28 negative qPCR controls (controls with which DNA should have no contact; see above). In addition, this value was well below DNA copy numbers that were obtained from all samples that were expected to generate positive results (see Results). Positive detections were then defined as samples that amplified a number of DNA copies that met or exceeded the determined minimum detection threshold.

v) Mean copy number

Mean copy number values were obtained by calculating the average of qPCR replicates for each site and then the averages across field replicates. Positive detections that were more than 11-fold greater than any other positive detection were omitted from the data, as they were likely the result of contamination (described in Results).

vi) Sequencing

A subset of samples (n=10) with positive qPCR detections, including at least one sample from each site with positive qPCR detections, was amplified using conventional PCR. Unincorporated primers and dNTPs were removed by incubating with 10U exonuclease I and 2U of shrimp alkaline phosphate for 15 minutes at 37°C followed by 15 minutes at 80°C. The cleaned product was sequenced in both directions using the amplification primers and Big Dye 3.1 cycle sequencing (ABI) and
then run through an ABI 3730 DNA Analyser (Applied Biosystems). The closest match for each sequence was identified using the BLAST tool on GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi).
Results

Two *matK* (*Sal_matK1* and *Sal_matK3*) and one *rbcL* (*Sal_rbcL2*) primer pairs amplified only target DNA, i.e., they generated DNA amplicons of the expected size from water soldier DNA extractions and not DNA extractions from sympatric plant species. All markers that successfully amplified product from water soldier tissue extractions also amplified positive water soldier eDNA controls using conventional PCR.

The *Sal_matK3* primers failed to amplify qPCR standards (positive DNA controls) at all concentrations ranging from $10^6$ to $10^0$ DNA copies per reaction. The *Sal_rbcL2* and *Sal_matK1* primers both amplified qPCR standards with an optimal efficiency of 106% and 80%, respectively.

Gel electrophoresis failed to identify any DNA following MoBio Power Water extraction kit, while bands were evident from eDNA samples that were extracted using the Qiagen DNeasy Blood and Tissue kit using the modified protocol (see methods).

PCR products from eDNA purified with phenol-chloroform extractions after Qiagen extractions produced brighter bands at the expected fragment length than Qiagen extractions that were not subjected to phenol-chloroform cleanup on a 1.5% agarose gel.
Using any of the three species-specific markers, conventional PCR inconsistently detected eDNA in field samples collected from Seymour Lake, the area with known water soldier populations. Using conventional PCR, only one or two (out of four) sample replicates collected in Seymour Lake yielded positive detections. Furthermore, samples which resulted in positive detections were re-assayed three to five times, only to fail and not amplify (i.e., complete failure after first run). Using qPCR, Sal_matK1 primers amplified qPCR standards but failed to detect eDNA in Seymour Lake samples. However, Sal_rbcL2 primers successfully amplified qPCR standards as well as Seymour Lake samples. In addition, three out of five sites in Seymour Lake had at least two of the four sample replicates yield positive results. Therefore, Sal_rbcL2 primers were used to quantify all field and control samples.

DNA copy numbers ranged from 0-3.976 per reaction in qPCR negative controls that were generated by running qPCR without including template DNA. The most frequent number of DNA copies found in qPCR negatives was 0 (Figure 3). Since the maximum number of DNA copies found in any qPCR negative was 3.976, the positive detection threshold was set at 4 DNA copies/5 μL of DNA extraction. Detections were defined as samples that amplified at or above the 4 DNA copies/5 μL of DNA extraction detection threshold (Figure 4).
Figure 3. Count of DNA copy numbers detected in qPCR negatives.
Figure 4. Positive qPCR results shown as mean estimated target DNA copy number/5 µL and associated standard error (+ SE). SL refers to the known water soldier population area (Seymour Lake), while Lock 5 represents Trent Lock 5, approximately 50 km downstream of Seymour Lake and 15 km upstream of the Bay of Quinte, Lake Ontario. The red dashed line represents the positive threshold cutoff value of 4 DNA copies/5 µL.
Water soldier DNA was quantified above the minimum detection threshold from samples collected at six of the forty sampling sites (15% of sites) in September, 2013 (Figure 4). No positive detections were obtained from samples collected in July, based on the predefined minimum detection threshold. Positive water soldier detections were found at sites 1-5 within the Seymour Lake sampling location and at Trent Lock 5 (Figure 5; Figure 6). No detections were obtained downstream at sites 6-10 in Seymour Lake, nor upstream at sites 1-10 in the Trent River. One set of qPCR triplicates from one extraction of a water sample taken at site 2 (SL2) in September with a mean value of 4057.206 DNA copies/5 μL were omitted. This is because the mean estimated target DNA copy number/5 μL was over 11-fold greater than any mean estimated target DNA copy number/5 μL and over 110-fold greater than the second largest mean estimated target DNA copy number/5 μL from SL2. This does not seem plausible and likely reflects contamination. However, three of the four samples and their qPCR replicates from site 2 were included in the results.

Copies of target DNA were detected at 5 sites outside the Seymour Lake sampling location including a positive detection obtained from Trent Lock 5. Copies of target DNA were obtained from Locks 19, 18, 13 and at site 1 in Rice Lake (Appendix C), however, these values were discarded as they were below the cutoff for accepting data as positive detections (4 DNA copies/5 μL DNA).
Figure 5. Positive and negative water soldier eDNA detections at sampling sites in Seymour Lake, Trent Hills, ON. SL= Seymour Lake and TR= Trent River.
Figure 6. Positive detection at Trent Lock 5 in Quinte West, Ontario, 15 kilometres north of the Bay of Quinte.
Variation in the number of DNA copies/5 μL was observed between sites, within sites (4 samples per site) and within samples (three qPCR replicates per sample). The maximum positive detection yielded from any site (excluding the single outlier described above), sample or qPCR triplicate was 506.795 DNA copies/5 μL obtained from Seymour Lake Site 3 while the minimum positive detection (above the minimum positive detection threshold) yielded from any site, sample or qPCR triplicate was 4.510 DNA copies/5 μL obtained from Trent Lock 5. Every positive detection had at least one out of four samples with all qPCR triplicates over the minimum detection threshold. Greater variation in positive qPCR detections was observed between and within sites when compared to variation within qPCR triplicates (Figure 7).

All sequenced eDNA positives had the closest match with the reference water soldier sequence from GenBank (www.ncbi.nlm.nih.gov/genbank; GenBank: HQ901565.1). The amplified sequence showed 99% homology with the reference water soldier sequence as four nucleotide mismatches existed between the two (0.92% sequence divergence).
**Figure 7.** Variation in the number of DNA copies/5 μL between sites, within sites and within samples from positive detections, with the outlier from site SL2.
removed. Sample refers to a water sample (a single jar that was filtered and extracted) and the three circles for each sample represent each of the three qPCR replicates (three superimposed circles reflect three identical qPCR replicates). The absence of DNA in a sample is shown as 0 DNA copies/5 μL. SL refers to the known water soldier population area (Seymour Lake), while Lock 5 represents Trent Lock, approximately 50 km downstream of Seymour Lake and 15 km upstream of the Bay of Quinte, Lake Ontario. Red dashed lines represent the positive threshold cutoff value of 4 DNA copies/5 μL.
Discussion

This study adds to the so far scant literature (Scriver et al., 2015; Fujiwara et al., 2016; Matsuhashi et al., 2016; Newton et al., 2016) on using eDNA to monitor invasive aquatic plant species. Species-specific PCR primers were designed for water soldier eDNA assays, and used to screen samples collected from a transect along the Trent-Severn Waterway spanning the site of an established population plus sites upstream and downstream where no water soldier has been observed. Quantitative PCR was a more effective method of detecting eDNA than conventional PCR. An expected amount of variation was found among samples between sites, within sites and between qPCR replicates. Positive samples were detected from sites with known water soldier populations in Seymour Lake, and were also detected from a site downstream where water soldier has not previously been identified. Despite the known limitations to detecting organisms from eDNA, this study has demonstrated the potential for using this tool to detect water soldier from eDNA and, by extension, additional invasive aquatic plant species.

Species-specific markers

Animal eDNA assays have targeted a range of mtDNA gene regions including cytochrome b, 12sr RNA and COI (e.g. Deiner and Altermatt, 2014; Piaggio et al., 2014). For plants, cpDNA segments including rbcL, matK and a trnH-psbA noncoding spacer have been recommended for barcoding (Little, 2009; Ghahramzadeh et al., 2013), however, there is no consensus on which cpDNA gene
regions are most suitable for eDNA studies as less attention has been given to developing plant eDNA techniques (Newton et al., 2016). To date, the number of eDNA studies that have used species-specific markers to detect aquatic plants from eDNA is limited. Two of these studies designed species-specific markers using cpDNA regions $matK$, $rbcL$, $trnH-psbA$ non-coding spacer and an intergenic spacer between $trnL$ and $trnF$ (Scriven et al., 2015; Fujiwara et al., 2016). A third study designed markers using the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (Newton et al., 2016). A study conducted by Fahner et al. (2016) evaluated the utility of ITS2, $matK$, $rbcL$ and the $trnL$ P6 loop DNA barcode regions for assessing the biodiversity of vascular plants from soil eDNA. The researchers concluded that using both $rbcL$ and ITS2 in metabarcoding was the most suitable as using markers from different linkage groups aids in resolution and both regions are supported by ongoing reference database development through Barcode of Life initiatives (Fahner et al., 2016).

In this study, we attempted to design water soldier species-specific markers using cpDNA gene regions $matK$, $rbcL$ and $trnH-psbA$. Species-specific markers using a $trnH-psbA$ non-coding spacer could not be developed as there was a lack of interspecific $trnH-psbA$ gene sequences available for comparison. Therefore, $trnH-psbA$ genes belonging to potentially sympatric plant species from the Trent-Severn Waterway would have to be sequenced before water soldier species-specific markers could be explored for that region. Three primer pairs from each of $matK$ and $rbcL$ gene regions were designed to anneal to sites that included mutations that
were putatively specific to water soldier. A set of \textit{rbcL} primers proved to be the most useful as they were the only primer pairs to successfully amplify water soldier eDNA from field samples using qPCR.

Although markers were designed to be species-specific in the Trent-Severn Waterway, researchers should keep in mind that this does not guarantee these markers will be species-specific in all locations. Regional studies must pay attention to sympatric plant species during the design and development of markers, and in pilot studies should sequence positive detections to ensure the amplified DNA fragments originate from the target species (Scr
ter \textit{et al.}, 2015). Furthermore, although water soldier in Ontario likely originated from a single source as there is no evidence of sexual reproduction, the genetic variation of water soldier \textit{cpDNA} has not been evaluated. Therefore, intraspecific polymorphisms may exist in geographical regions where there have been invasions by multiple genetic lineages. If so, primer pairs may not amplify some lineages if mutations have arisen in the primer binding sites. For example, Goldberg \textit{et al.} (2013) showed the potential impact of intraspecific mutations on eDNA assays in a study of New Zealand mudsnails (\textit{Potamopyrgus antipodarum}) as researchers found a substitution in the 5' primer binding region once primers were developed. Fortunately, the mutation did not prevent primer binding as mismatches located in the 5' end normally have little to no effect on priming efficiency compared to mismatches in the 3' end (Kwok \textit{et al.}, 1990; Christopherson \textit{et al.}, 1997; Whiley and Sloots, 2005; Bru \textit{et al.}, 2008), which may disrupt the nearby DNA polymerase active site (Beard \textit{et al.}, 2004;
Johnson and Beese, 2004). Investigations into new areas of water soldier invasions should therefore include direct sequencing of DNA extracted from plants if the eDNA assay does not generate positive results from sites of known invasion in order to investigate the possibility that mutations in primer binding sites may be limiting the usefulness of primers.

**Variation in DNA copy numbers**

Considerable variation was observed in the number of DNA copies collected between and within sites from which water soldier eDNA was detected. However, less variation was observed in the number of DNA copies at the qPCR triplicate level from the same sample. For example, positive sample replicates varied from 4-1699%; however, if a set of triplicates from site SL2 is excluded, all replicates varied by less than five-fold. In addition, within-sample replicates varied by no more than 18 copies/5 μL in 65% of positive replicate sets. Hunter et al. (2015) also reported heterogeneity in the average concentration of eDNA among samples within locations, but found similar concentrations of eDNA in qPCR replicates taken from the same sample. The greatest variation in the numbers of copies of DNA obtained from positive samples was found from samples collected at different sites, which were a minimum of 2 km apart. These sites surrounded a major patch of water soldier located immediately east of Hardy Island, which is in the middle of the known major population. Therefore, high concentrations of eDNA were expected at these sites. Variation in the number of DNA copies also existed between samples collected at the same site and this variation likely existed due to environmental
factors as well as extraction of samples prior to quantification via qPCR. For example, previous studies have found that rapid flow rates and strong dilution potential of eDNA due to the large volume of water may cause variation in amount of eDNA collected within each sample at a site (Thomsen et al., 2012a; Deiner and Altermatt, 2014). Another possible explanation for variation in number of DNA copies detected from each of the samples collected at a single site may exist due to varying DNA recovery post-extraction. Various studies have reported an absolute extraction efficiency of 16-33% after comparing the original amount of DNA to the amount of DNA recovered post-extraction (Kishore et al., 2006; Colussi et al., 2009; Swaran and Welch, 2012). In this study, one sample of four samples collected at site SL3 yielded a positive detection above the minimum detection threshold. Additionally, only two of four samples collected at sites SL4 and SL5 yielded positive detections above the minimum detection threshold. Furthermore, three of four samples collected at Trent Lock 5 yielded positive detections above the minimum detection threshold. The remaining two positive detections (SL1 and SL2) in Seymour Lake were obtained from all four samples collected at their corresponding sites (Figure 7). With the exception of samples from Trent Lock 5, all sites with positive detections were collected in Seymour Lake, where the invasive water soldier population is well established, and all values exceeded the previously identified detection threshold; therefore, these results were exceedingly unlikely to have been false positives (see below). The variation in the number of DNA copies demonstrates the importance of replicates for both sample collection and testing.
**Unexpected positive detection**

In this study, an unexpected positive detection was obtained at Trent Lock 5. This lock is located in Quinte West, Ontario approximately 50 km downstream from Seymour Lake and 15 km from the Bay of Quinte, Lake Ontario. As the Bay of Quinte is part of Lake Ontario; this finding is potentially of concern as it may indicate downstream expansion of the established population into Lake Ontario where control or containment efforts will not be possible. No known populations of water soldier exist at Trent Lock 5 where the positive eDNA detection was obtained, nor have water soldier sightings been reported at this location (EDDmapS, 2016). For this reason, it is important to consider the possibility that the samples from Trent Lock 5 may represent a false positive detection. False positive detections occur when a sample detects the presence of target eDNA, when in fact, the target species eDNA was not present at the sample collection site.

False positive detections may arise through the detection of non-target species. If primers demonstrate insufficient specificity, DNA from sympatric species may amplify (Darling and Mahon, 2011; Wilcox et al., 2013; Deiner and Altermatt, 2014; Newton et al., 2016). In this study, non-specific priming did not create false positives, as amplicons were verified as water soldier by sequencing, including amplicons derived from Trent Lock 5. Although hybridization between species may cause an incorrect conclusion that the target species is present (Wilcox et al., 2013), water soldier does not hybridize with any species and is not known to reproduce sexually in its introduced range (Snyder et al., 2016). Contamination of samples
may occur in the field and the laboratory, giving rise to false positive detections. Contamination may occur in the field if sites are visited one after another and eDNA is carried from one site to the next. In this study, contamination in the field was prevented by wearing fresh nitrile gloves at each sample collection site and the snare pole used to collect samples was rinsed in a 10% bleach solution between sites. Furthermore, there was no evidence of contamination in sites immediately downstream from Lake Seymour.

Contamination may also occur in the laboratory due to PCR, which generates billions of copies of the target sequence which can be spread easily throughout the lab (Darling and Mahon, 2011; Wilcox et al., 2013). Following clean lab protocols and including DNA extraction, PCR and cooler negative controls is essential to minimize contamination risk. In this study, PCR negative controls yielded low levels of contamination, however, the minimum positive detection threshold was set slightly above these values (4 DNA copies/5 μL DNA extraction) to increase confidence in positive detections and safeguard against false positives (Darling and Mahon, 2011). Water soldier produces offsets, which are smaller juvenile plants that bud off the adult through deteriorated lateral shoots (Erixon, 1979; Kornatowski, 1979). Offsets allow water soldier populations to expand as the smaller plants may be swept downstream with river current. If a transient water soldier offset or fragment existed at Trent Lock 5 at the time of sample collection, it may have resulted in a positive eDNA detection. However, transient water soldier offsets or fragments may not necessarily lead to an established population. Positive
eDNA detections also do not necessarily mean the organism is present at the sample collection site. For example, if a boat took up water in Seymour Lake containing water soldier eDNA and released it at Trent Lock 5, this could result in a positive eDNA detection. Trent Lock 5 and immediately upstream continue to be monitored without water soldier sightings, despite obtaining the positive eDNA detection (R. McGowan, OFAH, pers. comm.). Resampling of this site could be useful as it may determine if the positive detection was merely transient.

**Unexpected negative detections**

Trent Lock 5 was the only site outside of Seymour Lake where a positive detection was obtained. This could mean either water soldier eDNA did not exist in any other sampled areas or false negatives were obtained. False negative detections may occur when molecular testing fails to detect target eDNA when in fact the target species eDNA was present at the sample collection site (Darling and Mahon, 2011). As no samples collected in Seymour Lake in July 2013 resulted in positive detections, it seems likely that most of these samples produced false negative results. July samples were collected at sites with known water soldier populations which yielded positive detections when resampled in September. The lack of positive detections in July samples may be due to the varying detectability of eDNA throughout seasons due to environmental changes such as fluctuating water temperatures. For example, Dejean et al. 2011 found that eDNA of anuran tadpoles was detectable for 25 days after the removal of the target species at 8–11°C but only
21 days at a warmer temperature of 17±1°C. This may be due to the fact that lower temperatures can slow down enzymatic and microbial activity resulting in slower DNA degradation (Zhu, 2006). Furthermore, a study conducted by Merkes et al. 2014 shows that dead organisms can shed large quantities of DNA. Water soldier forms new leaves in the spring, which eventually die back in the fall (Forbes, 2000). Decomposing water soldier leaves may have led to higher eDNA concentrations that resulted in positive water soldier detections from samples collected in September. The lack of positive detections in July samples may also be due to eDNA degradation caused by sample degradation as these samples were stored at -20°C for several months prior to storage at -80°C. Smith and Morin (2005) found a significant reduction in eDNA quality when samples were stored at -20°C. The degree and speed of degradation depends on different factors including the concentration, base composition, length and sequence of a sample (Shewale and Liu, 2013). Despite there being many studies examining eDNA degradation rates in water (Zhu, 2006; Dejean et al., 2011; Thomsen et al., 2012a; Barnes et al., 2014; Pilliod et al., 2014), extracted eDNA degradation rates in storage have not been explored and should be further examined for eDNA studies.

In addition to the false negative detections from samples collected in July, 2013, false negative detections may have also been obtained downstream from positive detections in Seymour Lake. No positive detections were obtained east of Nappan Island, which is a large island approximately 3 kilometres downstream from Hardy Island in Seymour Lake. Negative samples were also obtained below Healey
Falls lock, however, sightings of water soldier have since been reported past this location as far as Percy Reach, which is approximately 20 km downstream from Seymour Lake and 40 kilometres from the Bay of Quinte, Lake Ontario (EDDmapS 2016). Even if there was no water soldier downstream from Seymour Lake in 2013, eDNA may have been dispersed by water current. Deiner and Altermatt (2014) observed downstream movement of eDNA in a river system but also suggested that transport distances for eDNA may vary between species. False negative detections downstream from positive detections in Seymour Lake may also reflect low sensitivity in assays. Although qPCR is more sensitive than conventional PCR (Wilcox et al., 2013; Turner et al., 2014; Amberg et al., 2015; Fukumoto et al., 2015), future research should aim to quantify the sensitivity of water soldier eDNA assays using known amounts of starting biomass.

Additional factors that may lead to false negatives include failure to collect eDNA and inhibitors. As noted earlier, the variation among samples taken from the same sites suggests that even if eDNA is present at a site, it may not be collected in all water samples. Schmidt et al. (2013) detected eDNA in only 45% of samples collected from sites where eDNA of the target species was known to occur. The number of samples taken per site varies across studies; however, collecting three samples per site is most commonly reported (Ficetola et al., 2008; Dejean et al., 2012; Takahara et al., 2012; Thomsen et al., 2012b; Hunter et al., 2015). Thomsen et al. (2012b) examined the probability of detecting the target species with up to three samples collected, and found that detection probabilities were significantly reduced
when based on only one or two samples per site. In this study, four samples were collected at each collection site to increase the likelihood of collecting water soldier eDNA. As previously mentioned, three sites yielded positive detections from less than three of four samples, suggesting that four samples per site is a more reliable sample size than three when surveying sites for water soldier eDNA.

PCR inhibition may also lead to false negative eDNA detections, however, this study assayed samples using qPCR rather than conventional PCR, which is often more sensitive than conventional PCR even in the presence of inhibitors (Gibson et al., 2012). In addition, post-extraction washes with phenol-chloroform were conducted to rid samples of non-target substances that typically cause inhibition of amplification that leads to false negative results. An internal positive control (IPC) can be added to samples for detection of inhibitors. For example, if samples collected from areas with known target eDNA occurrences yield negative detections, IPCs can be added to these samples to distinguish between true negatives and inhibition. If inhibitors are absent, the exogenous DNA included in the reagent will be co-amplified along with the target gene (Herwegh et al., 2005). On the other hand, if samples with IPCs fail to amplify, then inhibitors are likely present within the sample. Due to the experimental design of this study, IPCs were not required. Multiple positive detections were obtained from Lake Seymour, an area where water soldier has established. If positive detections were not obtained in Seymour Lake, there would be reason to believe inhibition may have occurred.
Future research

The detection of organisms from eDNA is a relatively new technique, and although it presents a potentially powerful tool for detection of aquatic invasive species, limitations to the method still exist. Limitations include the inability of this method to give insight on population size, distance from source, temporal factors and viability of the source of eDNA. Studies have demonstrated that eDNA abundance positively correlates with species’ density and biomass (Dejean et al., 2011; Takahara et al., 2012; Schmidt et al., 2013; Kelly et al., 2014; Moyer et al., 2014; Matsuhashi et al., 2016); however, limited field-based surveys are inconsistent with this finding (Takahara et al., 2012, 2013). Regardless, inferring population sizes from species’ biomass remains problematic in taxa, particularly those with pronounced size differences between life history stages, as one large organism may shed more DNA than many smaller organisms. Further water soldier eDNA research could assay samples collected from aquaria with known, increasing water soldier biomass. This could establish whether water soldier biomass or population size is positively correlated with eDNA concentrations. In addition, the precise location of a detected target organism cannot be made with eDNA monitoring due to the distribution of species’ eDNA in aquatic environments. As previously mentioned, Deiner and Altermatt (2014) first demonstrated that eDNA can be detected downstream from a source population. They reported significant decrease in detectability with increasing distance from the source, when detectability was determined as the number of positive amplifications of target DNA in PCR triplicates. However, eDNA concentrations should not be used to infer
proximity of source as no consistent relationship exists between eDNA concentrations and distance from target species (Lamarie et al., 2015; Spear et al., 2015). In addition, many other factors such as stream velocity, depth, water temperature, wind speed, wind direction and local changes in currents impact the potential distance that eDNA can be transported in aquatic systems (Minshall et al., 2000; Thomsen et al., 2012). To better understand this limitation of eDNA research, samples could be collected in regular intervals downstream from a mesh bag containing water soldier plants in a river with no known water soldier occurrences. The plants would be contained within the mesh sac to prevent their escape and potential establishment. This would give some insight on the downstream transport capability of water soldier eDNA. Furthermore, eDNA can persist in water after a species has left the aquatic environment (Ficetola et al., 2008). This can lead to the detection of a species that was not present at the time of sample collection. Many studies have assessed the rate of eDNA degradation in aquatic environments, which generally occurs over hours to days (Barnes et al., 2014; Pilliod et al., 2014; Strickler et al., 2015; Fujiwara et al., 2016). The DNA degradation rates or the residence time of detectable target DNA varies widely due to environmental conditions such as UV exposure, temperature and pH levels (Pilliod et al., 2013; Strickler et al., 2015). To better understand this limitation of eDNA research, samples collected prior to and in regular intervals following the removal of water soldier biomass may be assayed. The data from these assays would provide insight on eDNA persistence. Finally, both living and deceased target organisms shed DNA into their environment, and therefore eDNA assays cannot distinguish between living and dead individuals.
(Mountfort and Hayden, 2006). Further research could assay eDNA samples collected in regular time intervals from aquaria holding dead water soldier plants to determine if dead plants are detectable and if so, for how long. This information would be useful in post-treatment studies to establish how long water soldier eDNA persists following herbicide treatment. Regardless of potential limitations and knowledge gaps, the use of eDNA to monitor organisms in aquatic environments has tremendous potential and its use should continue to be explored.
References


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Winter, M., Schweiger, O., Klotz, S., Nentwig, W., Andriopoulos, P., Arianoutsou, M.,


Appendix A. Source of sequences for water soldier and 17 sympatric plant species from 7 families.

<table>
<thead>
<tr>
<th>Species</th>
<th>Family</th>
<th>matK</th>
<th>rbcl</th>
<th>trnH-psbA</th>
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<tr>
<td>Water soldier <em>Stratiotes aloides</em></td>
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<td>GenBank HQ901565.1</td>
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<td>Coontail <em>Ceratophyllum demersum</em></td>
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<td>GenBank AF543732.1</td>
<td>GenBank KC584879.1</td>
<td>GenBank AB331297.1</td>
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<td>Curled pondweed <em>Potamogeton crispus</em></td>
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<td>GenBank JF955609.1</td>
<td>GenBank AB196847.1</td>
<td>GenBank DQ786528.1</td>
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<td>GenBank DQ786557.1</td>
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<td>Canadian waterweed <em>Elodea canadensis</em></td>
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<td>Water nymph <em>Najas flexilis</em></td>
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Appendix B. The global positioning system (GPS) coordinates of all 40 sample sites in decimal degrees of longitude and latitude.

<table>
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<th>Name</th>
<th>Longitude (°)</th>
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**Appendix C.** Summary of water soldier eDNA data from sampling location in the lower Trent-Severn Waterway in July and September, 2013, showing mean qPCR results with standard deviation in brackets for each sample and site. RL= Rice Lake, TR= Trent River and SL= Seymour Lake.

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