DISSERTATION

XENOBIOTICS TRANSLOCATE IN AQUATIC PLANTS: A CASE STUDY USING THREE AQUATIC HERBICIDES

Submitted by

Mirella F. Ortiz

Department of Agricultural Biology

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Doctoral Committee:

Advisor: Franck Dayan
Co-Advisor: Scott Nissen

Patricia Bedinger
Mark Heilman

ABSTRACT

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When invasive aquatic weeds dominate aquatic ecosystems there are numerous negative impacts. Milfoil (*Myriophyllum* spp.) and hydrilla [*Hydrilla verticillata* (L.f.) Royle] are the most costly aquatic plants to manage in the U.S. per year. These invasive plants form extensive surface canopies that negatively affect water quality and native plant communities, and can also impact recreational uses such as swimming, fishing, and boating. Synthetic auxins, such as 2,4-dichlorophenoxyacetic acid (2,4-D), have been widely used for selective control of milfoil since 1959. Since then, several populations of hybrid watermilfoil (*M. sibiricum* × *M. spicatum*; HWM) have showed lower sensitivity to this herbicide.

In 2015, a HWM population with lower sensitivity to 2,4-D was found in Idaho, USA. Using the same 2,4-D-resistant population and a known susceptible Eurasian watermilfoil (*M. spicatum*; EWM) population from Colorado, the mechanism of 2,4-D resistance was examined by conducting ^{14}C-2,4-D absorption, translocation, desorption, and metabolism experiments. 2,4-D resistance in HWM is not due to non-target-site resistance as no differences in herbicide absorption, translocation, desorption and/or metabolism were identified; therefore, target-site resistance is the most likely resistance mechanism. More research is needed to identify the molecular basis for the 2,4-D-resistant trait in HWM.

Herbicide combinations are widely recommended to alleviate the evolution of herbicide resistance. The aquatic herbicide endothall is often used in combination with 2,4-D for HWM management as an effective control option and a resistance management strategy, but it is still unknown how combining herbicides might impact the behavior of each herbicide. Experiments combining radiolabeled with non-radiolabeled herbicides were conducted to evaluate herbicide absorption, accumulation, and translocation from shoots to roots in HWM. Endothall accumulation was not impacted when these herbicides were applied in combination, but its translocation from shoots to roots was reduced by 50% when applied in combination with 2,4-D. When 2,4-D, was applied in combination with endothall shoot absorption increased by 80%; however, 2,4-D movement from shoots to roots was reduced from 24.8% ± 2.6 to only 3.93% ± 0.4 when in the presence of endothall.

The overreliance on a single mode of action resulted in evolved fluridone resistance in hydrilla in the late 1990s. 2,4-D is not effective for hydrilla control at label rates, but the most recently registered auxinic herbicide, florpyrauxifen-benzyl, is highly active against hydrilla. Where fluridone-resistant hydrilla is present, endothall is being used in combination with florpyrauxifen-benzyl for its control. In order to test experiments combining radiolabeled and non-radiolabeled endothall and florpyrauxifen-benzyl were conducted to evaluate herbicide absorption, accumulation, and translocation in two hydrilla biotypes, monoecious (MHV) and dioecious (DHV). Herbicide accumulation in both biotypes was not impacted when these herbicides were applied in combination. Endothall translocation from shoots to roots in DHV did not appear to be impacted (alone = 18.7% ± 1.4; combination = 23.2% ± 2.2); however, endothall shoot-to-root translocation in MHV was reduced from 16.2% ± 1.3 to 2.2% ± 0.1 when applied in combination with florpyrauxifen-benzyl. Florpyrauxifen-benzyl shoot-to-root

translocation was reduced by 16 and 6 times in DHV and MHV when applied in combination with endothall, respectively.

These data highlight differences in herbicide behavior when herbicides are applied in combination. Future research is needed to determine if these differences negatively impact the operational effectiveness when herbicides are applied in combination.

Lastly, endothall and 2,4-D have been used to control aquatic weeds for more than 60 years, and still there is very little information available about the *in planta* behavior of these herbicides in aquatic weed species. 2,4-D is purportedly systemic in aquatic plants based almost entirely on its behavior in terrestrial plants. It was demonstrated in this dissertation that radioactive 2,4-D and endothall can translocate from shoots to root systems; however, it was not determined if the radioactivity in the roots was parent herbicide or a metabolite(s). Therefore, the last chapter of this dissertation used multiple analytical methods to answer the question if 2,4-D and endothall are truly systemic in aquatic plants. The intact 2,4-D detected in HWM shoots was 1.31 $\mu g\ g^{-1}$ dry weight (DW) and 0.11 $\mu g\ g^{-1}$ DW was detected in the roots. For endothall, 1.08 and 0.12 $\mu g\ g^{-1}$ DW was detected in DHV shoots and roots, respectively. We therefore conclude that 2,4-D and endothall have similar *in planta* behavior, with about 8-10% of absorbed intact active ingredient translocating to the roots of these aquatic plants.

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DEDICATION

Para minha mãe, Nancy, que apesar de a distância machucar todos os dias, nunca deixou de me apoiar.

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Aquatic weeds can negatively impact native aquatic plant species, affect fish habitat, and interfere with numerous human activities. Managing these weeds often relies on the use of herbicides; however, working within an aquatic ecosystem presents unique challenges.

Introduction

Aquatic weeds can negatively impact native aquatic plant species, affect fish habitat, and interfere with numerous human activities. Managing these weeds often relies on the use of herbicides; however, working within an aquatic ecosystem presents unique challenges.

Native aquatic plants are an instrumental component of aquatic ecosystems because they provide valuable habitat for fish and wildlife, improve water quality and clarity by stabilizing sediments, and reduce shoreline erosion (Savino & Stein 1982; Heitmeyer & Vohs Jr 1984). Native plants also improve water quality by absorbing excess nutrients and trapping certain water pollutants (Smart *et al.* 1998).

Aquatic plants can only grow in the shallow zones of lakes, ponds or rivers, known as the littoral zone. Littoral zones consist of areas transitioning between dry land and the open water area where sufficient light reaches the bottom of the lake, and where a sufficiently nutrient-rich sediment provide favorable conditions for plant growth. The width of the littoral zone will vary based on the topography of the area. In places where the slope of a lake bottom is steep, the littoral area may be limited. In contrast, a shallow lake where the bottom slopes gradually may have a littoral zone extending many meters into the lake or may even cover it entirely.

Aquatic weeds that grow in littoral zones are divided into three groups: emergent plants (also called emersed), floating plants and submersed plants (Figure 1.1). Emergent plants [e.g. cattail (*Typha latifolia* L.) and arrowhead (*Sagittaria* spp.)] inhabit the shallowest water and are rooted in the sediment with their leaves and stems extending above the water's surface. Floating plants [e.g. waterlily (*Nymphaea alba* L.) and waterlettuce (*Pistia stratiotes* L.)] grow at intermediate depths and include species that are rooted in the sediment, as well as free-floating species with roots that hang unanchored in the water column. Lastly, submersed plants [e.g. milfoil species (*Myriophyllum* spp.) and hydrilla (*Hydrilla verticillata* (L.f.) Royle)] are rooted in sediment and grow entirely under water, with no plant parts emerging from the water, except for flowers when present. Submersed plants inhabit the deepest fringe of the littoral zone (Figure 1.2).

When compared to native plants, aquatic weeds often have negative impacts on the aquatic ecosystems and disrupt many human activities, while native aquatic plants may also reach nuisance densities under ideal growing conditions. Through competition and displacement, invasive aquatic weeds commonly overwhelm native plants, reducing diversity by forming dense monotypic stands. These extensive plant canopies can: reduce native fish and macroinvertebrate populations and diversity (Hardin 1960; Madsen *et al.* 1991; Schultz & Dibble 2012); impede the natural flow of water through an ecosystem and its overall productivity (Nikora *et al.* 2008; Schultz & Dibble 2012); negatively impact irrigation canals and water supplies; impact recreational uses of waterbodies, such as swimming, fishing, and boating; reduce property value; and create habitat suitable for disease carrying vectors (Wilde *et al.* 2005; Zhang & Boyle 2010).

The total economic impact of aquatic weeds is estimated at US$14.2 billions/yr, and the cost of managing them was estimated to be over US$800 million/yr in 2005, with Eurasian

watermilfoil alone accounting for nearly US$400 million/yr (Pimentel 2005). For another major invasive aquatic plant, a 2019 risk assessment for potential hydrilla invasion in the Great Lakes concluded 'the economic losses associated with the impacts on recreational fishing, beach use, recreational boating, and commercial navigation are expected to range between $70 million and $500 million annually if hydrilla were to become established in the Great Lakes' (Great lakes hydrilla risk assessment 2019).

Early approaches to manage aquatic weeds were limited to cultural and biological control, and mechanical removal; however, since 2,4-D's commercialization in the late 1950s, chemical control has become the most common and cost-effective method for selective aquatic weed management in the United States. Chemical control of aquatic weeds can range in scale from a backpack sprayer for spot treat individual plants and localized problems, to large-scale treatments targeting entire lakes using boats and/or helicopters. For successful control of submersed aquatic weeds and also certain floating and emergent weeds not optimally treated via foliar application, herbicide applications are made directly to water around the target plants. The herbicide must remain at a particular concentration within the water column and the weeds must be exposed to the herbicide for a certain time period, ranging from a few hours up to several months (Gettys *et al.* 2014). These two factors have been defined as the concentration and exposure time (CET) relationship, and it is different for each herbicide and plant species (Getsinger & Netherland 1997).

While aquatic weed species can be removed using traditional chemical control strategies, these treatments can also negatively impact desirable native plants; therefore, it is important to know all the plant species present in the ecosystem in order to make the treatment as selective as possible. Different rates, timing and placement can result in the control of the target weed, while

enhancing the growth of desirable, beneficial plants. Using sound, scientifically-supported methods for selective aquatic weed management with herbicides, waterbodies with monospecific infestations can be managed to provide a more healthy, diverse, and ecologically-balanced aquatic community and variety of other important economic and water-use benefits (Getsinger *et al.* 2014).

There is a need for more active ingredients to be registered for use in aquatic systems in order to further enhance selectivity, reduce use rates, and to mitigate the risk of potential herbicide resistance. The development of new aquatic herbicides through specific aquatic research and development—including screening, development and potential registration of current terrestrial herbicides for aquatic use—may further expand the options available for controlling invasive aquatic weed species and possible new threats.

Overview of Chemical Aquatic Weed Management

Chemical management of aquatic weeds has progressed from broad-spectrum inorganic compounds such as sodium arsenite (1900-1930s) and copper (1900s and still widely used for bluegreen algae control), to conventional herbicides such as 2,4-D, endothall, diquat and glyphosate (1960-1970s). Various forms of copper and these older synthetic herbicides are still valuable tools for aquatic weed management today (Getsinger *et al.* 2008).

The first inorganic herbicide registered for aquatic use was copper sulfate in the 1950s. Copper is a micronutrient needed for plant growth that is used as a fungicide in agricultural systems and it has been widely used as an algaecide and for aquatic weed control since the early 1900s (even though it was not officially registered since the EPA was not created until 1970) (Gettys *et al.* 2014). Between 1970 and the early 2000s, only six conventional herbicides with distinct mechanisms of action (MOA) had been registered for aquatic use (Getsinger *et al.* 2008)

(Table 1.1). 2,4-D is an Herbicide Resistance Action Committee (HRAC) group O (WSSA group 4) plant growth regulator herbicide that mimics the action of the natural plant hormone indole-3-acetic acid (IAA). Endothall is in its own chemical class and inhibits plant serine/threonine protein phosphatases (Tresch *et al.* 2011; Bajsa *et al.* 2012). Diquat inhibits photosynthesis by diverting electrons from photosystem I (PSI) (HRAC group D, WSSA group 22). Glyphosate targets 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a key enzyme in aromatic amino acid biosynthesis (HRAC group G, WSSA group 9). Fluridone inhibits phytoene desaturase (PDS), a key enzyme committed to carotenoid biosynthesis (HRAC group F1, WSSA group 12). Acrolein is in its own chemical class with no known MOA and general biocidal properties not specific to aquatic weeds.

In the last several decades, government, university, and private company research scientists have worked collaboratively to discover, develop, and register with USEPA eight additional active ingredients for aquatic use (Table 1.1). Triclopyr and florpyrauxifen-benzyl are auxinic herbicides with the same general mode of action as 2,4-D but belong to different chemical families and possess different properties for aquatic weed control. Imazapyr, penoxsulam, imazamox and bispyribac-sodium all target acetolactate synthase (ALS), a key enzyme in branched-chain amino acid biosynthesis (HRAC group B, WSSA group 2). Carfentrazone and flumioxazin inhibit protoporphyrinogen oxidase (PPO), a key enzyme in chlorophyll and heme biosynthesis (HRAC group E, WSSA group 14), while topramezone inhibits hydroxyphenylpyruvate dioxygenase (HPPD), an enzyme involved in plastoquinone biosynthesis resulting in bleaching (Dayan *et al.* 2020) (HRAC group F1, WSSA group 12).

Herbicide Resistance in Aquatic Weeds

Similar to terrestrial weeds, herbicide resistance management in aquatic weeds consists primarily of herbicide rotations and mixtures, with mixtures being more effective than rotation at preventing evolution of resistance (Beckie & Reboud 2009). Also, early detection and rapid response methods are also recommended to stop the spread of aquatic weeds through human activities, and a similar proactive response to suspected resistant weeds in small areas can contain them before they become a problem on a large scale.

Registration of fluridone in the mid-1980s for whole-lake and large-scale management stimulated research on reduction of use rates, plant selectivity, residue monitoring, and impacts on fisheries. Fluridone was a game-changer in aquatic weed management because it was so effective at very low rates; however, the selection pressure exerted by fluridone resulted in the first case of herbicide resistance in an aquatic weed.

Hydrilla is one of the most serious invasive aquatic weed problems in the USA and is very sensitive to fluridone, being controlled at rates as low as 2 - 4 $\mu g\ L^{-1}$ depending on biotype (Netherland *et al.* 1997; Netherland 2015). Due to extensive hydrilla infestations in large, shallow public lakes in Florida, fluridone was intensively used in the late 1980s through the early 2000s in large public lakes in Florida. After years of repetitive treatments, several fluridone resistant hydrilla populations were identified in Florida. Each of these populations had one of three independent mutations in the gene encoding for the phytoene desaturase enzyme, fluridone's target site (Michel *et al.* 2004). A few noteworthy observations must be made about this herbicide resistance case. First, both monoecious and dioecious hydrilla biotypes were introduced in the US, but fluridone resistance in hydrilla has only been documented in the dioecious biotype and almost exclusively in select populations of dioecious hydrilla located in

Florida. For dioecious hydrilla, only the female plant is found in the US. Therefore, fluridone resistance has been the result of a somatic mutation and its spread is limited to vegetative reproduction. This is the only case of resistance not relying on pollen or seed movement (Dayan & Netherland 2005).

Second, the three natural mutations imparting fluridone resistance also provide cross-resistance to other non-aquatic PDS inhibitors such as norflurazon, but negative cross-resistance to beflubutamid, picolinafen and diflufenican (Arias *et al.* 2006). Finally, these mutations do not have any fitness cost. Consequently, resistant biotypes are the dominant populations in Florida after nearly a decade without treatment with fluridone (Netherland & Jones 2015). While resistance to fluridone is rare, fluridone-resistant hybrid watermilfoil populations have been detected in other locations such as in some Michigan lakes (Thum *et al.* 2012; Berger *et al.* 2012; Berger *et al.* 2015); however, this resistance mechanism remains to be determined. Identifying fluridone-resistant hydrilla has sparked renewed interest by industry and university researchers to find alternative modes of action for aquatic use (Getsinger *et al.* 2008).

Eurasian watermilfoil (*Myriophyllum spicatum* L.) (EWM) is a widespread aquatic invasive weed native to Eurasia introduced in the US in the 1940s. Similarly to hydrilla, this aquatic weed can rapidly spread around through autofragmentation and is one of the most troublesome aquatic weeds to manage (Pimentel 2009). A few years ago, commercial applicators in the upper Midwestern US identified EWM populations with reduced sensitivity to 2,4-D. Genetic analysis determined that these plants were not EWM, but a hybrid between EWM and the native northern watermilfoil (*M. sibiricum* Komarov) (Larue *et al.* 2013). While EWM is normally controlled by 2,4-D, these hybrid watermilfoil (HWM) are not only less sensitive to

2,4-D, but also are more invasive, with significantly higher growth rates compared to either parent (Moody & Les 2002).

A dotted duckweed population (*Landoltia punctata* [G. Mey.] Les & D.J. Crawford) from Florida also developed resistance to diquat and cross resistance to paraquat (Koschnick *et al.* 2006) and several other cases of herbicide resistance in aquatic weeds are reported in Asia and Australia in rice production fields (Graham *et al.* 1996).

Limitations

Most of the stakeholders interested in aquatic plant management recognize the need for more herbicide active ingredients to be registered, but there are several factors that limit development and registration of aquatic herbicides. The present cost of discovery, development, and registration of any new pesticidal active ingredient is several hundred million US$. Given the relatively niche use of aquatic weed control relative to terrestrial/agricultural herbicide uses, no herbicide will be exclusively developed for aquatic use, and the cost of registering an herbicide for additional aquatic use along with other uses is high with commonly a long return on such investment. To register an already existing herbicide active ingredient for aquatic use requires extensive research to demonstrate that the herbicide has minimal risk in aquatic environments with several key characteristics: 1) does not bioaccumulate, 2) does not affect native plant species, fishes, and waterfowl, and 3) has a relatively short half-life in the aquatic environment.

Overall, aquatic plant management is a niche market with significant upfront costs and a long return on investment with additional important technical criteria for herbicide use in water. These are the primary impediments to development and registration of new herbicides for aquatic uses.

Alternative to Herbicide in Aquatic Weed Management

In addition to chemical control, integrated management options of aquatic weeds include cultural, physical, mechanical, and biological practices that may also address needs or goals of stakeholders (Stallings *et al.* 2015).

Cultural suppression practices usually focus on education and preventing the introduction of invasive weeds. It includes boat inspections and modifications in the environment to make it less suitable for weed growth (Whetstone 2004). These include aeration to increase removal of ammonium and phosphorus from the water column or the use of dyes to limit light penetration, inhibiting photosynthesis and plant growth under water.

Physical control includes non-chemical, non-motorized techniques that are used to control aquatic weeds and range from hand-pulling to water-level drawdowns, or via use of benthic barriers (weed barriers) for localized issues. Mechanical suppression practices consist mainly of mechanical removal of the biomass utilizing large power-driven equipment and are common alternative methods for aquatic weed control.

Biological control practices include the classical approach of introducing a natural enemy (e.g., insects, fish, and pathogens) of the nonnative invasive weed. For example, a small Australian weevil (*Bageous hydrillae*) that feeds on hydrilla in its native environment was introduced in the US to manage hydrilla infestation. Nonclassical biological control approaches consist of introducing naturalized nonnative biocontrol agents. For example, the Chinese grass carp (*Ctenopharyngodon idella*) was introduced for use in lakes to reduce the biomass of undesired aquatic vegetation (Hanlon *et al.* 2000). While alternative aquatic weed management practices may work in some very specific circumstances, these approaches are often difficult to

implement on a large scale and may be less effective or less predictable in control outcomes than chemical weed management.

Future Prospects

There is increasing recognition among aquatic weed managers that different populations of aquatic weeds can vary significantly in their potential for nuisance growth, spread, impacts, and most important for herbicide management, response to control efforts (Thum & McNair 2018). Population-centered, as opposed to species-centered, approaches to understanding and managing aquatic weeds are important, since populations are ultimately the unit of management for most invasive species (Thum & McNair 2018; Reichard *et al.* 2015).

The registration of herbicide active ingredients for aquatic use is generally an afterthought for most chemical companies; therefore, older herbicides may be re-evaluated for activity against important aquatic weeds. Sometimes this search does identify herbicides that have potential to control aquatic weeds. One example is the herbicide quinclorac (HRAC group O, WSSA group 4). Quinclorac demonstrated significant activity against Eurasian watermilfoil at concentrations as low as 40 ppb; unfortunately, field evaluations determined that the half-life was longer than 160 days. This long half-life would not be viewed favorably by the USEPA, so the aquatic use of quinclorac was never pursued. This example illustrates that any new active ingredient and even older products should be evaluated for potential aquatic uses. In another example, (Sartain & Mudge 2018) evaluated the efficacy of 12 herbicides against giant salvinia (*Salvinia molesta),* a free-floating aquatic weed, and documented that metsulfuron and sulfometuron would control giant salvinia. Additional studies also demonstrated that metsulfuron had low toxicity to nontarget aquatic plants (Prevost 2019). This research resulted in USEPA Section 24(c) Special Local Need (SLN) labels being requested for metsulfuron foliar

applications to giant salvinia by state natural resource management agencies in Louisiana and Texas. A more narrow use of herbicides without full aquatic registrations for control of select aquatic weed species via SLN registrations has been utilized in the past. This can be an appropriate mechanism to allow limited aquatic use where there is no other fully aquatic-registered herbicide that can control the target weed.

In conclusion, aquatic herbicides have been important tools for many decades in integrated management strategies for control of aquatic nuisance and invasive plants. Improving existing aquatic herbicide use patterns and searching for new and evaluating old herbicide active ingredients for activity against important aquatic weeds is absolutely critical to 1) minimize risk of herbicide resistance in aquatic weeds populations, 2) restore and maintain critical wildlife habitat, and 3) protect the diverse water uses of aquatic environments.

Table 1.1: Herbicides currently registered for aquatic use listed by year of registration (Adapted from Gettys *et al.* (2014))

Herbicide	Year of Registration	Primary use				Mode of Action
		Submersed	Floating	Emergent	Algae	
Copper	1950s	X	X		X	Contact; Plant cell toxicant
2,4-D ester	1959	X	X	X		Systemic; PGR
Endothall	1960	X	X		X	Contact; Protein phosphatase
Diquat	1962	X	X	X		Contact; PSI
2,4-D amine	1976	X	X	X		Systemic; PGR
Glyphosate	1977			X		Systemic; EPSPS
Fluridone	1986	X	X			Systemic; PDS
Triclopyr	2002	X	X	X		Systemic; PGR
Imazapyr	2003			X		Systemic; ALS
Carfentrazone	2004	X	X	X		Contact; PPO
Penoxsulam	2007	X	X			Systemic; ALS
Imazamox	2008	X	X	X		Systemic; ALS
Flumioxazin	2011	X	X	X		Contact; PPO
Bispyribac	2012	X	X			Systemic; ALS
Topramezone	2013	X	X			Systemic; HPPD
Florpyrauxifen-benzyl	2018	X	X	X		Systemic; PGR

Please refer to HRAC website for listing of herbicides based on their HRAC classification:

https://hracglobal.com/tools/hrac-moa-2020-revision-description-and-master-herbicide-list

PGR = Plant growth regulator

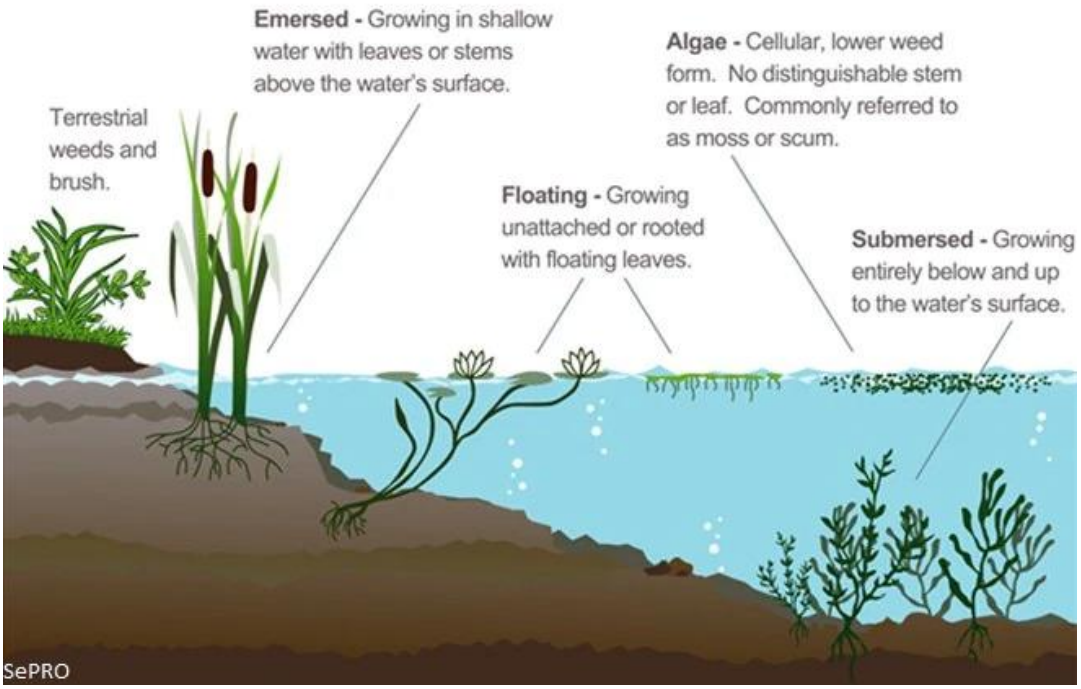

Figure 1.1. Illustration of the various aquatic plants and growth pattern.

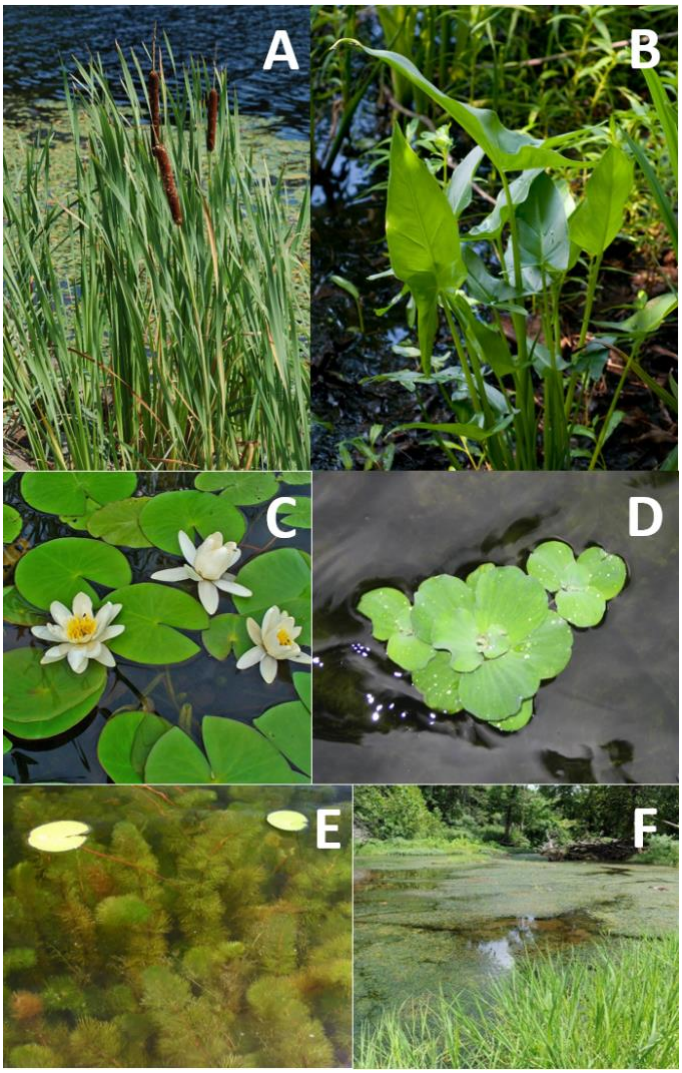

Figure 1.2. Aquatic weeds include emergent plants (A) cattail (*Typha latifolia* L.) (photo from Jacquelyn Boyt #170B27EAD5) and (B) arrowhead (*Sagittaria cuneata*) (photo from J. Harry Rich State Forest #44BA94E776), floating plants (C) waterlily (*Nymphaea alba* L.) (photo from H. Zell) and (D) waterlettuce (*Pistia stratiotes* L.) (photo by Tim Chandler #AFFB7B27CD), and submersed plants (E) twoleaf watermilfoil (*Myriophyllum heterophyllum Michx*) (photo from Leslie J. Mehrhoff) and (F) hydrilla (*Hydrilla verticillata* (Lf) Royle) (photo from Charlotte Wray).

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CHAPTER 2: 2,4-D AND 2,4-D BUTOXYETHYL ESTER BEHAVIOR IN EURASIAN AND HYBRID WATERMILFOIL (*Myriophyllum* spp.)

Introduction

Eurasian watermilfoil (*Myriophyllum spicatum* L.; EWM) is an invasive aquatic plant that has spread throughout the United States (US)[1]. Infestations often have numerous negative environmental and economic impacts. A severe infestation results in dense monotypic stands of submerged vegetation[2] that extends to the water surface, impairing recreational uses (fishing, boating, swimming),[3] reducing property values,[4] affecting natural water flow,[5] altering native aquatic habitats, and reducing native fish and macroinvertebrate diversity.[6,7]

EWM management is one of the most extensive aquatic weed control endeavors in the US.[8] This species and its hybrids are especially problematic across the upper Midwest states of Wisconsin, Michigan, and Minnesota. In lakes where EWM and the native northern watermilfoil (*M. sibiricum* Komarov) co-occur, they can hybridize and these hybrids (*M. spicatum* × *M. sibiricum*; HWM) grow more aggressively than either parent,[9-11] requiring intensive management.[12] One of the main herbicides used to manage EWM and HWM is the synthetic auxin, 2,4-dichlorophenoxy acetic acid (2,4-D).[13,14] 2,4-D is one of the least expensive management options and at low use rates provides selective control; however, several HWM populations have showed reduced susceptibility to 2,4-D.[15,16]

The amine and ester (butoxyethyl ester; BEE) formulations of 2,4-D are registered for aquatic weed management. The first granular 2,4-D BEE formulation was labeled for aquatic use in 1959. The BEE of 2,4-D was impregnated onto granules and the granules spread over the infested areas, which allowed for a prolonged release of 2,4-D BEE from the granules. Due to its

lipophilic nature, the 2,4-D BEE was then rapidly absorbed by the target species.[17] 2,4-D BEE is a proherbicide that is bioactivated into its active form, 2,4-D acid, by the plant via the activity of esterase enzymes.[18] Depending on water chemistry and temperature, 2,4-D BEE can also be hydrolyzed to the free acid in the water column and absorbed by plants.[17] Successful 2,4-D-BEE treatments often provided multiple years of EWM control; however, the registration of liquid amine formulations of 2,4-D in 1976 provided another management option, while the granular ester formulation is used for submersed plant control only, the liquid amine formulation is used to control both emergent and submersed plants.[1] For over 45 years, 2,4-D was the only auxin herbicide labeled for aquatic use.[19]

Auxins are a group of plant growth hormones that regulates developmental processes in plant growth and morphogenesis, such as root formation and leaf initiation, tropic responses, cell division and shoot growth, plant development and apical dominance, among other essential plant growth processes.[20] In susceptible plants, synthetic auxins mimic the effects of natural auxin and induce strong changes in gene expression that ultimately lead to lethal plant growth responses,[21] however, synthetic auxins are more stable within plants and less susceptible to metabolic inactivation compared to the naturally produced auxins.[20] 2,4-D interferes with the plant's ability to maintain proper hormone balance, posing limited risk to wildlife, 2,4-D is readily absorbed and translocated throughout sensitive plants accumulating mainly in the growing points of shoots and roots, and is generally selective for control dicots, with minimal impact to monocots.[1]

Since the introduction of 2,4-D as the first synthetic auxin herbicide in 1945, resistance to this class of herbicides has been reported in 41 terrestrial weed species (Heap, I. (2021). The international survey of herbicide resistant weeds; available at www.weedscience.com; accessed July 19, 2020) and HWM is the first aquatic weed species to evolve resistance to 2,4-D.

Resistance mechanisms to 2,4-D in other species involve mutations in the gene encoding the target site protein(s) as well as non-target site changes that alter herbicide movement or degradation rates to non-phytotoxic forms.[18] The origin of resistance (or lower susceptibility) to 2,4-D by HWM is not clear. Early studies showed that both EWM and HWM were equally sensitive to 2,4-D;[22] however, more recent research reported lower susceptibility to 2,4-D in HWM but not in EWM.[15,16] The HWM population used in our studies was collected from Hayden Lake in Idaho after several years of treatments with recommended rates of 2,4-D.

At this time, it is difficult to determine whether this population has a higher natural tolerance to this herbicide or has evolved resistance. Therefore, we conducted a series of experiments to identify a possible basis for the differential response to 2,4-D in this population.

Additionally, no information is available regarding the behavior of the two 2,4-D formulations in milfoil spp., including whether 2,4-D BEE is efficacious against the HWM population resistant to 2,4-D; therefore, the objectives of this research were to examine differences in 2,4-D and 2,4-D BEE absorption, translocation, desorption, and metabolism by EWM and HWM to determine if differences in these processes were the possible 2,4-D resistance mechanisms in HWM.

Material and Methods

Plant Material

EWM shoot fragments were collected from the Leggett Canal, north of Boulder, CO (4013’ N, 10508’ W) in fall 2006. HWM shoot fragments were collected from Hayden Lake, Idaho, in spring 2015. This population was selected after several years of poor control by 2,4-D. The genotype of both species were confirmed by a KASP assay.[23] The shoot fragments have been cultured under optimum greenhouse conditions since collection.

To produce uniform plant material for absorption and translocation experiments, 10 cm apical meristem sections from the previously propagated plants were harvested and the distal end of the cutting planted in 16 cm × 12 cm × 6 cm (1,152 cm^3) plastic pots filled with soil collected from Colorado State University's organic research farm and covered by a 1 cm layer of unwashed silica sand. Each pot was fertilized with 2 g of slow-release fertilizer (Osmocote Classic 15-9-12, Everris NA, Inc., USA) and four apical meristem shoots were planted in each pot. Transplanted apical meristem shoots were grown in de-chlorinated tap water in 1.2 m × 1 m × 0.9 m (1,041 L) plastic tanks in the greenhouse until they produced roots (approximately 3 weeks). The photoperiod was 14:10 h light:dark, supplemental lighting was provided with 400-watt sodium halide light bulbs, and the greenhouse temperature was set at 24°C during the day and 18°C at night.

When shoots reached 13-15 cm in length, they were removed from their original pots and the most uniform plants with well-developed root systems were selected for absorption and translocation experiments. Even though HWM plants were transplanted 5 days prior to EWM, HWM were in general 3-5 cm longer than EWM plants at the time of transplants due to its faster growth rate. Roots of selected plants were rinsed with tap water to remove any soil residue and transplanted into 15 mL plastic conical centrifuge test tubes (Thermo Fisher Scientific, USA). Tubes were filled with unwashed silica sand and a layer of low melting point eicosane wax (Eicosane 99%, Fisher Scientific, USA) was applied to the surface to isolate the root system from water column.[24,25] Transplanted plants were kept in the laboratory for two days prior to treatment to acclimate. For desorption and metabolism experiments, non-rooted 10 cm apical shoots were collected from EWM and HWM stock populations. After collection plants were also moved to the laboratory to acclimate for several days.

Absorption and Translocation

Six clear 4 L glass beakers (25 cm tall × 15 cm diam.) were filled with 3 L of dechlorinated tap water (pH 7.1). Three beakers were treated with 1 mg L^{-1} formulated 2,4-D amine (Clean Amine®, Loveland Products) spiked with 37 KBq L^{-1} of ^{14}C-2,4-D amine ring-labeled (55 mCi $mmol^{-1}$ specific activity, American Radiolabeled Chemicals, Inc.). The other three beakers received 1 mg L^{-1} formulated 2,4-D BEE (Navigate®, Applied Biochemists) spiked with 37 KBq of ^{14}C-2,4-D BEE ring-labeled (54 mCi $mmol^{-1}$ specific activity, American Radiolabeled Chemicals, Inc.). The amount of radioactivity present in each beaker was confirmed by collecting a 5 mL sample for each tank, adding 10 mL of scintillation cocktail (Ecoscint XR, National Diagnostics, USA), and analyzed by liquid scintillation spectroscopy (Packard 2300 TR, PerkinElmer, USA; LSS).

Six sealed EWM and 6 sealed HWM plants were added to each tank and maintained in the laboratory at 28°C with 12:12 h light:dark photoperiod, supplemented with two fluorescent grow lights. The solutions were gently stirred two times a day for 2 min each time. Plants were harvested at 6, 12, 24, 48, 96 and 192 h after treatment (HAT). Three replicates were randomly harvested from a different beaker at each time point, triple rinsed in non-treated water, divided into above and belowground tissue, dried at 60°C for at least 48 h to achieve constant moisture, weighted, and oxidized in a biological oxidizer (OX500, R.J. Harvey Instrument Co., USA). The absorbed ^{14}C was collected by a ^{14}C trapping cocktail (OX161, R.J. Harvey Instrument Co., USA). After oxidation, radioactivity was quantified by LSS. The study was repeated in time.

Due to 2,4-D BEE's lipophilicity (log Kow = 5.3), a short time point exposure experiment was conducted with EWM non-rooted 10 cm apical shoots. EWM plants were exposed to ^{14}C-herbicide treatments as previously described and harvested at 2, 4, 8, 16, 32, 64

min after treatment (MAT). Radioactivity absorbed was determined by LSS as previously described.

Desorption

Six clear 1 L glass beakers (15.5 cm tall × 12 cm diam.) were filled with 0.5 L of tap water with pH 6.8 and were treated as previously described. The beakers were stirred for 2 min and the amount of radioactivity in each solution was confirmed as described above using 1 mL aliquots. Six 10 cm apical meristem shoots of each species were exposed to either ^{14}C-2,4-D or ^{14}C-2,4-D BEE for 24 h. Three plants from each tank were harvested, triple rinsed, weighted, and dried, and the other three were triple rinsed and placed each in one 50 mL glass tube containing 40 mL of de-chlorinated tap water. The amount of ^{14}C-2,4-D or ^{14}C-2,4-D BEE desorbing from treated plants was determined by taking 1 mL subsamples at 1, 2, 4, 6, 12, 24, 48 and 72 HAT, and radioactivity was determined by LSS as previously described. After 72 h in the nontreated water and plants were collected, dried, weighted, and oxidized to determine the amount of radioactivity remaining in the plant. Three replicate water samples were collected at each time point and the study was repeated in time.

Metabolism

2,4-D metabolism was adapted from Figueiredo et al (2018)[26]. Briefly, EWM and HWM plants were treated with the same procedures and conditions as used in the desorption experiments. Three plants of each species were harvested at 12, 24, 48, 96, and 192 HAT and at each time point, plant tissue was triple rinsed, rapidly frozen in liquid nitrogen and stored at -20°C. Metabolite extraction was performed by grinding the 10 cm apical meristem shoot with a pestle in a 50 mL tube, then digesting tissue with a 5 mL solution of acetic acid:acetonitrile:water (1:10:89 v/v) on a table shaker for 15 min. Extracts were transferred into

50 mL centrifuge filters with 25 mL microfiltration membranes (pore size of 0.45 μm) and centrifuged at 2,000 *g*. The extraction procedure was repeated two more times. Filters and tissue larger than 0.45 μm were dried at 60°C for oxidation to quantify the non-extracted metabolites. A final extracted volume of 15 mL was passed through to a C18 solid-phase extraction (SPE) cartridge, and a 5 mL aliquot of digestion solution that passed through the cartridge was quantified by LSS. For both EWM and HWM about 99% of radioactivity was retained by the silica matrix and 87% was recovered with 4 mL of acetonitrile and dried under vacuum (Labconco Corporation, Kansas City, MO, USA) at 40°C. Entire extracts were suspended in 500 μL of high-performance liquid chromatography (HPLC) solvent A and filtered into a 1.5 mL centrifuge tubes using a 0.4 μm nylon syringe filter. Filtered solution was transferred to HPLC vials and 200 μL was used for HPLC (Hitachi Instruments, Inc., San Jose, CA, USA) analysis using a 4.6 mm by 150 mm column (C18 Column; Zorbax Eclipse XDB-C18; Agilent Technologies, Santa Clara, CA, USA). Parent compounds and radioactive metabolites were detected using an in-line radioactivity-detector (FlowStar LB 513; Berthold Technologies GmbH & Co., Bad Wildbad, Germany) with a YG-150-U5D solid cell YG-Scintillator flow cell (150 μL, Berthold Technologies). The gradient elution started at 100% mobile phase A containing formic acid:acetonitrile:water (0.1:10:89.9 v/v) to 75 % phase B containing formic acid:acetonitrile (0.1:99.9 v/v) at 15 min. The column was allowed to re-equilibrate for 15 min at a flow rate of 1 mL min^{-1}.

For 2,4-D BEE metabolism EWM and HWM plants were treated as described for 2,4-D metabolism. Three plants of each species were harvested and stored as described previously at 1, 6, 12, and 24 HAT. Metabolite extraction was performed by grinding the 10 cm apical meristem shoot with a pestle in a 50 mL glass tube, then suspending tissue with a 5 mL solution of acetic

acid:water (1:99 v/v) and shaking it vigorously. For liquid-liquid extraction 5 mL of ether was added to the glass tube, vortexed for one min and centrifuged for one min at 1,000 *g*, then the organic phase was transferred to a 20 mL vial. The liquid-liquid extraction process was repeated four times and the organic extract was dried overnight in the fume hood. For both EWM and HWM about 93% of radioactivity were extracted and metabolites not extracted (polar metabolites) were filtered as described before, filter papers were oxidized, and filtered solution were quantified by LSS. Extracted non-polar metabolites were filtered through glass wool into HPLC vials and 200 μL was injected in the HPLC as described before, except the gradient started at 90% A to 90 % B at 10 min. The column was allowed to re-equilibrate for 10 min at a flow rate of 1 mL min^{-1}.

Statistical Analyses

Prior to combining data from repeated experiments for statistical analyses, Levene's test for homogeneity of variance (α = 0.05 level of significance) were performed using R (Version 4.0.0, R Project). Means and standard errors for each experiment were back calculated from dry weight, assuming 90% of water content using MS Excel (MS Office 2016). Data collected from these experiments were analyzed using the MS Excel and plotted with Prism 9 (GraphPad Software, Inc., USA). Absorption, translocation, desorption, and metabolism over time were analyzed using a nonlinear regression analysis to fit a hyperbolic function[27], where *y* is the predicted absorption at time *x*, and *a* and *b* are constants.

$$y = \frac{ax}{b+x} \quad [1]$$

The plant concentration factor (PCF) was calculated to determine bioaccumulation, a metric often used in aquatic plants research to compare absorption across different herbicide

concentrations and in different species. The equation used to calculate PCF was adapted from de Carvalho et al. (2007)[28] and can be defined as:

$$\text{PCF} = \frac{Herbicide\ concentration\ in\ plant\ (\text{ng}/g\ fresh\ biomass)}{Herbicide\ concentration\ in\ water\ (\text{ng}/mL)} \quad [2]$$

The nonlinear regression equations resulting from these analyses were used to calculate two other values (A_{192} and t_{90}). Predicted absorption at 192 HAT (A_{192}) and the predicted time required for 90% of that absorption (t_{90}). The A_{192} value was used to compare the theoretical maximum absorption among different plant parts, plant species, and herbicides, and t_{90} was used to compare the rate of absorption or how quickly the plant absorbed to its maximum.

Results and Discussion

Herbicide Absorption and Translocation

The eicosane wax barrier (previously described)[24] was effective in isolating plant roots from the radiolabeled 2,4-D and 2,4-D BEE treatment solutions. The concentration of ^{14}C-2,4-D and ^{14}C-2,4-D BEE in the treatment solution was 40.71 ± 0.70 Bq mL^{-1} and 38.64 ± 0.13 Bq mL^{-1} respectively, while the amount found in waxed, non-plant test tubes 192 HAT was only 0.27 ± 0.03 Bq mL^{-1} for 2,4-D (0.66 ± 0.07 %) (n=6) and 0.05 ± 0.02 Bq mL^{-1} for 2,4-D BEE (0.13 ± 0.05 %) (n=5). This is the combination of all the samples per experiment and there was no detected radioactivity in five of the combined 11 non-plant test tubes. Based on these data, this small amount of radioactivity did not impact our results or data analyses.

Our primary objective was to investigate possible 2,4-D resistance mechanisms in HWM. We first determined if reduced absorption or translocation of ^{14}C-2,4-D and ^{14}C-2,4-D BEE might have contributed to differential plant response to 2,4-D and if there were any differences between herbicide formulations. Both 2,4-D formulations bioaccumulated in the plant tissues at

concentrations greater than the water column concentration (Figure 2.1A); however, 2,4-D BEE's high n-octanol/water partition coefficient (log K_{ow} 5.3) resulted in 6.3 and 6.6 times greater accumulation of this 2,4-D formulation in EWM and HWM relative to 2,4-D acid (log K_{ow} 2.81), respectively (Table 2.1). Florpyrauxifen-benzyl, another synthetic auxin herbicide used for aquatic plant management, has a log K_{ow} very similar to 2,4-BEE; however, florpyrauxifen-benzyl's PCFs in EWM and HWM 192 HAT were 120 ± 34 and 62 ± 11, respectively.[29] Triclopyr is also an auxin mimic herbicide with aquatic uses, but with a log K_{ow} of -0.44. Interestingly, triclopyr's PCF 192 HAT in EWM was 34.6 ± 5.6.[30] The mechanism responsible for triclopyr's high PCF is unknown.

2,4-D BEE absorption was very rapid and this was reflected by the predicted t_{90} value of 29 and 15 h for EWM and HWM, respectively (Table 2.1); therefore, a short exposure time experiment was conducted with EWM to better understand 2,4-D and 2,4-D BEE bioaccumulation rates (Figure 2.1B). After only 1 h 2,4-D BEE PCF was 16.78 ± 0.32, 30 times greater than 2,4-D's PCF at the same time (0.53 ± 0.01). Even though 2,4-D and 2,4-D BEE bioaccumulation was greater in HWM than in EWM, increased herbicide accumulation does not necessarily equate to better control[25] and therefore, herbicide absorption does not contribute to 2,4-D resistance in HWM. One possible explanation for herbicide greater accumulation in HWM could be due to its faster growth rate and lower herbicidal impacts on plant growth.

Shoot-to-root translocation was approximately three times lower in HWM compared to EWM for both herbicides formulations (Figure 2.2), with only 9.2 ± 0.4 % and 3.8 ± 1.61% of total absorbed radioactivity present in HWM roots 192 HAT for 2,4-D and 2,4-D BEE, respectively. More translocation occurred in EWM, with 26.0 ± 1.68 %, and 11.3 ± 0.88 % of total absorbed radioactivity found in the roots 192 HAT for 2,4-D and 2,4-D BEE, respectively.

Even though triclopyr bioaccumulation was greater than 2,4-D and 2,4-D BEE in EWM, Vassios et. al (2017) found only 2.6 ± 0.3 % radioactivity in its roots. Similarly, florpyrauxifen-benzyl bioaccumulation was greater in EWM and HWM, but translocation from shoots-to-roots was very limited, 0.48 ± 0.28 % and 1.11 ± 0.28 %, respectively.[29] Limited herbicide translocation from shoots-to-roots may be involved in HWM resistance to 2,4-D; however, it is important to note that under field conditions shoots and roots are not separated and depending on the sediment type roots can be more or less exposed to the treated water column.

Herbicide Desorption

Herbicide absorption continued to increase over time in controlled environment experiments because there is no herbicide dilution or degradation; however, it is important to note that herbicides can diffuse out of the plant when external herbicide concentrations decrease due to water exchange or herbicide degradation.[31] When treated plants were transferred to non-treated water, radioactivity was detected in the clean water. Herbicide desorption was slightly greater in EWM than HWM for both herbicide formulations (Figure 2.3), with 55.07 ± 0.99 % and 63.41 ± 1.47% of total absorbed radioactive herbicide being desorbed 72 HAT for 2,4-D and 2,4-D BEE, respectively. Less desorption occurred in HWM, with 45.34 ± 1.63 %, and 59.27 ± 1.28 % of total absorbed radioactive herbicide desorbing 72 HAT for 2,4-D and 2,4-D BEE, respectively. Similarly, the systemic herbicide imazamox (log K_{ow} 0.73) is quickly desorbed from EWM, with 46% of absorbed imazamox readily moving out of the plant into the water column by 12 HAT.[31] Another study looking at aquatic herbicide desorption demonstrated that although endothall's log K_{ow} is -0.55, it binds irreversibly to several serine/threonine protein phosphateses; therefore, endothall's desorption in EWM by 96 HAT was only 28.92 ± 15.63 % well below equilibrium with the water column.[25]

Even though HWM desorption was slightly lower than EWM, 2,4-D and 2,4-D BEE desorption in both plant species is driven mainly by a concentration gradient, and the establishment of a dynamic equilibrium between the shoots and water column; therefore, herbicide desorption does not appear to play a role in 2,4-D resistance in HWM.

Metabolism

To determine if 2,4-D metabolism was a factor in the resistance mechanism of this HWM population, we measured 2,4-D metabolism over 192 h. In the 2,4-D HPLC protocol, 2,4-D acid analytical standard eluted as a single peak with a retention time (RT) of 13.2 min. 2,4-D BEE metabolism was also measured over 24 h time course. In the 2,4-D/2,4-D BEE HPLC protocol, 2,4-D and 2,4-D BEE had RT of 8.3 min and 12.6 min, respectively, with no other peaks observed. Any other radioactive peaks that did not correspond to the standard herbicide RT are products assumed to be derived from herbicide metabolism.

At 192 HAT, the same proportions of 2,4-D and one main metabolite (metabolite 1) with RT of 10.5 min were detected in EWM and HWM (Figure 2.4A and 2.4B). No attempt was made to identify the metabolite. Similarly, Figueiredo et al (2018) found a peak with the same RT for a main metabolite in a susceptible and 2,4-D resistant common waterhemp (*Amaranthus tuberculatus*) populations, where at 264 HAT the same amount of 2,4-D and metabolite 1 were detected in the susceptible population. For 2,4-D BEE by 1 HAT most of the ester compound was rapidly converted into the free acid (Figure 2.4C) and by 24 HAT no 2,4-D BEE was detected in either plant species. 2,4-D BEE absorption occurs very rapidly and by 24 h the herbicide absorption reached a maximum asymptote (Figure 2.1B), therefore the absence of 2,4-D BEE in plants by 24 h is not surprising and the reason why we only analyzed plants 24 HAT. After the *in planta* conversion of the ester into the free acid form, 2,4-D's metabolism in both

plant species occurs in the same manner as it does when the 2,4-D acid formulation is applied (data not shown). Both herbicide formulations were metabolized at the same rate in both plant species; therefore, metabolism does not appear to contribute to 2,4-D resistance in HWM.

Conclusions

Herbicide resistance mechanisms have been categorized into two types, (a) non-target-site resistance (NTSR), involving decreased absorption or translocation and/or enhanced herbicide sequestration or metabolism to inactive metabolites, and (b) target-site resistance (TSR), resulting from mutations in the genes encoding the protein targets of the herbicides or increases in levels of the target protein through gene amplification or transcriptional upregulation.[32]

We examined four possible NTSR mechanisms, and while decreased absorption is not a common NTSR mechanism, it has been reported with resistance of prickly lettuce (*Lactuca serriola*) to 2,4-D.[33] When reduced herbicide absorption is implicated, it is most often associated to another factor, such as reduced translocation, and many systemic herbicides such as 2,4-D rely on translocation through the phloem for optimal activity. Reduced 2,4-D translocation was observed in the 2,4-D-resistant population of *L. serriola*, relative to a susceptible population,[33] as well as in 2,4-D-resistant wild radish (*Raphanus raphanistrum*).[34] Naturally occurring auxins such as IAA are polar and readily translocate in the symplast, so it is reasonable that a synthetic auxin such as 2,4-D also requires adequate translocation for optimal activity. Lastly, plants contain large numbers of genes encoding enzymes that detoxify xenobiotic compounds, and some of these gene families can also detoxify herbicides by rapid metabolism of the active ingredients into non-herbicidal products.[32] More rapid 2,4-D metabolism was observed in a 2,4-D-resistant population of *A. tuberculatus*, relative to a susceptible population.[26]

Auxinic herbicide resistance in wild mustard (*Sinapis arvensis* L.),[35] false cleavers (*Galium spurium* L.),[36] kochia (*Bassia scoparia* L.),[37] and yellow starthistle (*Centaurea solstitialis* L.)[38,39] was also not due to differences in herbicide absorption, translocation and/or metabolism. Auxin herbicide resistance in these species probably results from other mechanisms, such TSR. More research is needed to identify the molecular basis for the 2,4-D-resistant trait and to assess other physiological aspects of 2,4-D resistance in HWM.

Table 2.1. Predicted plant concentration factor 192 HAT (PCF_{192}), herbicide absorption ($\mu g\ g^{-1}$) at 192 HAT (A_{192}), and the time in hours required to reach 90% of A_{192} (t_{90}). Values represent the mean, and error terms represent the standard error of the mean (n = 6).

Herbicide	Species	Plant Part	PCF_{192}	A_{192}	t_{90}
2,4-D	EWM	Aboveground	5.66 ± 0.22	49.5 ± 2.81	145
		Belowground		14.2 ± 1.03	148
	HWM	Aboveground	7.88 ± 0.23	75.1 ± 4.65	155
		Belowground		8.51 ± 0.65	136
BEE	EWM	Aboveground	35.61 ± 1.33	132.9 ± 7.04	29
		Belowground		10.16 ± 1.46	100
	HWM	Aboveground	52.11 ± 1.09	206.4 ± 8.81	15
		Belowground		8.49 ± 0.57	125

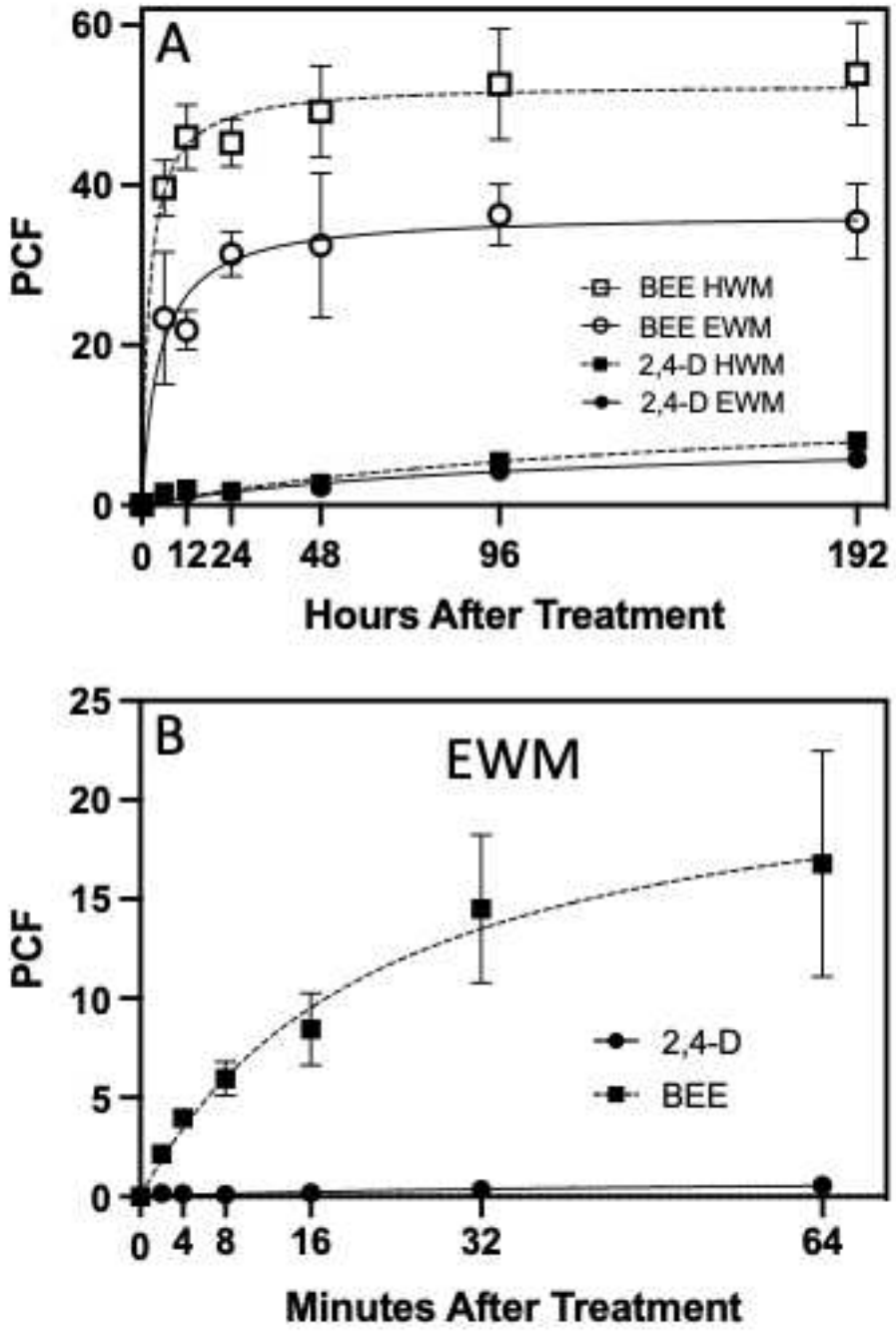

Figure 2.1. Herbicide concentration in plants, expressed as plant concentration factor (PCF). Herbicide bioaccumulation in EWM and HWM over 192 h time course for (A) ^{14}C-2,4-D and ^{14}C-2,4-D BEE. (B) ^{14}C-2,4-D and ^{14}C-2,4-D BEE bioaccumulation in EWM over 64 m time course. Data presented are means, and error bars are the standard error of the mean (n=6).

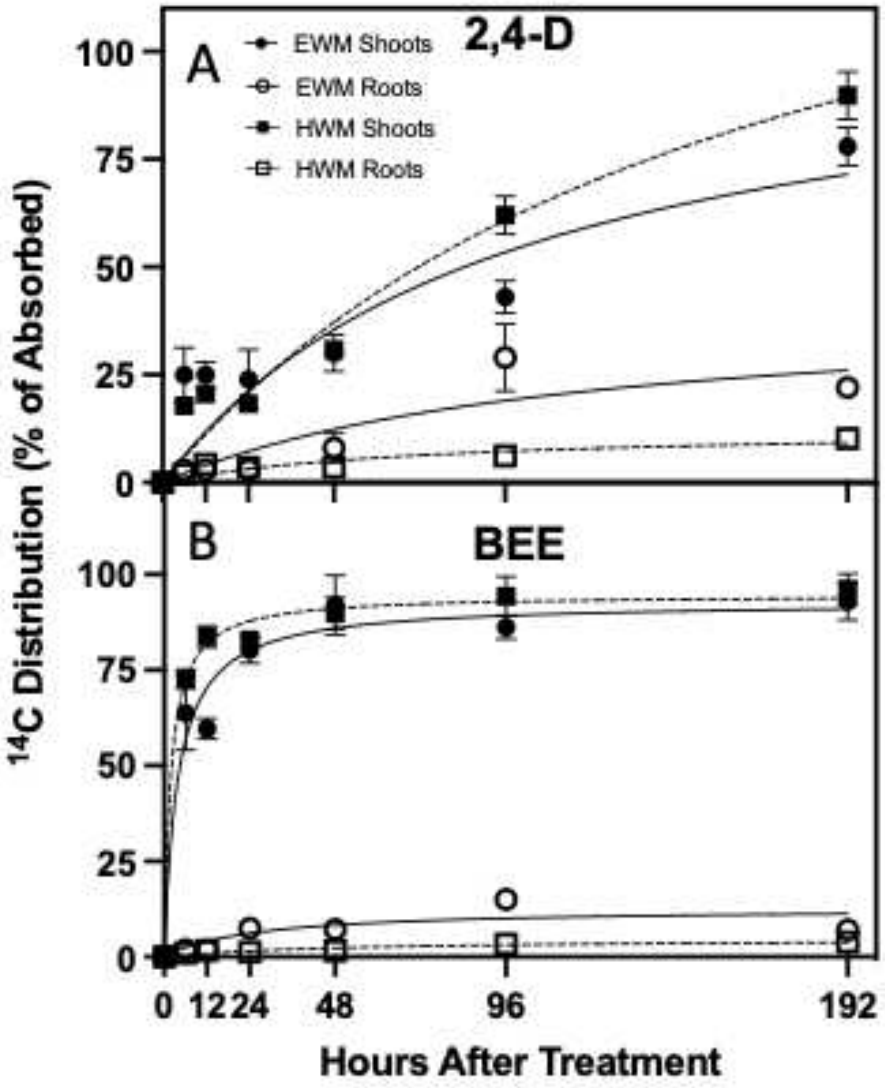

Figure 2.2. ^{14}C distribution in plants over 192 h following exposure to (A) ^{14}C-2,4-D or (B) ^{14}C-2,4-D BEE, expressed as percentage of total herbicide absorbed. Closed circles and squares are the percentage of herbicide in the shoots; open circles and squares are the percentage of herbicide in the roots. Data presented are means, and error bars are the standard error of the mean (n=6).

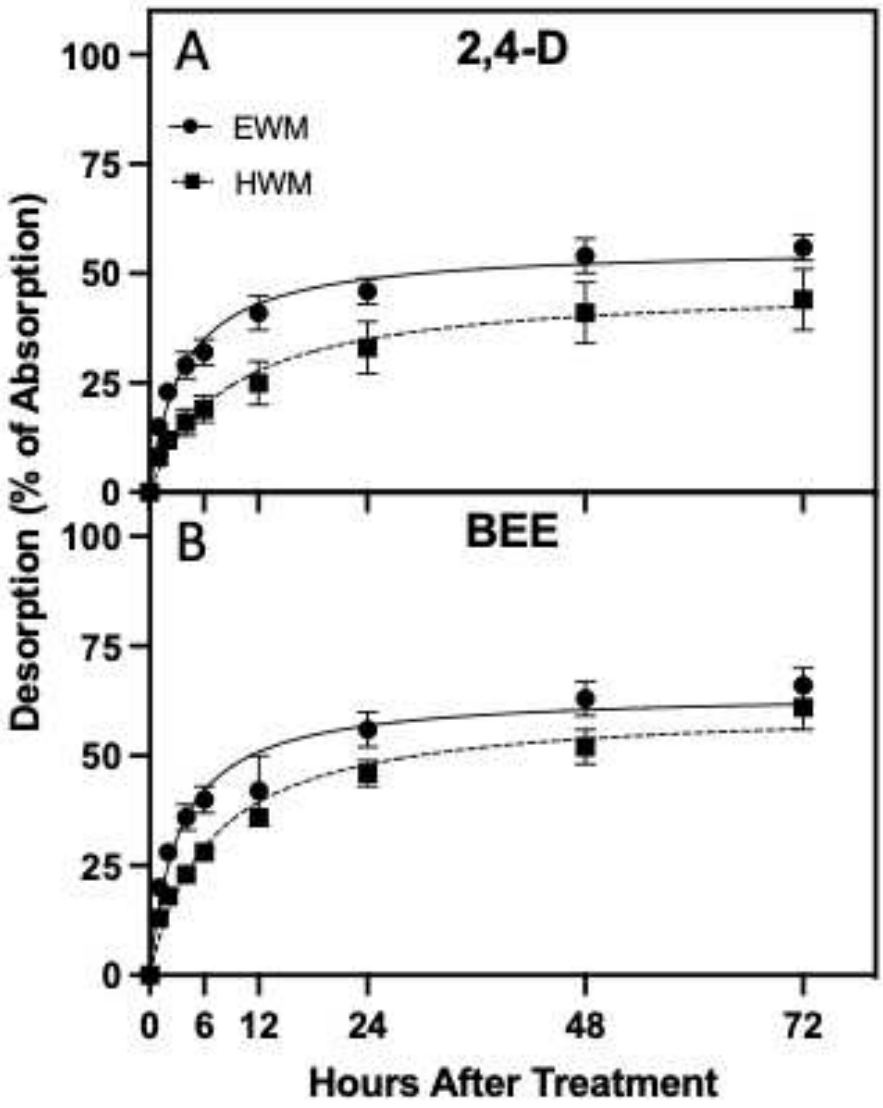

Figure 2.3. Desorption of (A) ^{14}C-2,4-D and (B) ^{14}C-2,4-D BEE over 72 h, expressed as a percentage of total absorbed ^{14}C following a 24 h initial exposure. Data presented are means, and error bars are the standard error of the mean (n=6).

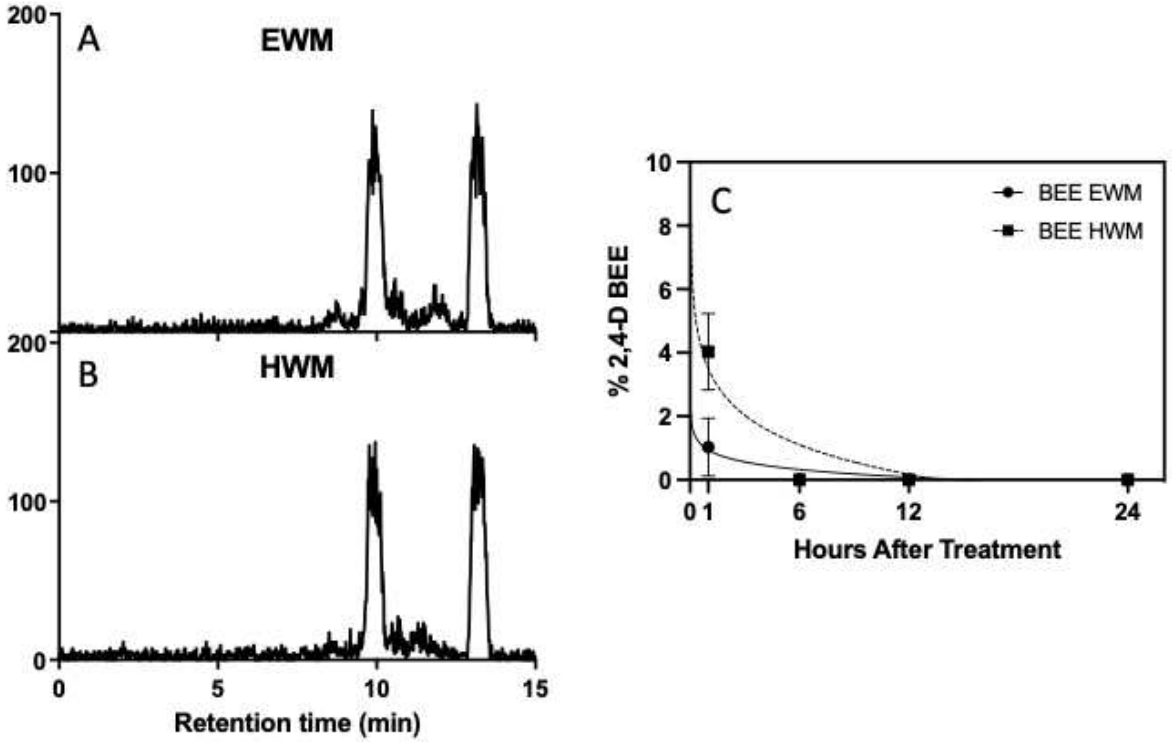

Figure 2.4. Metabolism of ^{14}C-2,4-D and ^{14}C-2,4-D BEE in EWM and HWM. Chromatograms of ^{14}C-2,4-D at 192 HAT in (A) EWM and (B) HWM (radioactive unites in Bq versus retention time in minutes). (C) Percentage of ^{14}C-2,4-D BEE in plants over 24 h time course.

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CHAPTER 3: ENDOTHALL AND 2,4-D BEHAVIOR IN HYBRID WATERMILFOIL WHEN APPLIED IN COMBINATION

Introduction

Eurasian watermilfoil (*Myriophyllum spicatum* L.; EWM) is a widespread invasive aquatic plant species across the United States (US). Its management is one of the most extensive and expensive among aquatic invasive plants (Gettys et al. 2020; Pimentel 2009). EWM can hybridize with native northern watermilfoil (*Myriophyllum sibiricum* Kom.), and many populations originally identified as invasive EWM were later confirmed to be hybrids (*M. spicatum* × *M. sibiricum*; HWM) (Moody and Les 2002; Sturtevant et al. 2009). HWM infestations can rapidly displace native plant communities resulting in a dense monotypic vegetation that forms undesirable surface canopies. A severe HWM infestation can negatively affect water quality, altering native aquatic habitats, reducing native fish and macroinvertebrate diversity, and impairing recreational uses of the water, such as fishing, boating, and swimming (Madsen et al. 1991; Newroth 1985; Schultz and Dibble 2012; Smith and Barko 1990).

HWM grows more aggressively than either parent, requiring intensive management (Glisson and Larkin 2021; Taylor et al. 2017; Thum and McNair 2018), and while there are several control strategies for aquatic invasive plants, the use of herbicides is one of the most important management options. The synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) and the serine/threonine protein phosphatase inhibitor, endothall (7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid) are herbicides typically used to manage HWM (Getsinger et al. 1982; Netherland et al. 1991; Wersal et al. 2010). The herbicide 2,4-D is intensively used for HWM control as it is one of the least expensive management options and is generally selective for

dicots, with minimal impacts to monocots. The extensive use of 2,4-D eventually selected for HWM with reduced sensitivity to 2,4-D (Ortiz et al. 2021). Similarly, endothall, a broad-spectrum herbicide, is widely used for large-scale and spot treatments of HWM. These two herbicides are also often used in combination at low concentrations to improve selective control in a single treatment event (Skogerboe and Getsinger 2006).

Combining herbicides with different modes of action (MOA) or using herbicide rotations is widely recommended to delay herbicide resistance evolution (Beckie and Reboud 2009). In terrestrial studies and computer simulations, mixtures were the most effective measure at delaying resistance evolution (Beckie and Reboud 2009; Busi et al. 2020; Evans et al. 2018). Herbicide mixtures ensure that sensitive weeds, terrestrial or aquatic, are treated with two different MOA and weeds resistant to one herbicide MOA are likely to be killed by another herbicide acting at a different site of action (Busi et al. 2020).

In addition to helping to delay herbicide resistance evolution, tank-mixes or pre-mixed herbicides can reduce application costs and may synergistically improve herbicide efficacy. Although this is an effective and popular strategy in terrestrial systems, herbicides must be compatible with each other. In some cases, herbicides may be antagonistic (ACCase with 2,4-D as an example) resulting in reduced weed control. In other cases, different herbicide formulations may not stay in solution or congeal upon mixing, for example when off-the-shelf formulations of endothall and 2,4-D are combined. Chinook® is a pre-mix of endothall and 2,4-D developed to alleviate this problem. With a formulation like Chinook®, the ratio of endothall and 2,4-D are fixed, but it is a great tool for applicators to reduce their application costs while still taking advantage of two different MOA.

To date, only 15 herbicides and nine different MOA are registered for aquatic use (Ortiz et al. 2020). Consequently, when resistance develops in aquatic weeds, the options for using alternative herbicide MOA are very limited. Applying herbicide mixtures is the recommended strategy to avoid or delay evolution of herbicide resistance (Bourguet et al. 2013; Lagator et al. 2013a; Lagator et al. 2013b). This concept is being implemented in aquatic weed management practices; yet there is very little information about how herbicide behavior might change when products are applied as mixtures. There are examples of both herbicide antagonism and synergism in terrestrial weed management, and the same can occur in aquatic systems (Kyser et al. 2021; Wersal and Madsen 2010; Wersal and Madsen 2012). To better understand how herbicide behavior might change when used in combination, we investigated the behavior of endothall and 2,4-D applied alone and in combination, therefore, the objectives of this research were to determine herbicide absorption, accumulation and translocation patterns alone and in combination over a 96 h time course in HWM.

Material and Methods

Plant Material

HWM plants used in the experiments were grown vegetatively from shoot fragments collected in 2015 from Hayden Lake, Idaho. The hybrid genotype of the plants was previously confirmed (Patterson et al. 2017). Uniform plant material was obtained by propagating 10 cm apical sections of these plants in 16 cm × 12 cm × 6 cm (1,152 cm^3) plastic pots filled with organic soil (Colorado State University Organic Research Farm). Each pot received 2 g of slow-release fertilizer (Osmocote Classic 15-9-12, Everris NA, Inc., USA) prior to transplanting apical meristem shoots in each pot. Pots were placed in 1.2 m × 1 m × 0.9 m (1,041 L) plastic tanks with unwashed play sand at the top and grown in de-chlorinated tap water under greenhouse

conditions. The photoperiod was 14:10 h light:dark, supplemental lighting was provided with 400-watt sodium halide light bulbs, and the greenhouse temperature was set at 24°C during the day and 18°C at night.

When apical shoots reached 15-18 cm in length (approximately two weeks after progagation), plants with well-developed roots were selected for absorption and translocation experiments. Roots were cleaned with tap water and transplanted into 15 mL plastic conical centrifuge tubes (Thermo Fisher Scientific, USA). The tubes were filled with unwashed silica sand and sealed with a low melting point eicosane wax (Eicosane 99%, ACROS Organics, USA) to isolate the root system from water column (Frank and Hodgson 1964; Ortiz et al. 2019). Plants were transferred to 4 L plastic tanks (22.7 cm tall × 17 cm diameter) filled with dechlorinated water for a 24 h acclimatization to the laboratory environment prior to initiating the labeling experiments.

Herbicide Exposure

Twelve 4 L glass beakers (25 cm tall × 15 cm diam.) were filled with 3.5 L of dechlorinated tap water (pH 6.8). Six beakers were treated with ^{14}C-endothall ring-labeled (56.6 mCi mmol^{-1} specific activity, Moravek, Inc.). Three of these beakers were treated with ^{14}C-endothall combined with formulated dipotassium salt of endothall (Cascade®, UPL NA Inc.) to achieve a final concentration of 0.75 mg L^{-1} in the water column and the other three beakers were treated with ^{14}C-endothall combined with formulated, non-radiolabeled pre-mix herbicide of endothall and 2,4-D (Chinook®, UPL NA Inc.) to achieve a final concentration of 0.75 and 0.3 mg L^{-1} of endothall and 2,4-D, respectively. The other six beakers were treated with ^{14}C-2,4-D ring-labeled (55 mCi mmol^{-1} specific activity, American Radiolabeled Chemicals, Inc.). Three of these beakers were treated with ^{14}C-2,4-D combined with formulated 2,4-D (Clean Amine®,

Loveland Products) to achieve a final concentration of 0.3 mg L^{-1} in the other water column and the other three beakers were treated with ^{14}C-2,4-D combined with formulated, non-radiolabeled pre-mix herbicide of endothall and 2,4-D as described before.

Each treatment was replicated 3 times for a total of 12 treatment beakers. Each ^{14}C-endothall treated tank contained 36.3 ± 1.0 KBq L^{-1}, while each ^{14}C-2,4-D treated tank 37.8 ± 1.7 KBq L^{-1}. The radioactivity in each treatment tank was confirmed using a liquid scintillation spectroscopy (LSS) (Packard 2500R, PerkinElmer, USA).

Each beaker contained 6 HWM plants, and one tube with a toothpick simulating a plant stem as a control to test the wax barrier efficacy. All plants were held by a round test tube rack (No-Wire Round Rack, Bel-Art Scienceware, USA). During the experiment, plants were maintained in the laboratory, at 22°C, with 14:10 h light:dark period, supplemented with a LED grow lights. Beakers were stirred once a day and treatment water volume was maintained by adding more water to the tanks daily. Plants were harvested at 6, 12, 24, 48, and 96 h after treatment (HAT). Three replicates were randomly harvested from a different tank at each time point, rinsed four times in clean, dechlorinated tap water, and divided into shoots and roots. After separation, plant parts were dried at 60°C for at least 48 h, dry biomass was recorded for each plant part, plant tissues were combusted in a biological oxidizer (OX500, R.J. Harvey Instrument Co., USA) for 2 minutes, and the resulting $^{14}CO_2$ was captured by a ^{14}C trapping cocktail (OX161, R.J. Harvey Instrument Co., USA). The efficiency of the oxidizer was tested before oxidizing plant parts and it was always greater than 98%. After oxidation, radioactivity was quantified by LSS. The study was repeated.

Statistical Analyses

Data collected from these experiments were analyzed using RStudio (Version 1.4.1717) and MS Excel and plotted with Prism 9 (GraphPad Software, Inc., USA). Levene's test for homogeneity of variance ($\alpha = 0.05$ level of significance) was performed using the car package in R (Version 4.0.0, R Project) to confirm that data from repeated experiments could be combined. For all experiments, fresh weight was converted to dry weight values assuming 90% water content. Absorption and translocation over time were analyzed using a nonlinear regression analysis to fit a hyperbolic function (Kniss et al. 2011) as described before.

Bioaccumulation of herbicides was estimated by calculating the plant concentration factor (PCF) using an equation was adapted from de Carvalho et al. (2007) presented by Vassios et al. (2017). PCF is often used to compare herbicide absorption across different herbicide concentrations and in different aquatic plant species. The predicted absorption at 96 HAT (A_{96}) and the predicted time required for 90% of that absorption (t_{90}) were derived from the nonlinear regression equations of these analyses. The A_{96} value is a measure of the theoretical maximum absorption among different plant parts, plant species, and herbicides. The t_{90} value is a measure of the rate of absorption.

Results and Discussion

Plant roots were effectively isolated from the radiolabeled treatment solutions through the eicosane wax barrier. Only 0.029 ± 0.009 Bq mL^{-1} (n=6) and 0.021 ± 0.008 Bq mL^{-1} (n=6) of radioactivity was detected in the non-plant toothpick test tubes 96 HAT for ^{14}C-endothall and ^{14}C-2,4-D treatments, respectively. There was no detected radioactivity in seven of the 12 combined test tubes. Based on these data, this small amount of radioactivity did not impact the results of the study.

Endothall absorption did not reach a maximum asymptote when applied alone or in the presence of 2,4-D (Figure 3.1). Although the asymptotic rise to max function is the most biologically relevant function to describe herbicide absorption (Kniss et al. 2011), previous research also demonstrated that endothall at 3 mg L^{-1} did not reach maximum asymptote in EWM or hydrilla (*Hydrilla verticillata* (L.f.) Royle) 192 HAT (Ortiz et al. 2019).

Bioaccumulation of ^{14}C-endothall did not change in the presence of 2,4-D. At 96 HAT the PCF_{96} was 12.0 ± 0.6 when applied alone and 13.2 ± 0.6 in the presence of 2,4-D (Figure 3.1). These values were not statistically different. Endothall bioaccumulation at 3 mg L^{-1} 192 h was only 3.3 ± 0.4 in EWM (Ortiz et al. 2019). The reason for greater herbicide accumulation in this study is likely due to be the difference in herbicide rate. The lower concentration may have allowed the plant to be more physiologically active, maintaining a stronger concentration gradient for a longer timer period. Based on HWM's increased growth rate compared EWM, HWM appears to be more physiologically active.

The n-octanol/water partition coefficient (log K_{ow}) of endothall is very similar to triclopyr and penoxsulam (−0.55, −0.45 and -0.35, respectively), which should translate to a similar PCF, but it varied greatly in EWM and hydrilla (Ortiz et al. 2019; Vassios et al. 2017). However, de Carvalho et al. (2007) demonstrated that in aquatic plants, log K_{ow} values <2 are not reliable predictors of herbicide accumulation and increased herbicide accumulation does not necessarily correlate to better plant control (Ortiz et al. 2019).

The PCF_{96} for ^{14}C-2,4-D alone at 0.3 mg L^{-1} was 6.9 ± 0.3 (Figure 3.1). Previous research reported that 2,4-D accumulation at 1 mg L^{-1} 192 HAT was 5.7± 0.2 and 7.88 ± 0.2 for EWM and HWM, respectively (Ortiz et al. 2021). When in the presence of 0.75 mg L^{-1} endothall, ^{14}C-2,4-D bioaccumulation in HWM increased to 12.5 ± 0.6 (Table 3.1). Endothall caused a similar

increase in foliar absorption of ethephon, another plant growth regulator, in bean leaves (Sterrett et al. 1974).

Endothall absorption was calculated at 96 HAT (A_{96}) by a correction factor based on the ratio of radiolabeled:non-radiolabeled herbicide. HWM A_{96} was 63.3 ± 1.9 µg g^{-1} (Table 3.1), and it was not impacted when in combination with 2,4-D (74.0 ± 2.0 µg g^{-1}). In contrast, 2,4-D absorption increased significantly in the presence of endothall, 16.9 ± 1.2 µg g^{-1} and 36.7 ± 1.9 µg g^{-1}, alone and in combination with endothall, respectively (Table 3.1).

Endothall shoot-to-root translocation, estimated by the presence of radioactivity, was 16.7% ± 2.6 when applied alone (Figure 3.2). This is approximately twice the amount of translocation previously reported for EWM (Ortiz et al 2019). The current study used a lower endothall concentration and the more aggressive HWM, so these differences are not unexpected. The combination of endothall plus 2,4-D reduced endothall translocation by 50% (9.2% ± 1.2) (Figure 3.2). While this difference is statistically significant, it may not have any significant impact on the biological and operational usefulness of endothall.

Shoot-to-root translocation of 2,4-D was 24.8% ± 2.6 when applied alone, but only 3.93% ± 0.4 when applied in combination with endothall (Figure 3.2). This is the first study to measure shoot-to-root herbicide translocation in aquatic plants when two herbicides were applied in combination. As previously mentioned, our research did not evaluate the biological impacts of these herbicide interactions. Endothall limits basipetal 2,4-D transport in detached bean leaves (Leonard and Glenn 1968); however, we can only speculate that a similar process might occur in aquatic plants.

In conclusion, both endothall and 2,4-D's behavior are significantly impacted by each other's presence. These differences included greater herbicide absorption and reduced herbicide

translocation to the roots of HWM. Future research needs to be conducted to determine if this reduced translocation negatively affects the overall effectiveness of this control strategy.

Table 3.1. Predicted plant concentration factor 96 hours after treatment (HAT) (PCF_{96}), herbicide absorption ($\mu g\ g^{-1}$) at 96 HAT (A_{96}), and the time in hours required to reach 90% of A_{96} (t_{90}). Values represent the mean, and error terms represent the standard error of the mean (n=6)

Treatment	PCF_{96}	Plant Part	A_{96} ($\mu g\ g^{-1}$)	t_{90} (hours)
^{14}C-endothall	12.0 ± 0.6	Shoots	63.3 ± 1.9	78.3
		Roots	12.2 ± 2.1	75
^{14}C-endothall + 2,4-D	13.2 ± 0.6	Shoots	74.0 ± 2.0	84.6
		Roots	7.9 ± 0.8	73.8
^{14}C-2,4-D	6.9 ± 0.3	Shoots	16.9 ± 1.2	70.7
		Roots	5.6 ± 0.7	81.8
^{14}C-2,4-D + endothall	12.5 ± 0.6	Shoots	36.7 ± 1.9	69.1
		Roots	1.3 ± 0.08	61

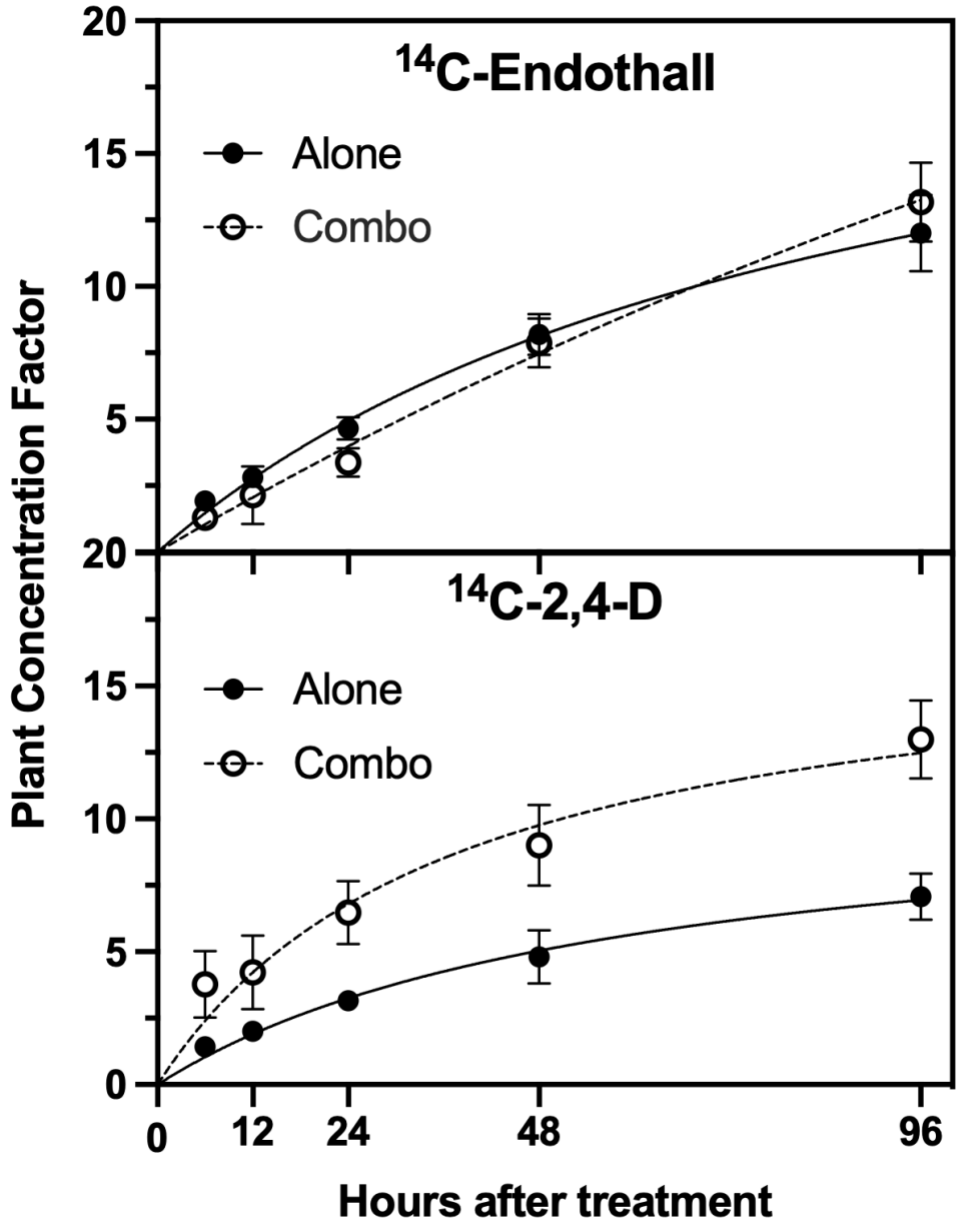

Figure 3.1. ^{14}C-endothall and ^{14}C-2,4-D bioaccumulation in HWM over 96 h time period expressed as plant concentration factor (PCF). Data presented are means and standard error of the mean (n=6)

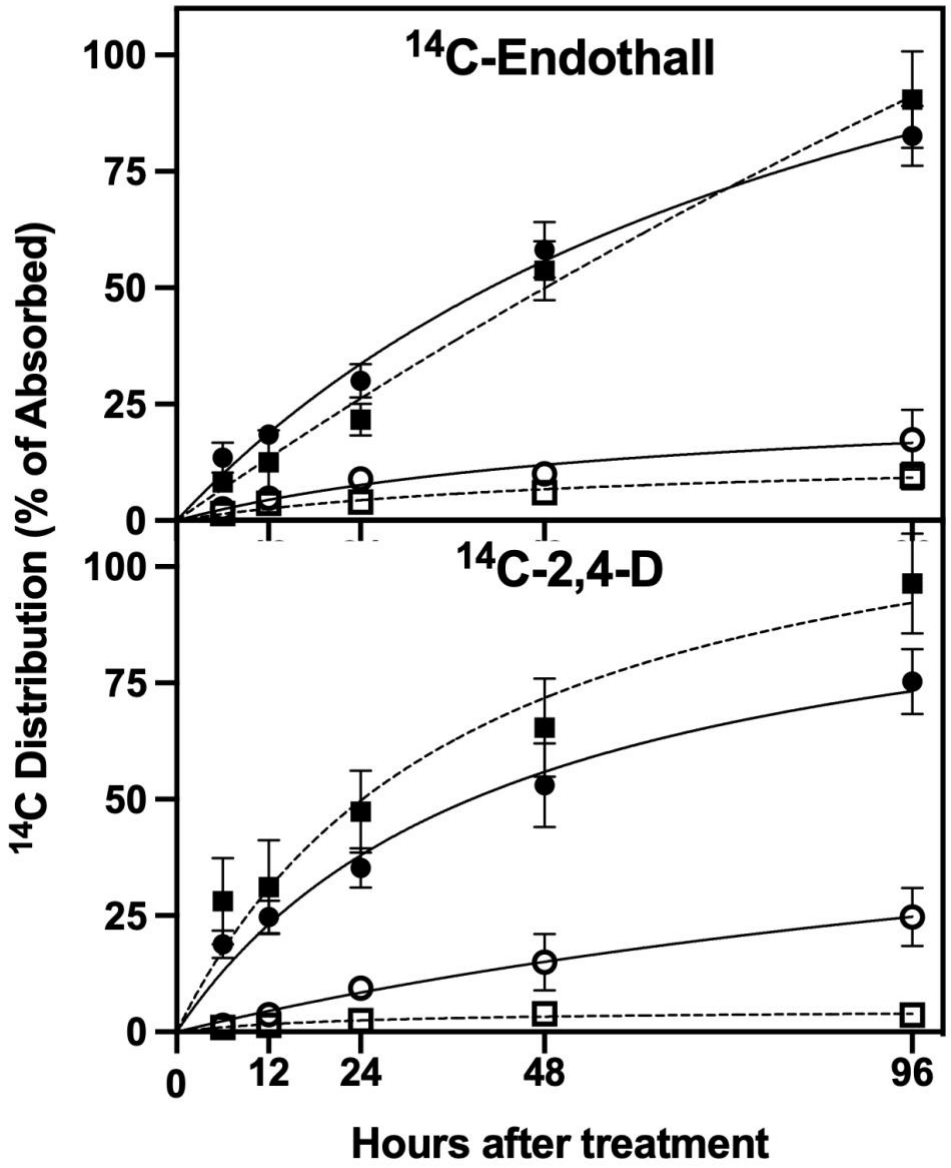

Figure 3.2. ^{14}C-herbicide distribution in plants over 96 h following exposure to ^{14}C-endothall or ^{14}C-2,4-D, expressed as percentage of total herbicide absorbed. ● = percentage of ^{14}C alone in shoots; ○ = percentage of ^{14}C alone in roots; ■ = percentage of ^{14}C-herbicide in combination with non-radiolabeled 2,4-D or endothall in shoots; □ = percentage of ^{14}C-herbicide in combination with non-radiolabeled 2,4-D or endothall in roots. Data presented are means, and error bars are the standard error of the mean (n=6).

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CHAPTER 4: ENDOTHALL AND FLORPYRAUXIFEN-BENZYL BEHAVIOR IN *Hydrilla verticillata* WHEN APPLIED IN COMBINATION

Introduction

Hydrilla [*Hydrilla verticillata* (L.f.) Royle] is a submersed, invasive plant that has been described as the "perfect aquatic weed" due to numerous physiological adaptations that make it highly aggressive and competitive (Langeland 1996). Both monoecious and dioecious hydrilla biotypes are present in the US and they have spread significantly from their initial introduction sites (Cook and Lüönd 1982). Dioecious hydrilla (DHV) was first introduced in Florida as an aquarium plant in the 1950s and is commonly found in the southern US, while monoecious hydrilla (MHV) was first reported near Washington DC and Raleigh NC in 1980 and occurs from North Carolina northward (Gettys et al. 2020; Gettys and Leon 2021).

Prior to 1986, the serine/threonine protein phosphatase inhibitor, endothall (7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid) (HRAC group 31; Bajsa et al. 2012) was frequently used for hydrilla control at concentrations of several mg L^{-1} (ppm) in the water column. Following its registration for aquatic use in 1986, fluridone (1-methyl-3-phenyl-5[3-(trifluoromethyl)phenyl]-4(1H)-pyridinone) (HRAC group 12) became the primary tool for hydrilla management because of its high efficacy and ability to control tuber production (Nawrocki et al. 2016). Fluridone is a bleaching herbicide that inhibits the carotenoid synthesis pathway by targeting phytoene desaturase. It is applied to the water column in μg L^{-1} (ppb) and it was used intensively for decades (Dayan and Netherland 2005; Nawrocki et al. 2016). Fluridone's extensive use in Florida, without any herbicide rotation or mixture, eventually selected for fluridone-resistant DHV in the late 1990s (Arias et al. 2005; Dayan and Netherland

2005; Michel et al. 2004; Netherland and Jones 2015; Puri et al. 2007). Only the female form of DHV is found in the US and its spread is limited to vegetative reproduction (Michel et al. 2004). Fluridone resistance has been the result of a somatic mutation, and this is the first case of evolved herbicide resistance in a plant that does not rely on sexual reproduction (Ortiz et al. 2020).

The development of fluridone-resistant DHV reverted management strategies back to including endothall as the standard for the last two decades (Sperry et al. 2021). The overreliance on a single, effective mode of action (MOA), without rotations or mixtures, has led to suspected endothall resistance in some hydrilla populations in central Florida as well (Giannotti et al. 2014).

The loss of fluridone for DHV management and the potential for new market opportunities were a driving force behind identifying and registering several new herbicide MOA for aquatic plant management (Getsinger et al. 2008; Ortiz et al. 2020). In 2018, florpyrauxifen-benzyl (2-pyridinecarboxylic acid, 4-amino-3-chloro-6-(4-chloro-2-fluoro-3-methocy-phenyl)-5-fluoro-, phenyl methyl ester) (HRAC group 4; Epp et al. 2016) was registered for aquatic use. Florpyrauxifen-benzyl is an auxin-mimic herbicide highly active in hydrilla, even though it is monocotyledons (Beets et al. 2019). Auxin-mimics herbicides are generally active on dicots, with minimal impact to monocots. Florpyrauxifen-benzyl is an excellent tool for hydrilla control as it was classified as a reduced-risk pesticide by the U.S. Environmental Protection Agency (Epp et al. 2016).

Herbicide rotations and mixtures are widely recommended to mitigate the evolution of herbicide resistance (Beckie and Reboud 2009). Terrestrial field research and computer simulation models suggest that combining herbicide modes of action as mixtures are the most

effective measure at delaying resistance evolution (Beckie and Reboud 2009; Busi et al. 2020; Evans et al. 2018). Herbicide mixtures ensure that weeds, terrestrial or aquatic, are treated with two different MOA and weeds potentially resistant to one herbicide mode of action are still controlled by another herbicide acting at a different site of action (Busi et al. 2020).

To date, only 15 herbicides and nine different MOA are registered for aquatic use (Ortiz et al. 2020). Consequently, when resistance develops in aquatic weeds, the options for alternative chemical controls are very limited. To reduce the opportunity for aquatic weeds to evolve resistance there has been more interest in applying herbicide mixtures; however, there is very little information about how herbicide behavior might change when products are applied as mixtures. There are examples of both herbicide antagonism and synergism in terrestrial weed management and the same could occur in aquatic systems (Kyser et al. 2021; Wersal and Madsen 2010; Wersal and Madsen 2012). To better understand how herbicide behavior might change when used in combination, we investigated the behaviors of endothall and florpyrauxifen-benzyl applied alone and in combination. The objectives of this research were to determine herbicide absorption and translocation patterns when these herbicides were applied alone and in combination in MHV and DHV over a 192-h time course.

Material and Methods

Plant Material

MHV and DHV tubers, were collected from Shearon Harris Lake, North Carolina (35.61°N, 78.95°W), and Orange Lake, Florida (29.46°N, 82.17°W), respectively in spring 2016 and cultured under greenhouse conditions for the last five years. To produce uniform plant material for this research, 10 cm apical sections were cut from the previously propagated plants and the distal end was planted in 16 cm × 12 cm × 6 cm (1,152 cm^3) plastic pots filled with soil

collected from Colorado State University's organic research farm. Each pot was fertilized with 2 g of slow-release fertilizer (Osmocote Smart Release® Plant Food 15-9-12, The Scotts Company LLC, 14111 Scottslawn Road, Marysville, OH 43040) and six apical meristem shoots were planted in each pot for a total of 16 pots. Pots were covered by a 1 cm layer of washed sand. Plants were grown in dechlorinated tap water in 1.2 m × 1 m × 0.9 m (1,041 L) plastic tanks under greenhouse conditions until they produced roots. The photoperiod was 14:10 h light:dark, supplemental lighting was provided with 400-watt sodium halide light bulbs, and the greenhouse temperature was set at 24°C during the day and 18°C at night.

Approximately three weeks propagated apical shoots reached 13-15 cm in length. Shoots were removed from their original pots and plants with well-developed roots were selected for absorption and translocation experiments. Roots were washed with tap water to remove soil residue and replanted into plastic test tubes (15 mL Conical Centrifuge Tubes, Thermo Fisher Scientific, USA). Test tubes were filled with unwashed silica sand. After transferring plants into test tubes, a low melting point eicosane wax (Eicosane 99%, ACROS Organics, USA) was used to seal the top of the tube to isolate the root system from water column (Frank and Hodgson 1964; Ortiz et al. 2019). Plants were moved to 4 L plastic tanks (22.7 cm tall × 17 cm diameter) filled with dechlorinated tap water and were allowed to acclimate for 24 h in the laboratory prior to herbicide treatment.

Herbicide Exposure

Twelve 4 L glass beakers (25 cm tall × 15 cm diam.) were filled with 3.5 L of dechlorinated tap water (pH 7.1). Six beakers were treated with ^{14}C-endothall (56.6 mCi mmol^{-1} specific activity, Moravek Biochemicals, Inc., 577 Mercury Lane, Brea, CA 92821) combined with formulated dipotassium salt of endothall (Cascade®, United Phosphorus, Inc., 630 Freedom

Business Center, Suite 402, King of Prussia, PA 19406) to achieve a final concentration of 2 mg L^{-1} in the water column. Three of these beakers were treated with endothall only and the other three beakers were treated with endothall as described plus 3.8 μg L^{-1} of formulated, non-radiolabeled florpyrauxifen-benzyl (ProcellaCOR™ EC, SePRO Corporation, 11550 North Meridian Street, Suite 600, Carmel, IN 46032). The other six beakers were treated with ^{14}C-florpyrauxifen-benzyl (57.1 mCi $mmol^{-1}$ specific activity, Moravek Biochemicals, Inc., 577 Mercury Lane, Brea, CA 92821) at 3.8 μg L^{-1}. Three of these beakers were treated with ^{14}C-florpyrauxifen-benzyl only and the other three beakers were treated with ^{14}C-florpyrauxifen-benzyl plus 2 mg L^{-1} of formulated, non-radiolabeled dipotassium salt of endothall (Cascade®).

Each treatment was replicated 3 times for a total of 12 treatment beakers. Each ^{14}C-endothall treated tank contained 33.8 ± 1.0 KBq L^{-1}, while each ^{14}C-florpyrauxifen-benzyl treated tank 12.4 ± 0.5 KBq L^{-1}. The radioactivity in each treatment tank was confirmed using a liquid scintillation spectroscopy (LSS) (Packard 2500R, PerkinElmer, USA).

Each beaker contained 6 MHV, 6 DHV plants, and one empty tube waxed with a toothpick simulating a plant stem as a control to test the wax barrier efficacy. All plants were held by a round test tube rack (No-Wire Round Rack, Bel-Art Scienceware, 661 Route 23 South, Wayne, NJ 07470). During the experiment, plants were maintained in the laboratory, at 22°C, with 14:10 h light:dark period, supplemented with a LED grow lights. Beakers were stirred once a day and treatment water volume was maintained by adding more water to the tanks daily. Plants were harvested at 6, 12, 24, 48, 96 and 192 h after treatment (HAT). Three replicates of each biotype were randomly harvested from a different tank at each time point, rinsed four times in clean, tap water, and divided into shoots and roots. After separation, plant parts were dried at 60°C for at least 48 h, dry biomass was recorded for each plant part, plant tissues were

combusted in a biological oxidizer (OX500, R.J. Harvey Instrument Co., 11 Jane Street, Tappan, NE 10983) for 2 minutes, and absorbed ^{14}C was collected by a ^{14}C trapping cocktail (OX161, R.J. Harvey Instrument Co.). The efficiency of the oxidizer was tested before oxidizing plant parts and it was always greater than 96%. After oxidation, radioactivity was quantified by LSS. The study was repeated twice.

Desorption

Six clear 1 L glass beakers (15.5 cm tall × 12 cm diam.) were filled with 0.5 L of tap water with pH 6.8 and were treated as previously described. The beakers were stirred for 2 min and the amount of radioactivity in each solution was confirmed as described above using 1 mL aliquots. Six 10 cm apical meristem shoots of each species were exposed to either ^{14}C-2,4-D or ^{14}C-2,4-D BEE for 24 h. Three plants from each tank were harvested, triple rinsed, weighted, and dried, and the other three were triple rinsed and placed each in one 50 mL glass tube containing 40 mL of de-chlorinated tap water. The amount of ^{14}C-2,4-D or ^{14}C-2,4-D BEE desorbing from treated plants was determined by taking 1 mL subsamples at 1, 2, 4, 6, 12, 24, 48 and 72 HAT, and radioactivity was determined by LSS as previously described. After 72 h in the nontreated water and plants were collected, dried, weighted, and oxidized to determine the amount of radioactivity remaining in the plant. Three replicate water samples were collected at each time point and the study was repeated in time.

Statistical Analysis

Prior to combining data from repeated experiments for statistical analyses, Levene's test for homogeneity of variance ($\alpha = 0.05$ level of significance) were performed using R (Version 4.0.0, R Project). Means and standard errors for each experiment were back calculated from dry weight, considering 90% of water content, determined based on 10 hydrilla plants. Data collected

from these experiments were analyzed using RStudio (Version 1.4.1717) and MS Excel and plotted with Prism 9 (GraphPad Software, Inc., USA). Absorption and translocation over time were analyzed using a nonlinear regression analysis to fit a hyperbolic function (Kniss et al. 2011), where *y* is the predicted absorption at time *x*, and *a* and *b* are constants.

$$y = \frac{ax}{b+x} \quad [1]$$

The plant concentration factor (PCF) was calculated to determine bioaccumulation, a metric often used in aquatic plants research to compare absorption across different herbicide concentrations and in different species. The equation used to calculate PCF was adapted from (de Carvalho et al. 2007) and can be defined as:

$$\text{PCF} = \frac{Herbicide\ concentration\ in\ plant\ (\text{ng}/g\ fresh\ biomass)}{Herbicide\ concentration\ in\ water\ (\text{ng}/mL)} \quad [2]$$

The nonlinear regression equations resulting from these analyses were used to calculate two other values, predicted absorption at 192 HAT (A_{192}) based on the ratio of radiolabeled/non-radiolabeled herbicide ratio in the water column, and the predicted time required for 90% of that absorption (t_{90}). The A_{192} value was used to compare the theoretical maximum absorption among different plant parts, plant species, and herbicides, and t_{90} was used to compare absorption rate or how quickly the plant reached maximum absorption.

Results and Discussion

Plant roots were effectively isolated from the radiolabeled treatment solutions through the eicosane wax barrier. Only 0.041 ± 0.012 Bq mL^{-1} (n=6) and 0.022 ± 0.007 Bq mL^{-1} (n=6) of radioactivity was found in the non-plant toothpick test tubes 192 HAT for ^{14}C-endothall and ^{14}C-florpyrauxifen-benzyl treatments, respectively. There was no detected radioactivity in five of the 12 combined test tubes. Based on these data, this insignificant amount of radioactivity did not impact the results of the study.

Endothall absorption did not reach a maximum asymptote in either biotype when applied alone or in the presence of florpyrauxifen-benzyl (Figure 4.1). Although the asymptotic rise to max function is the most biologically relevant function to describe herbicide absorption (Kniss et al. 2011), previous research also demonstrated that endothall at 3 mg L^{-1} did not reach maximum asymptote for DHV and MHV (Ortiz et al. 2019).

The ratio between ^{14}C-endothall alone in the whole plant and in the water column at 2 mg L^{-1} 192 HAT (PCF_{192}) was 20.2 ± 1.3 and 25.8 ± 0.7 in DHV and MHV, respectively, while in the presence of 3.8 µg L^{-1} florpyrauxifen-benzyl, ^{14}C-endothall accumulation in DHV and MHV was 19.0 ± 1.5 and 30.2 ± 0.9, respectively (Figure 4.1 and Table 4.1), which was not statistically different than ^{14}C-endothall alone. The accumulation of this herbicide at 3 mg L^{-1} was 11.0 ± 0.9 and 6.6 ± 0.7 for DHV and MHV, respectively (Ortiz et al. 2019). The reason for greater herbicide accumulation in this study may be due to the difference in herbicide rate, as a lower concentration allows the plant to be more physiologically active and maintaining a stronger concentration gradient longer.

Based on their n-octanol/water partition coefficient (log K_{ow}), endothall (1.91) and fluridone (1.87) accumulation should be very similar, but for both Eurasian watermilfoil (*Myriophyllum spicatum*) and hydrilla their PCF varied greatly (Ortiz et al. 2019; Vassios et al. 2017). Unlike terrestrial plants, log K_{ow} values <2 are not reliable predictors of herbicide accumulation in aquatic plants (de Carvalho et al. 2007) and increased herbicide accumulation does not necessarily correlate to improved control (Ortiz et al. 2019).

The PCF_{192} for ^{14}C-florpyrauxifen-benzyl alone at 3.8 µg L^{-1} was 299.4 ± 21.3 and 433.5 ± 25.4 in DHV and MHV, respectively (Figure 4.1). Previous research reported that florpyrauxifen-benzyl accumulation at 10 µg L^{-1} was 90 ± 20 and 10 ± 2 for DHV and MHV,

respectively (Haug et al. 2021). In this case, the reasons for greater herbicide accumulation in the current study are likely due to differences in ratio of radiolabeled and non-radiolabeled herbicide and herbicide rate, but could also be related the numbers of plants per treatment tank. Haug et al. (2021) exposed 30 plants per treatment tank to a mixture of 25% radiolabeled to 75% non-radiolabeled florpyrauxifen-benzyl, which could have limited the accumulation of radioactivity in the plant. In this study only 12 plants were added per tank and 100% radiolabeled herbicide was used. In the presence of 2 mg L^{-1} endothall, ^{14}C-florpyrauxifen-benzyl accumulation in DHV and MHV was 219 ± 12.8 and 364.0 ± 17.0, respectively (Table 4.1) and was not statistically different than ^{14}C-florpyrauxifen-benzyl alone.

Florpyrauxifen-benzyl high log K_{ow} (5.5) is very similar to 2,4-D butoxyethyl ester's (BEE) (log K_{ow} 5.3), another auxin-mimic herbicide used for aquatic plant management. 2,4-D BEE PCF_{192} in Eurasian watermilfoil and hybrid watermilfoil (*M. spicatum* × *M. sibiricum*) was 35.11 ± 1.33 and 52.11 ± 1.09, respectively, substantially lower than florpyrauxifen-benzyl in DHV and MHV. Both florpyrauxifen-benzyl and 2,4-D BEE are pro-herbicides that are bioactivated into their acid forms once they are absorbed by the plant (Nandula et al. 2019). Depending on water chemistry and temperature, both herbicides can be hydrolyzed to the free acid in the water column and absorbed by plants. The acid form of florpyrauxifen-benzyl is significantly more lipophilic than 2,4-D acid, which could explain its higher accumulation.

Endothall absorption 192 HAT (A_{192}) in DHV and MHV by the shoots was similar, 307.9 ± 20.2 µg g^{-1} and 382.3 ± 13.6 µg g^{-1}, respectively (Table 4.1), and when in combination with florpyrauxifen-benzyl it was only impacted in MHV, with a 40% increase in herbicide absorption (DHV = 260.9 ± 15.1 µg g^{-1}; MHV = 535.6 ± 25.6 µg g^{-1}). In contrast, while florpyrauxifen-benzyl A_{192} was not impacted in presence of endothall, its absorption was significantly lower

than endothall's, which was not unexpected considering the rates at which each herbicide is applied (endothall = 2 mg L^{-1} and florpyrauxifen-benzyl = 3.8 µL^{-1}). Florpyrauxifen-benzyl absorption was very similar between the two biotypes, 3.1 ± 0.4 µg g^{-1} and 4.9 ± 0.3 µg g^{-1}, in DHV and MHV, respectively (Table 4.1). Previous study indicated similar florpyrauxifen-benzyl absorption in MHV, but 4 times more in DHV (Haug et al. 2021), the reason for discrepancy between the two studies is unknown.

Endothall shoot-to-root translocation was 18.7% ± 1.4 and 16.2% ± 1.5 when applied alone in DHV and MHV, respectively, supporting previously published data that endothall can translocate to the roots of both hydrilla biotypes (Figure 4.2) (Ortiz et al. 2019). In combination with florpyrauxifen-benzyl, endothall translocation in DHV was not impacted (23.2% ± 2.2), but it was greatly reduced in MHV (2.20% ± 0.09) (Figure 4.2). This reduced translocation in MHV could be due to the fast-acting properties of florpyrauxifen-benzyl, affecting the plant's vascular tissue rapidly enough to reduce movement to belowground tissues. Florpyrauxifen-benzyl shoot-to-root translocation was 9.1% ± 1.1 and 1.3% ± 0.1 of total absorbed herbicide found in the roots of DHV and MHV, respectively (Figure 4.3). In combination with endothall, florpyrauxifen-benzyl translocation was reduced in both hydrilla biotypes (DHV = 0.5% ± 0.1; MHV = 0.2% ± 0.03) (Figure 4.3). Although florpyrauxifen-benzyl is highly active in both hydrilla biotypes, the dose that corresponds to 50% inhibition of growth (GR_{50}) is lower in MHV than DHV (Netherland and Richardson 2016; Richardson et al. 2016). As both hydrilla biotypes were exposed to the same dose of florpyrauxifen-benzyl (3.8 µg L^{-1}) it could be that MHV vascular tissue was affected faster than DHV, affecting herbicide translocation to the roots.

In conclusion, although radioactive herbicide translocation to the roots was impaired when the herbicides were used in combination, this is the first radiolabeled study looking at

herbicide combination in aquatic plants, therefore future research needs to be conducted to determine if this reduced translocation negatively affects the overall effectiveness of this control strategy in the field. This study suggests that endothall and florpyrauxifen-benzyl have no negative impacts on each other's absorption and accumulation in hydrilla and can be a good tool to delay development of herbicide resistant aquatic weeds when used in combination.

Table 4.1. Predicted plant concentration factor 192 hours after treatment (HAT) (PCF_{192}), herbicide absorption ($\mu g\ g^{-1}$) at 192 HAT (A_{192}), and the time in hours required to reach 90% of A_{192} (t_{90}). Values represent the mean, and error terms represent the standard error of the mean (n=6).

Biotype	Treatment	PCF_{192}	Plant Part	A_{192}	t_{90}
DHV	**^{14}C-endothall**	20.2 ± 1.3	Shoots	307.9 ± 20.2	159
			Roots	65.9 ± 3.7	150
	^{14}C-endothall + florpyrauxifen-benzyl	19.0 ± 1.5	Shoots	260.9 ± 15.1	150
			Roots	72.4 ± 5.2	154
	^{14}C-florpyrauxifen-benzyl	299.4 ± 21.3	Shoots	3.11 ± 0.39	112
			Roots	0.53 ± 0.08	147
	^{14}C-florpyrauxifen-benzyl + endothall	219.4 ± 12.8	Shoots	2.94 ± 0.18	111
			Roots	0.018 ± 0.003	80
MHV	**^{14}C-endothall**	25.8 ± 0.7	Shoots	382.3 ± 13.6	135
			Roots	87.5 ± 8.4	145
	^{14}C-endothall + florpyrauxifen-benzyl	30.2 ± 0.9	Shoots	535.6 ± 25.6	160
			Roots	12.3 ± 0.8	135
	^{14}C-florpyrauxifen-benzyl	433.5 ± 25.4	Shoots	4.91 ± 0.26	110
			Roots	0.097 ± 0.008	141
	^{14}C-florpyrauxifen-benzyl + endothall	364.0 ± 17.0	Shoots	4.32 ± 0.23	115
			Roots	0.010 ± 0.002	114

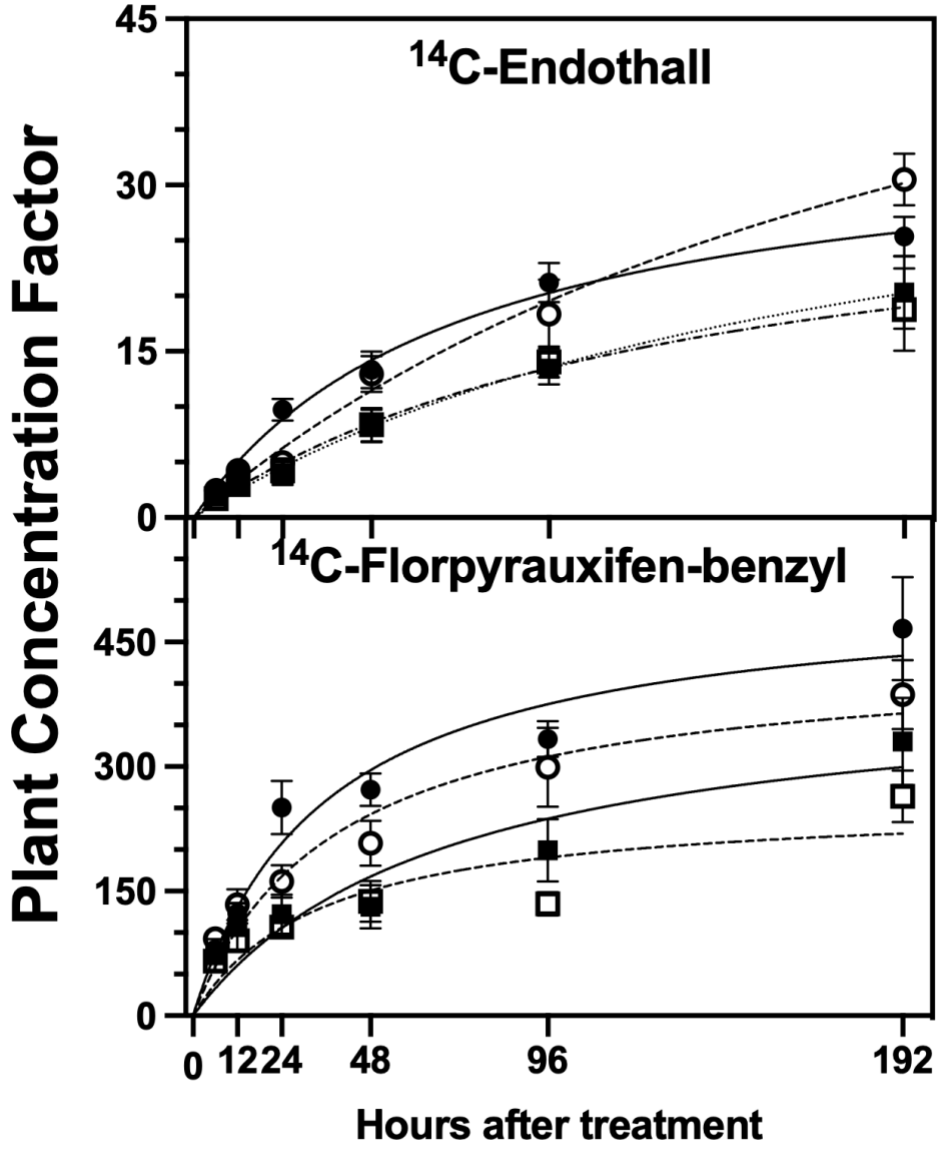

Figure 4.1. ^{14}C-endothall and ^{14}C-florpyrauxifen-benzyl bioaccumulation in MHV and DHV over 192-h time period expressed as plant concentration factor (PCF). ● = herbicide bioaccumulation in MHV alone; ○ = bioaccumulation in MHV in combination; ■ = herbicide bioaccumulation in DHV alone; □ = bioaccumulation in DHV in combination. Data presented are means and standard error of the mean (n=6).

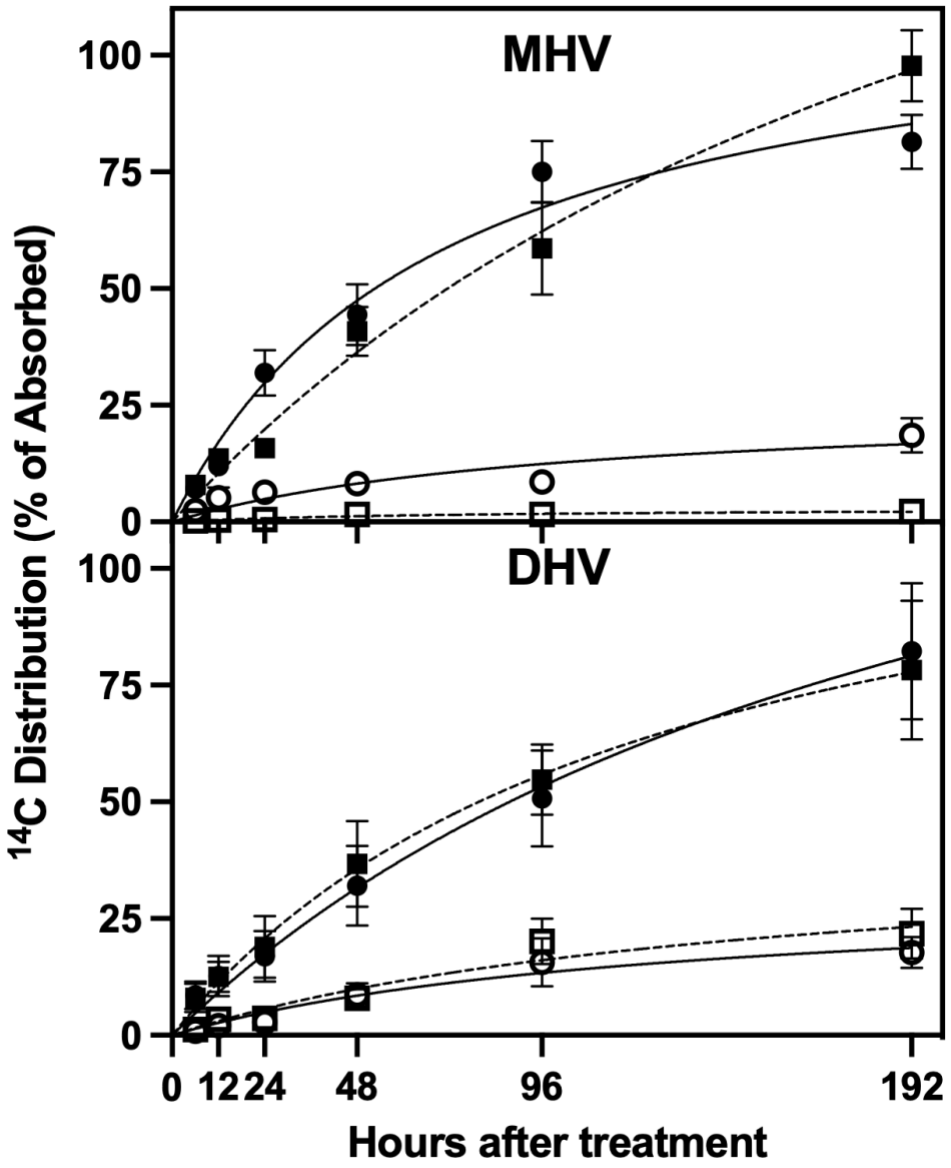

Figure 4.2. ^{14}C distribution in plants over 192 h following exposure to ^{14}C-endothall, expressed as percentage of total herbicide absorbed. ● = percentage of ^{14}C-endothall alone in shoots; ○ = percentage of ^{14}C-endothall alone in roots; ■ = percentage of ^{14}C-endothall in combination with florpyrauxifen-benzyl in shoots; □ = percentage of ^{14}C-endothall in combination with florpyrauxifen-benzyl in roots, respectively. Data presented are means, and error bars are the standard error of the mean (n=6).

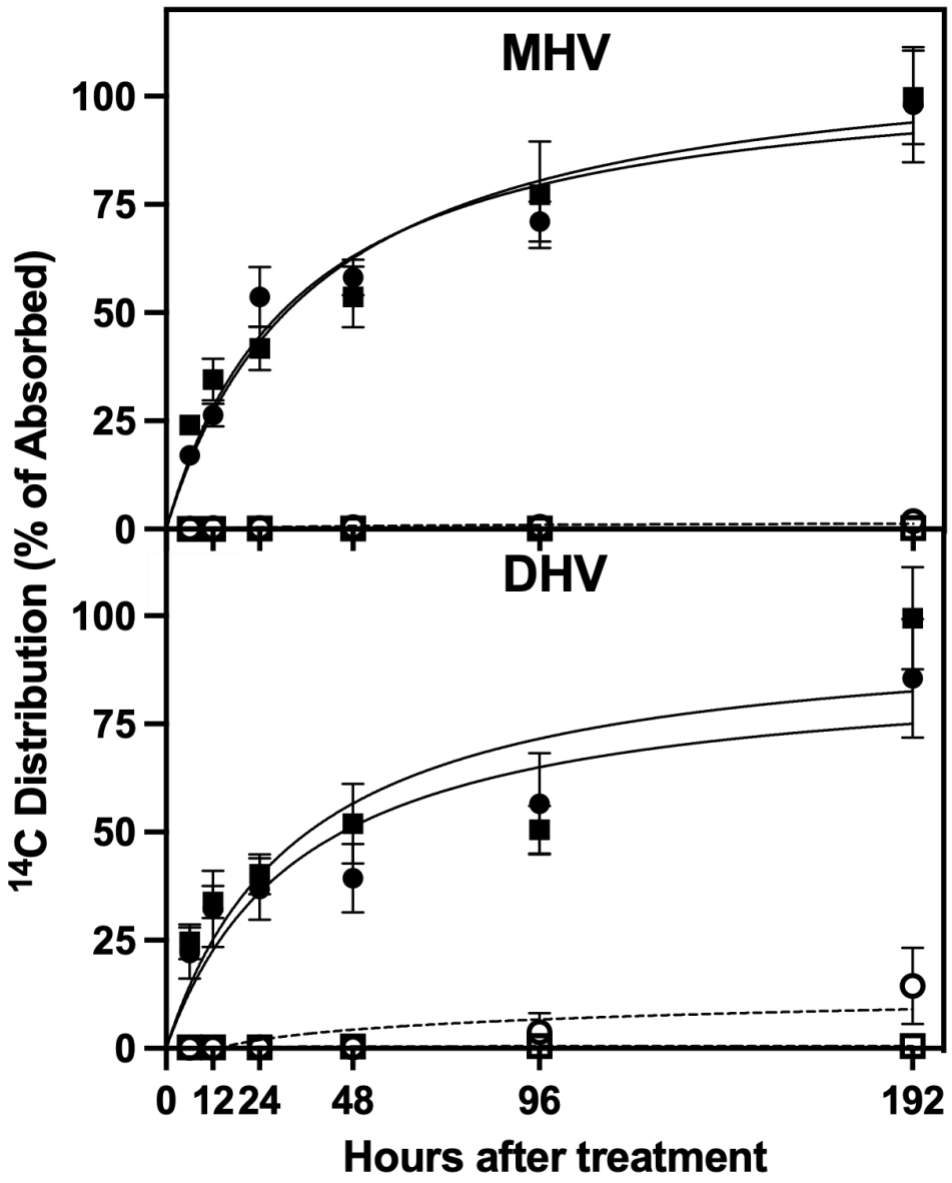

Figure 4.3. ^{14}C distribution in plants over 192 h following exposure to ^{14}C-florpyrauxifen-benzyl, expressed as percentage of total herbicide absorbed. ● = percentage of ^{14}C-florpyrauxifen-benzyl alone in shoots; ○ = percentage of ^{14}C-florpyrauxifen-benzyl alone in roots; ■ = percentage of ^{14}C-florpyrauxifen-benzyl in combination with endothall in shoots; □ = percentage of ^{14}C-florpyrauxifen-benzyl in combination with endothall in roots. Data presented are means, and error bars are the standard error of the mean (n=6).

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CHAPTER 5: ENDOTHALL AND 2,4-D METABOLISM IN THE ROOTS OF AQUATIC PLANTS

Introduction

Invasive and nuisance aquatic weeds have significant impacts on water quality, aquatic ecosystem services, the efficient conveyance of water, and all types of recreation (fishing, boating, swimming) (Madsen et al. 1991; Newroth 1985). Significant aquatic weed infestations can also impact property values, reducing property tax revenue for lake associations (Horsch and Lewis 2009). Herbicides have been employed as important tools for aquatic plant management since the 1960s as they represent the most cost effective and selective management strategy (Ortiz et al. 2020). Endothall (7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid) and 2,4-dichlorophenoxy acetic acid (2,4-D) have been available for aquatic plant management longer than any other herbicides and there is a wealth of information about their behavior when applied in large scale management operations (Cason and Roost 2011; Gyselinck and Courter 2015; Nault et al. 2014; Parsons et al. 2004; Skogerboe and Getsinger 2006). Unfortunately, there is relatively little information available about their behavior once these herbicides are absorbed by aquatic weed species.

The synthetic auxin herbicide, 2,4-D, mimics the effects of the natural auxin, indole-3-acetic acid (IAA), which regulates developmental processes in plant growth and morphogenesis, and induces significant changes in gene expression that ultimately lead to lethal plant growth responses (Dayan et al. 2020). 2,4-D interferes with the plant's ability to maintain a proper hormone balance, and it is readily absorbed and translocated throughout sensitive plants,

accumulating mainly in the growing points of shoots and roots of terrestrial plants. This systemic property of 2,4-D has not been studied in aquatic plants.

Endothall has been classified as a contact herbicide; however, there is growing evidence that it has systemic properties (Ortiz et al. 2019). Its mode of action was unknown for over 60 years, but its activity as a serine/threonine protein phosphatase inhibitor has been demonstrated (Bajsa et al. 2012; Tresch et al. 2011). Protein phosphatases and kinases maintain a sensitive balance between phosphorylated and dephosphorylated forms of proteins playing important roles in signal transduction pathways (Farkas et al. 2007). It represents the only herbicide with this mode of action in the new group 31 herbicide class (HRAC 2021).

Endothall and 2,4-D, like most other herbicides, interfere with normal physiological and biochemical functions that are essential for plant survival (Dayan et al. 2020; Dayan et al. 2010). To reach their target sites, these herbicides must penetrate the plant cuticle and cross other lipophilic barriers, if the target site is within a subcellular compartment (Takano et al. 2019). In terrestrial weed management, these herbicides are formulated with, or require the addition of, surfactants to aid in herbicide penetration of the cuticle to reach the underlying mesophyll cells. Aquatic plants do not have a cuticle because there is no need for moisture conservation, so when used for aquatic applications, these herbicides come into direct contact with the mesophyll cells from the water column.

Terrestrial and aquatic applications of 2,4-D and endothall still require that these herbicides cross one or more biological membranes, i.e., the plasma membrane and organellar membranes (Sterling 1994). Biological membranes consist primarily of phospholipids that self-assemble into a bilayer with a hydrophobic core, giving them unique chemical properties. Membranes are semipermeable with properties that allow for the selective movement of

molecules based on both charge and size (Kronzucker and Britto 2011; Rodríguez-Navarro and Rubio 2006). Endothall and 2,4-D could cross plant's biological membranes through several different pathways to reach their target sites. They could simply diffuse into the cell passively by dissolving through the membrane's hydrophobic core driven by a concentration gradient (Hsu and Kleier 1996; Vassios et al. 2017), or they could be actively transported via a protein transporter (Ge et al. 2014).

Many auxinic herbicides, like 2,4-D, dicamba, picloram, triclopyr, and clopyralid are also weak acids with phloem mobility. This observation lead to the theory that phloem translocation required a weak acid functional group, usually a carboxylic acid, that would become protonated at low pH and diffuse through the lipophilic phloem membrane (Bromilow et al. 1990). The phloem's alkaline pH would then cause the molecules to dissociate and the polar anions would be unable to diffuse back through the membrane (Tyree et al. 1979). This process would allow herbicides, like 2,4-D, to translocate long distances and accumulate in root and shoot meristems. This phenomenon is known as acid trapping (Hsu and Kleier 1996; Kleier 1988). The driving force behind this process is the fact that different plant cellular compartments are maintained at different pH levels. The cell wall pH ranges between 3 and 5; therefore, herbicides with pK_a values in that physiological range could be more subject to acid trapping (Hsu and Kleier 1996).

The theory that weak acid functionality was essential for phloem mobility was challenged with the commercialization for glyphosate (Roundup). Glyphosate has three ionizable groups, one with a pK_a of 2.6, meaning that glyphosate is negatively charged at all times in the plant. Glyphosate is highly mobile and controls many perennial species, especially grasses, because of its ability to accumulate in root and shoot meristems. Endothall is similar to glyphosate in that it has more than one pK_a, one at pH 3.4 and one at pH 6.7, but unlike glyphosate, both of

endothall's pK_a are in the physiological range. Because of herbicides like glyphosate, Hsu and Kleier (1996) proposed that phloem mobility was really a function of two parameters, log K_{ow} and pK_a. Using both of these parameters provided a better prediction of an herbicide's phloem mobility. The model proposed by Hsu and Klier (1996) predicts that endothall would be a systemic herbicide and would fall within the range of log K_{ow} and pK_a values that are predicted to allow for maximum translocation (Figure 5.1).

After 60 years of commercial use of endothall and 2,4-D to control aquatic weeds, there is very little evidence support that either active ingredients behave as systemic herbicides. Several recent studies using radiolabeled 2,4-D and endothall demonstrated that 15% to 25% of the absorbed ^{14}C-2,4-D or ^{14}C-endothall translocated to the root systems of Eurasian watermilfoil (*Myriophyllum spicatum* L.) and diecious hydrilla (*Hydrilla verticillata* (L.f) Royle), respectively (Ortiz et al. 2021; Ortiz et al. 2019). Since these values were generated by biological sample oxidation there was no way to determine if this radioactivity was parent herbicide or a metabolite. So, the question of true systemic behavior for 2,4-D or endothall could not be definitively answered. The main objective of this research was to use multiple analytical methods to answer the question: Are 2,4-D and/or endothall truly systemic in aquatic plants?

Material and Methods

Plant Material

Hybrid watermilfoil plants used in the experiments were from shoot fragments collected in August 2021 from Colorado Youth Outdoors, Fort Collins, CO. Its hybrid genotype was confirmed through a KASP assay (Patterson et al. 2017). Uniform plant material was obtained by propagating 10 cm apical sections of these plants in 16 cm × 12 cm × 6 cm (1,152 cm^3) plastic pots filled with organic soil (Colorado State University Organic Research Farm). Each pot

received 2 g of slow-release fertilizer (Osmocote Classic 15-15-15, Everris NA, Inc., USA) prior to transplanting apical meristem shoots in each pot. Pots were placed in 1.2 m × 1 m × 0.9 m (1,041 L) plastic tanks with washed play sand and grown in de-chlorinated tap water under greenhouse conditions. The photoperiod was 14:10 h light:dark, supplemental lighting was provided with 400-watt sodium halide light bulbs, and the greenhouse temperature was set at 24°C during the day and 18°C at night.

When apical shoots reached 85-100 cm in length (approximately three months after propagation), plants of similar size and root length (12-15 cm) were selected for radiolabeled and non-radiolabeled experiments. Roots were cleaned with tap water and transplanted into 30 mL plastic pots. The pots were filled with unwashed silica sand and sealed with a low melting point eicosane wax (Eicosane 99%, ACROS Organics, USA) to isolate the root system from water column (Frank and Hodgson 1964; Ortiz et al. 2019). Plants for radiolabeled experiments were transferred to 18.9 L plastic buckets (36.5 cm tall × 28.5 cm diam.) filled with de-chlorinated water for a 24 h acclimatization to the laboratory environment prior to initiating the labeling experiments. Plants for non-radiolabeled experiments were kept in the greenhouse.

Dioecious hydrilla plants were propagated from tubers collected from Orange Lake, Florida in 2016. Tubers were kept in de-chlorinated tap water in the greenhouse for 2 weeks, and sprouted tubers were planted in field soil as previously described to encourage root development. Approximately three months after tuber propagation, plants were transferred to individual plastic pots, sealed at the top and either moved to the laboratory or kept in the greenhouse as previously described. Hydrilla plants were overall bigger than HWM plants (100-125 cm).

Radiolabeled Experiment

Eight 2.4 L plastic containers (22.9 cm tall × 12.7 cm diam.) were filled with either 1.8 or 2 L of dechlorinated tap water (pH 6.8) for ^{14}C-2,4-D and ^{14}C-endothall experiments, respectively. Four containers were treated with ^{14}C-endothall (56.6 mCi mmol^{-1} specific activity, Moravek, Inc.) combined with formulated dipotassium salt of endothall (Cascade®, United Phosphorus, Inc.) to achieve a final concentration of 4 mg L^{-1} in the water column. The other four containers were treated with ^{14}C-2,4-D carboxyl-labeled (50 mCi mmol^{-1} specific activity, American Radiolabeled Chemicals, Inc.) combined with formulated 2,4-D (Clean Amine®, Loveland Products) to achieve a final concentration of 4 mg L^{-1} in the other water column.

Each treatment was replicated 4 times for a total of 8 treatment containers. Each ^{14}C-endothall treated tank contained 1722.3 ± 0.003 KBq L^{-1}, while each ^{14}C-2,4-D treated tank 209.1 ± 0.0009 KBq L^{-1}. The radioactivity in each treatment tank was confirmed using a liquid scintillation spectroscopy (LSS) (Packard 2500R, PerkinElmer, USA). Each ^{14}C-endothall container contained 2 hydrilla plants and each ^{14}C-2,4-D container contained 3 HWM plants. During the experiment, plants were maintained in the laboratory, at 22C, with 14:10 h light:dark period, supplemented with a LED grow lights. Containers were stirred once a day and treatment water volume was maintained by covering the containers with a plastic wrap to avoid evaporation. All the plants were harvested at 96 h after treatment (HAT), rinsed five times in clean, dechlorinated tap water, and divided into shoots and roots. After separation, plant parts were rapidly frozen in liquid nitrogen and stored at -20°C. To increase the radioactivity in each plant part, shoots and roots from plants in the same treatment container were combined into one sample.

^{14}C-2,4-D metabolism was presented by Ortiz et al. (2021), adapted from Figueiredo et al. (Figueiredo et al. 2018). Briefly, metabolite extraction was performed by grinding the entire root biomass or a 1 g of shoot, with a pestle in a 50 mL tube, then digesting tissue with a 5 mL solution of acetic acid:acetonitrile:water (1:10:89 v/v) on a table shaker for 30 min. Extracts were transferred into 50 mL centrifuge filters with 25 mL microfiltration membranes (pore size of 0.45 μm) and centrifuged at 570 *g* for 5 min. The extraction procedure was repeated two more times for each sample. Filters and tissue larger than 0.45 μm were dried at 60°C and oxidized in a biological oxidizer for 2 min (OX500, R.J. Harvey Instrument Co., USA). The absorbed ^{14}C was collected by a ^{14}C trapping cocktail (OX161, R.J. Harvey Instrument Co., USA) and non-extracted metabolites were quantified. A final extracted volume of 15 mL was passed through to a C18 solid-phase extraction (SPE) cartridge, and a 5 mL aliquot of digestion solution that passed through the cartridge was quantified by LSS. About 97% of radioactivity was retained by the silica matrix and 76% was recovered with 4 mL of acetonitrile and dried under vacuum (Labconco Corporation, Kansas City, MO, USA) at 40°C. Entire extracts were suspended in 500 μL of high-performance liquid chromatography (HPLC) solvent A and filtered into a 1.5 mL centrifuge tubes using a 0.2 μm nylon syringe filter. Filtered solution was transferred to HPLC vials and 200 μL was used for HPLC (Hitachi Instruments, Inc., San Jose, CA, USA) analysis using a 4.6 mm by 150 mm column (C18 Column; Zorbax Eclipse XDB-C18; Agilent Technologies, Santa Clara, CA, USA). Parent compounds and radioactive metabolites were detected using an in-line radioactivity-detector (FlowStar LB 513; Berthold Technologies GmbH & Co., Bad Wildbad, Germany) with a YG-150-U5D solid cell YG-Scintillator flow cell (150 μL, Berthold Technologies). The gradient elution started at 100% mobile phase A containing formic acid:acetonitrile:water (0.1:10:89.9 v/v) to 75 % phase B containing formic

acid:acetonitrile (0.1:99.9 v/v) at 15 min. The column was allowed to re-equilibrate for 15 min at a flow rate of 1 mL min^{-1}.

^{14}C-endothall metabolite extraction was performed by grinding the sample as previously described, then digesting tissue with a 5 mL solution of trifluoroacetic acid:water (0.1:99.9 v/v) on a table shaker for 30 min. Extracts were filtered as previously described and the extraction procedure was repeated two more times using 2.5 mL of extraction solution each time. Filters papers were oxidized, filtered solution were quantified by LSS as previously described, and a final extracted volume of 10 mL was dried under vacuum at 40°C. Entire extracts were suspended in 500 μL of HPLC solvent A, filtered and transferred to HPLC vials was previously described. HPLC injections consisted of 200 μL and the gradient elution started at 100% mobile phase A containing trifluoroacetic acid:water (0.1:99.9 v/v) to 95% phase B containing trifluoroacetic acid:acetonitrile (0.1:99.9 v/v) at 15 min. The column was allowed to re-equilibrate for 10 min at a flow rate of 1 mL min^{-1}.

Non-radiolabeled Experiment

Eight 18.9 L plastic tanks (36.5 cm tall × 28.5 cm diam.) were filled with 16 L of dechlorinated tap water (pH 6.8). Four tanks were treated with dipotassium salt of endothall (Cascade®, United Phosphorus, Inc.) and the other four containers were treated with 2,4-D (Clean Amine®, Loveland Products) to achieve a final concentration of 4 mg L^{-1} in the other water column.

Each treatment was replicated 4 times for a total of 8 treatment tanks. Each endothall container contained 2 hydrilla plants and each 2,4-D container contained 3 HWM plants. During the experiment, plants were maintained in the greenhouse, at the same settings of during plant growth. All experiments using cold materials were carried out under similar conditions as the

radiolabeled studies and plants were harvested at 96 HAT, rinsed, divided into shoots and roots, rapidly frozen in liquid nitrogen and stored at -20°C. Metabolites extraction was the same as previously described for 2,4-D and endothall prior to injection for liquid chromatography – tandem mass spectrometry (LC-MS/MS) analysis.

All samples were analyzed by LC-MS/MS system (Shimadzu Scientific Instruments, Columbia, MD, USA). consisting of a Nexera X2 UPLC with 2 LC-30AD pumps, a SIL-30AC MP autosampler, a DGU-20A5 Prominence degasser, a CTO-30A column oven, and SPD-M30A diode array detector coupled to an 8040-quadrupole mass spectrometer. For 2,4-D, the MS was in negative mode with a MRM of 219.05 > 161.0 and set for 100 ms dwell time with a Q1 prebias of 23.0 V, a collision energy of 10.0 V, and a Q3 prebias of 30 V. For endothall, the MS was in negative mode with a MRM of 185.1 > 141.1 and set for 100 ms dwell time with a Q1 prebias of 19.0 V, a collision energy of 11.0 V, and a Q3 prebias of 24 V. For both herbicides extracts, the samples were chromatographed on a 100 × 4.6 mm Phenomenex Kinetex® 2.6 µm Biphenyl 100 Å column maintained at 40 °C. Solvent A consisted of formic acid:water (0.1:99.9 v/v) and solvent B was formic acid:methanol (0.1:99.9 v/v). For 2,4-D the gradient started at 30% B and increased linearly to 100% B until 3.5 min. The mobile phase remained at 100% B until 6 min, then returned to 30% B at 6.5 min, and maintained at 30% until the end of the run at 10 min. For endothall the gradient started at 65% B for the first min and increased linearly to 90% B until 4 min. The mobile phase remained at 90% B until 5 min, then returned to 65% B at 5.1 min, and maintained at 65% until the end of the run at 8 min. The flow rate was set at 0.4 mL/min for both herbicide protocols and each sample was analyzed as 5 µL injection volumes. Retention times for 2,4-D and endothall were 5.7 and 2.9, respectively. A standard curve of serial dilutions of authentic 2,4-D and endothall standards were used for quantification.

Results were reported as percentage of the total disintegrations per minute or amount detected adjusted by the dry weight of the plant samples. All experiments were conducted twice with four replications each.

Results and Discussion

Different plant species were selected for 2,4-D and endothall exposure based on previously published data on herbicide absorption and translocation (Ortiz et al. 2021; Ortiz et al. 2019). We selected a single time point, 96 HAT, because for both herbicides and species 96 HAT was the time point where maximum translocation was achieved. Hybrid watermilfoil and dioecious hydrilla plants were selected for 2,4-D and endothall exposure, respectively, as previous studies demonstrated higher translocation of 2,4-D in milfoil, and endothall in hydrilla (Ortiz et al. 2021; Ortiz et al. 2019). By using radiolabeled herbicides, it was possible to determine the amount of extractable and non-extractable radioactivity in the shoots and roots of both species. HWM treated with ^{14}C-2,4-D had 68.2% ± 4.3 and 57.0% ± 12.0 extractable radioactivity in shoots and roots, respectively, while non-extractable, bound metabolites represented 27.7% ± 3.3 and 26.0% ± 4.4 in the shoots and roots, respectively (Figures 5.2A and 5.2B). For hydrilla treated with ^{14}C-endothall, the percentage of extracted radioactivity was 61.7% ± 12.1 in the shoots, but a much higher percent of 86.0% ± 12.8 in the roots, while non-extractable bound metabolites represented 38.3% ± 4.1 and 14.1 ± 2.7 in the shoots and roots, respectively (Figures 5.2C and 5.2D).

Non-extractable ^{14}C was quantified using a biological oxidizer and LSC, while the extractable/soluble ^{14}C fraction could be further analyzed by HPLC coupled with in-line radioactivity detection to determine the amount of radioactivity that was still present as intact 2,4-D acid or intact endothall. The ^{14}C-2,4-D standard eluted as a single peak with a retention

time (RT) of 13.0 min, while ^{14}C-endothall eluted with RT of 11.2 min, with no other peaks observed. For plant extracts, other radioactive peaks that did not correspond to the standard herbicide RT were metabolites derived from the ^{14}C-endothall parent. In milfoil shoots, 83% of the radioactivity eluted at a retention time of 13.0 min as the intact 2,4-D acid. There was a single metabolite peak at 10.4 min that accounted for 17% of the injected radioactivity (Figure 5.3). No attempt was made to identify the metabolite; however, Figueiredo et al. (2018) and Ortiz et al. (2021) found a peak with the same RT as this main metabolite in common waterhemp (*Amaranthus tuberculatus*) and milfoil, respectively.

For ^{14}C-endothall in hydrilla, 59% of the radioactivity eluted at 11.2 minutes as intact endothall. There was a single metabolite with a RT of 1.8 min that accounted for 41% of the injected radioactivity (Figure 5.3). The endothall metabolite was not retained at all by the reverse phase column and eluted at the void volume. That means that this metabolite was very polar and had no weak acid functional activity. No attempt was made to identify the metabolite. In previous studies, only the total amount of radioactivity was reported (Ortiz et al. 2019), but from this research it was possible to establish that a significant amount of the total radioactivity was still intact herbicide 96 h after the initial treatment was applied. There was more intact 2,4-D in milfoil compared to endothall in hydrilla, but in both cases a significant amount of herbicide remained intact. This is an important finding that helps to support the idea that intact herbicide would be available to translocate to milfoil and hydrilla roots for at least 96 HAT.

The presence of 2,4-D or endothall could not be quantified by the radioactivity detector in the root system of milfoil or hydrilla. However, these analyses were successful using LC-MS/MS. The quantities of intact 2,4-D and endothall were present in a shoot:root ratio of approximately 10:1 for both species, meaning there was about 10 times more herbicide in the

shoots of both species compared to the roots. The intact 2,4-D detected in milfoil shoots was 1.31 µg g^{-1} dry weight (DW) and 0.11 µg g^{-1} DW was detected in the roots (Figure 5.4A). For endothall, 1.08 and 0.12 µg g^{-1} DW was detected in hydrilla shoots and roots, respectively (Figure 5.4B). Previous studies reported 9.2% ± 0.4 of total absorbed ^{14}C-2,4-D in the roots of milfoil at 192 HAT and 16% ± 2.3 of total absorbed ^{14}C-endothall in the roots of hydrilla at 192 HAT (Ortiz et al. 2021; Ortiz et al. 2019). These previous studies did not investigate in which form the radioactive herbicide was being translocated to the roots of the plants, but our results provide additional support to endothall's systemic activity by demonstrating that 5.7% of the total intact endothall detected per gram of fresh weight is in the roots of hydrilla, while only 4.7% of the total intact systemic 2,4-D detected per gram of fresh weight is in the roots of milfoil.

This is the first study reporting the translocation and quantification of intact aquatic herbicides in the roots system of any aquatic weed. This research may also have implications related to environmental remediation by aquatic plants. Chemical pollutants could be moved into the benthic environment by shoot to root translocation of aquatic plants where it could be sequestered for the long term and subjected to microbial degradation.

Based on years of research in terrestrial species, 2,4-D was thought to translocate in aquatic plants in the same manner; however, no data has ever been presented to substantiate this paradigm. The assumed *in planta* behavior of 2,4-D is often attributed to its weak acid chemistry. At the same time, endothall has always been considered a contact herbicide that does not translocate in terrestrial or aquatic species. Even though endothall has two ionizable groups (both weak acids), it chromatographs well using ion suppression, reverse phase HPLC just like 2,4-D. Endothall behaves like a weak acid herbicide, again just like 2,4-D. These data clearly

demonstrate that if 2,4-D is considered systemic based on the current research, then endothall should be afforded the same designation. In fact, based the percentage of herbicide absorbed, endothall translocation is higher than 2.4-D.

In conclusion, using a combination of ^{14}C-labeled studies and analysis of unlabeled herbicides by LC-MS/MS, we can conclude that both 2,4-D and endothall have similar *in planta* behavior, with about 8-10% of absorbed intact active ingredient translocating to the roots of aquatic plants. Therefore, endothall should be classified as a systemic herbicide rather than a contact herbicide.

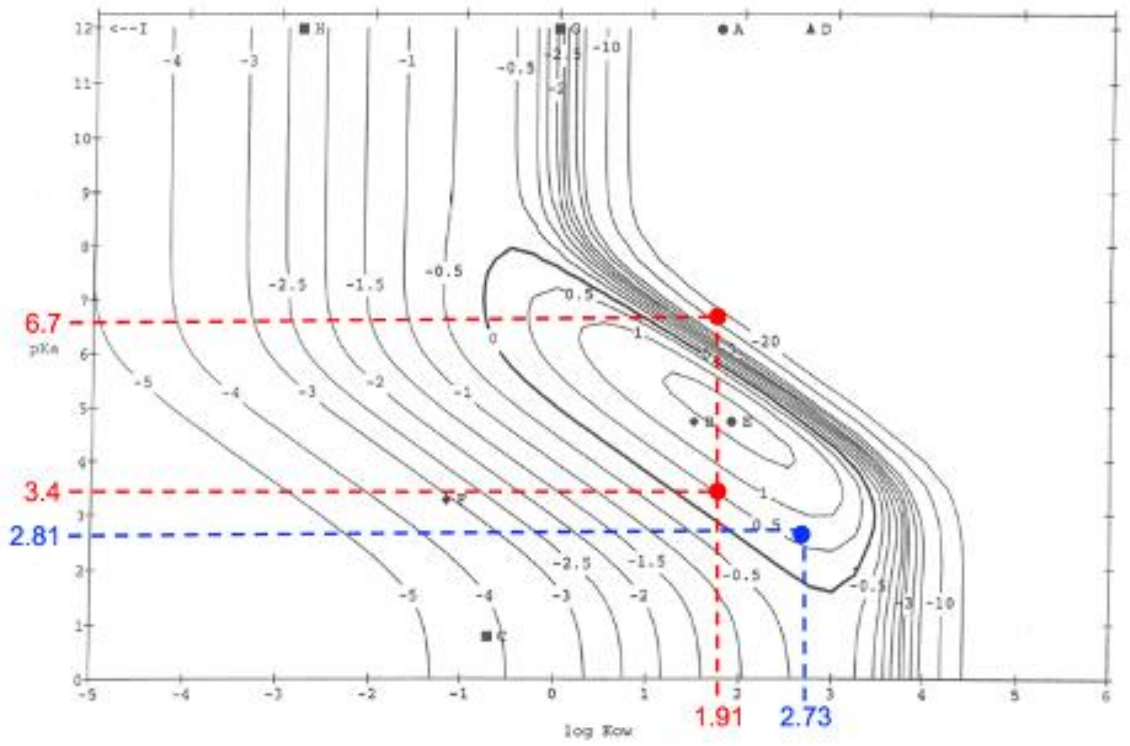

Figure 5.1. Adapted from Hsu and Kleier (1996). Contour plot of the log K_{ow} and pk_a for maximum phloem mobility. Dashed lines in red represents endothall's physicochemical properties, and in blue 2,4-D's.

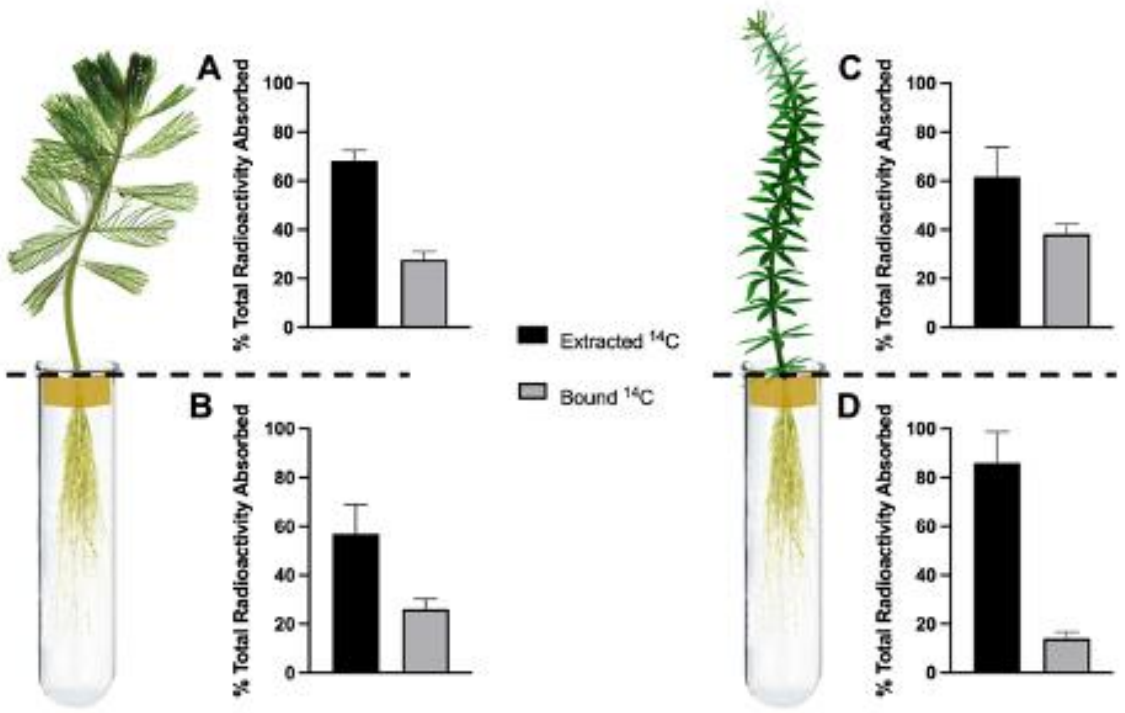

Figure 5.2. Schematic diagram of how plants were exposed to the herbicide treatments and the respective radioactivity recovered for extracted and bound ^{14}C. ^{14}C-2,4-D extraction in the shoots (A) and roots (B) of milfoil, and ^{14}C-endothall extraction in the shoots (C) and roots (D) of hydrilla. Values represent the mean, and error terms represent the standard error of the mean (n = 8).

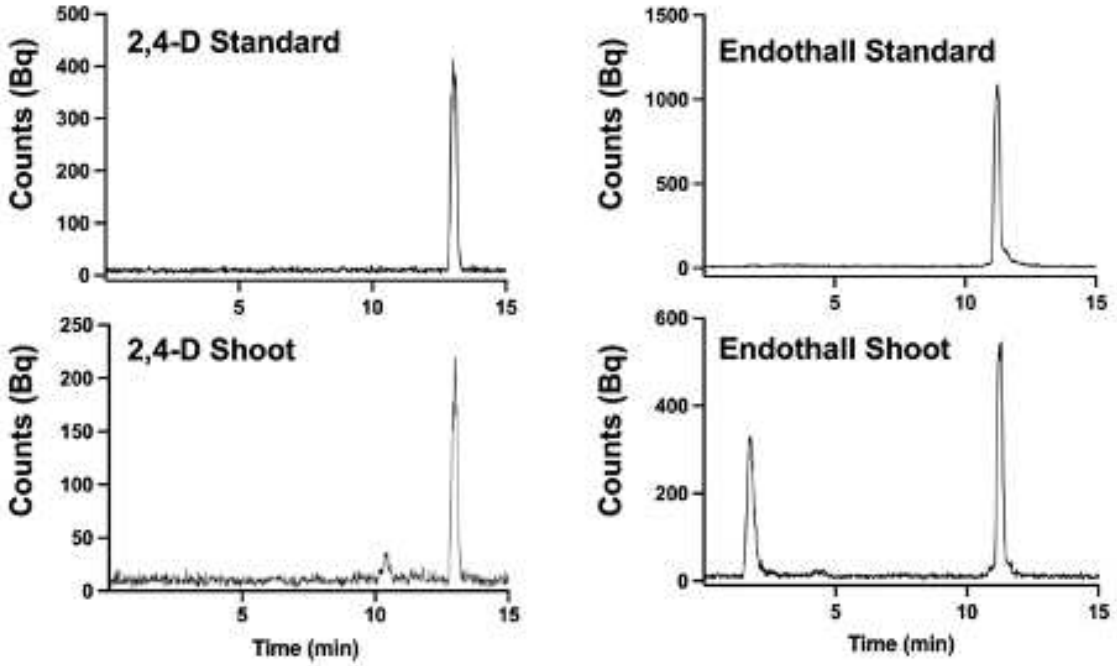

Figure 5.3. Metabolism of ^{14}C-endothall and ^{14}C-2,4-D in plant shoots. Chromatograms of ^{14}C-endothall and ^{14}C-2,4-D standard and at 96 HAT (radioactive units in Bq versus retention time in minutes).

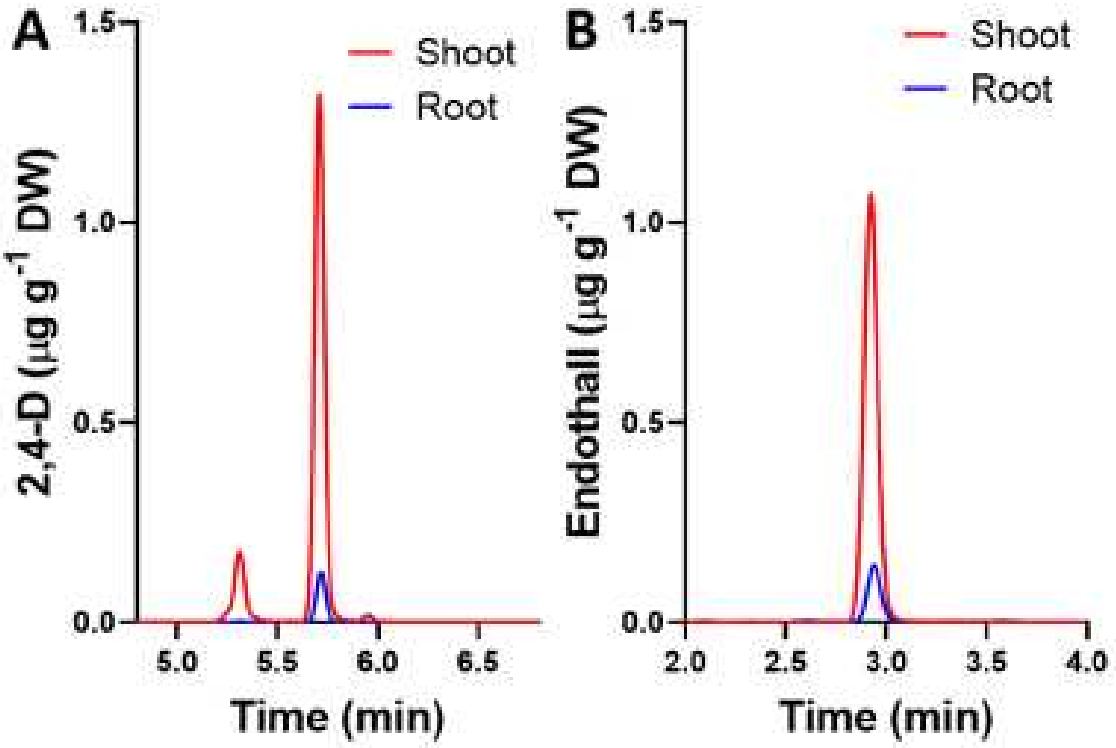

Figure 5.4. Intact endothall and 2,4-D in shoots and roots of hydrilla and milfoil at 96 HAT, respectively.

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