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Herbicide assays for predicting or determining plant responses in aquatic systems

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INTRODUCTION

Herbicides are an important component of aquatic plant management, but represent a relatively small sector of herbicide use compared to agriculture and other commercial production systems. There are over 150 active herbicide ingredients registered for use in cropping systems, but only 13 are registered in aquatic systems in the United States. There is a considerable amount of information regarding the activity and responses of terrestrial plant species (both weeds and crops) to herbicides, but comparatively little for aquatic plants and aquatic herbicides. With this in mind, the purpose of this article is to provide a framework for researchers and practitioners who seek to better understand and evaluate the response of both invasive and native aquatic plants to herbicides.

We will focus on herbicides registered for use in aquatic systems, with an operational definition of a chemical/herbicide used to control unwanted vegetation growing in the littoral zone. This zone is further defined with plants rooted in the sediment and growing up through and out of the water (emergent), plants rooted in the sediment with floating leaves on the water surface, free-floating plants on the water surface, and completely submersed plants rooted in the sediment (Madsen 2009). We will not attempt to provide information on the activity of individual herbicides to specific plants; a good overview of aquatic herbicides and their spectrum of activity can be found in Netherland 2009. Our goal is to provide a general overview of the herbicide mode/mechanism of action as it relates to plant injury, emphasizing those assays used to detect activity and measure impact.

A list of those herbicides currently registered for use in aquatic systems can be found in Table 1. Also included is the herbicide mode of action, specific mechanism of activity (target site), and the typical phenological response. Where the mode of action differs depending on application technique (i.e., target plant from different habitats within the littoral zone), both are listed. Assays for measuring activity will largely take into account these parameters. The assays discussed represent methods to ascertain activity/response, whether that be new target species for control, target species to assess unwanted damage/injury, or populations that might be exhibiting resistance/enhanced tolerance.

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GROWTH RESPONSE STUDIES

Growth studies can provide useful information, particularly as it relates to activity on a whole plant basis. In addition to visual assessment, differential response to a herbicide can be measured through stem length, leaf number, plant biomass, growth rates, reproductive development (propagule number and viability), etc. Regression analysis to model the response can be used to generate inhibition (I_{50}, I_{90} values) to a particular parameter as a function of herbicide rate. These studies are useful to evaluate the response of different populations of the same species to assess changes or differences in herbicide response. such as the development of resistance (Puri et al. 2007). Growth experiments are also useful in determining the level, as function of herbicide rate, of off-target damage to desirable species. Irrigation restrictions for aquatic herbicides can also be ascertained using growth experiments but should be coupled with visual evaluations (Andrew et al. 2003). Growth response studies can also be used to evaluate the impacts of herbicides across a range of plant species, providing an assessment of selectivity.

Although these studies can provide very useful and practical information, care must be taken when applying results to real-world situations. Aquatic plants, especially completely submersed species, can be difficult to culture and maintain over the duration of the study period. Fluridone, for example requires 56 d (8 wk) to fully evaluate activity (W.T. Haller pers. comm.). Issues with water quality, appropriate nutrient levels, light intensity and quality, gas exchange, algae growth, and other abiotic and biotic stresses can complicate results. Untreated reference plants can help to minimize these effects, but differences between untreated and treated can be drastically underestimated if plants are grown under suboptimal conditions. Therefore a considerable amount of time and effort should be invested to develop an adequate culture system that closely simulates natural conditions prior to evaluation.

PIGMENT ANALYSIS

Plants produce a range of pigments that can be easily measured to assess herbicide responses. Chlorophyll A and B represent the most common pigments, and their respective levels often reflect photosynthetic capacity and overall plant health. Chlorophyll can be readily extracted using several methods including acetone (Arnon 1949), dimethyl sulfoxide (Hiscox and Israelstam 1979), and chloroform : methanol (Lichtenthaler and Wellburn 1983). The latter method also extracts carotenoids simultaneously (Doong et al. 1993, Puri et al. 2008). Other methods to

Table 1. Herbicides for use in aquatic systems with mode of action, specific mechanism of activity and typical phenotypic response.

Herbicide	Mode of action	Mechanism of action[1]	Phenotypic response
Glyphosate	Absorbed by emergent leaf and green stem tissues only, lack of activity in water, systemic (phloem-mobile to areas of active growth in leaf tips, buds)	Inhibition of EPSP[2] synthase, resulting in loss of aromatic amino acid production	Cessation of growth, yellowing of affected tissues, slow plant death occurring over 1–4 wk
Triclopyr	Rapidly absorbed by abovewater and submersed leaf and stem tissues, systemic (phloem-mobile to areas of active growth in leaf tips, buds)	Disruption in plant hormone levels and distribution, specific increases in auxin-like responses	Rapid stem twisting and epinasty, leaf malformation, cessation of growth, slow death occurring over 1–3 wk
2, 4-D	Rapidly absorbed by abovewater and submersed leaf and stem tissues, systemic (phloem-mobile to areas of active growth in leaf tips, buds)	Disruption in plant hormone levels and distribution, specific increases in auxin-like responses	Rapid stem twisting and epinasty, leaf malformation, cessation of growth, slow death occurring over 1–3 wk
Endothall	Absorption through leaf and stem tissue of emerged and submersed plants, contact with little to no translocation	Cell membrane disruption through an inhibition of serine/threonine phosphatase, resulting in rapid cell leakage	Rapid leaf and tissue damage with water-soaked lesions followed by necrosis in emerged leaf tissues; submersed tissues can exhibit discoloration followed by rapid degradation
Diquat	Absorption through leaf and stem tissue of emerged and submersed plants, contact with little to no translocation	Cell membrane disruption through a diversion in electron flow in photosystem I resulting in overproduction of radical oxygen and cell leakage	Rapid leaf and tissue damage with water-soaked lesions followed by necrosis in emerged leaf tissues; submersed tissues can exhibit discoloration followed by rapid degradation
Fluridone	Moderate uptake by leaf tissues from submersed applications, with no translocation; leaf uptake on floating species appears to be from diffusion from water	Inhibition of phytoene desaturase resulting in loss of carotenoid production; subsequent degradation of chlorophyll	Bleaching or whitening of new leaf and bud tissues, slowing of growth and gradual degradation of plant tissues. Lack of translocation dictates long exposure times to exhaust plant's ability to regenerate from meristematic tissue
Penoxsulam	Rapid uptake by leaf, root and stem tissues, systemic, (phloem-mobile to areas of active growth in leaf tips, buds)	Inhibition of acetolactate synthase resulting in loss of branched chain amino acid production	Cessation of growth, yellowing of affected tissues, slow plant death occurring over 1–4 wk
Carfentrazone	Rapid uptake by leaf and stem tissues, limited root uptake; limited translocation	Inhibition of protoporphyrinogen oxidase resulting in rapid buildup of photoactive protoporphrin IX. leading to excessive radical production and subsequent cell leakage	Rapid leaf and tissue damage with water-soaked lesions followed by necrosis in emerged leaf tissues; submersed tissues can exhibit discoloration followed by rapid degradation
Flumioxazin	Rapid uptake by leaf and stem tissues, limited root uptake; limited translocation	Inhibition of protoporphyrinogen oxidase resulting in rapid buildup of photoactive protoporphrin IX, leading to excessive radical production and subsequent cell leakage	Rapid leaf and tissue damage with water-soaked lesions followed by necrosis in emerged leaf tissues; submersed tissues can exhibit discoloration followed by rapid degradation
Imazapyr	Rapid uptake by leaf and stem tissues, systemic (phloem-mobile to areas of active growth in leaf tips, buds)	Inhibition of acetolactate synthase resulting in loss of branched chain amino acid production	Cessation of growth, yellowing of affected tissues, slow plant death occurring over 1–4 wk
Imazamox	Rapid uptake by leaf and stem tissues, systemic (phloem-mobile to areas of active growth in leaf tips, buds)	Inhibition of acetolactate synthase resulting in loss of branched chain amino acid production	Cessation of growth, yellowing of affected tissues, slow plant death occurring over 1–4 wk
Copper Chelates Copper sulfate	Rapid uptake by leaf tissues and algae; uptake relating to activity appears to be proportionate to surface area; contact with no translocation	Cell membrane disruption through unknown mechanisms, resulting in rapid cell leakage	Rapid degradation of algae cells, limited activity on emerged and submersed leaf tissue, but enhances activity of certain submersed herbicides such as diquat, but mechanism is unclear
Bispyribac sodium	Rapid uptake by leaf and stem tissues, systemic (phloem-mobile to areas of active growth in leaf tip, bud)s	Inhibition of acetolactate synthase resulting in loss of branched chain amino acid production	Cessation of growth, yellowing of affected tissues, slow plant death occurring over 1–4 wk
Topramezone	Moderate uptake by leaf tissues from submersed applications with no translocation; leaf uptake on floating species appears to be from diffusion from water	Inhibition of cofactors that block phytoene desaturase resulting in loss of carotenoid production; subsequent degradation of chlorophyll	Bleaching or whitening of new leaf and bud tissues, slowing of growth and gradual degradation of plant tissues. Lack of translocation dictates long exposure times to exhaust the plant ability to regenerate from meristematic tissue

[1]Mechanism of action adapted from the Herbicide Handbook (Weed Science Society of America 2014) and Dayan et al, 2015.
[2]Abbreviation: EPSP, 5-enolpyruvylshikimate-3-phosphate.

determine carotenoid levels include the methanolic-based extraction from Sandman and Boger (1983). Anthocyanin levels also provide a measure of plant stress, as these pigments commonly increase after plants are exposed to certain herbicides. Doong et al. (1993) used the anthocyanin extraction method of acidified methanol (Mancinelli 1990) to assess hydrilla responses to fluridone. Pigment analysis should be performed under dim light conditions and with samples stored in the absence of light, because chlorophyll is easily photodegraded.

Fluridone and topramezone inhibit carotenoid production through the inhibition of phytoene desaturase, which is a critical component to the terpenoid biosynthetic pathway. In the presence of either herbicide, phytoene levels increase and β-carotene levels decrease. Both of these compounds can be quantified through UV spectroscopy (Sprecher et al. 1998) because extinction coefficients are available for both compounds. Phytofluene can also be measured with similar methodology, but first requires the careful development of a standard curve. These pigments can be extracted from tissue using basic methanol (6% potassium hydroxide [KOH] in methanol), centrifuged to remove solids, then the supernatant added to petroleum benzine for quantification. Interestingly, Dayan et al. (2015) note that fluridone and topramezone show differences in the tissues affected due to water solubility. The highly lipophilic fluridone shows activity in terms of bleaching directly on the treated leaf, whereas the less lipophilic topramezone translocates via the phloem to display activity (bleaching) in meristematic tissues away from the treated leaf. Whether these differences are displayed in aquatic plants, especially submersed species, is unclear.

Pigment analysis can provide valuable information regarding the overall health of plant tissues and impacts of herbicide treatments. Pigment level comparisons to untreated or known modes of action can help elucidate the activity of herbicides in aquatic plants, or provide insight into causal effects from off-target or unintentional applications. For example, the carotenoid-inhibiting herbicides fluridone and topramezone show a decrease in carotenoids and chlorophyll in new leaf tissue, but not in older (prior to treatment) tissues. An increase in anthocyanin has also been observed with herbicides (Doong et al. 1993). Flumioxazin and diquat can actually display an increase in carotenoids due to their mechanism of action that generates excess electrons in both new and older tissues.

Although pigment analysis can provide insight into basic mechanistic effects, it is very useful for researchers and managers on a large scale operational basis. For example, changes in pigment levels of a particular target species should reflect a certain concentration of herbicide, coupled with exposure time of that herbicide. As such, managers can use pigment analysis as a quick and relatively easy method to predict efficacy of a herbicide treatment. This methodology is often coupled with immunoassays to determine herbicide concentrations and provides a relatively accurate assessment of activity and ultimately effectiveness of treatments.

Pigment analysis provides a good complement to growth studies and other measurements, and is generally expressed as mg pigment gram^{-1} of plant tissue fresh weight. Samples should be taken from leaves that are approximately the same age and stage of development (growth before/after herbicide treatment), and avoid lignified tissue such as stems, petioles, and larger leaf veins. Algae present on the leaves also contain considerable amounts of chlorophyll, so leaf tissue should be cleaned prior to extraction. Also, sample leaves from similar light environments, which will be full sun/maximum light for most emergent and floating plants should be used. For submersed plants, entire shoot tips are often used. Pigment levels in submersed tissues are highly variable due to the light absorption and attenuation properties of water. Due to the decrease in light within the water column, chlorophyll levels increase, and the ratio of chlorophyll A to B decreases, along with decreased levels of carotenoids. Therefore it is essential to sample comparative tissues as a function of leaf age (relationship to treatment timing), stage of development, and placement with the water column.

One final consideration is the derivation of fresh weight, especially with submersed plants. Because pigments are expressed on the basis of fresh weight, the removal of water adhered to the outside of the plant without initiating desiccation is important to maintain uniformity and relativeness of measurements. Most researchers use blotting onto absorbant paper, although centrifugation appears to be more accurate and repeatable (Duivenvoorden 1987). Pigments can also be derived from dry tissues, but freeze-drying should be employed to minimize losses from degradation; once again, tissues should not be exposed to light prior to and during extraction.

CHLOROPHYLL FLUORESCENCE

Chlorophyll fluorescence is a measure of quantum efficiency of the light reactions of photosynthesis. During normal photosynthesis, chlorophyll molecules absorb light energy and direct this energy to reaction centers (P680 and P700) that use electrons derived from the splitting of water to reduce $NADP^+$ to $NADPH_2$. The proton gradient that is formed as a result of this electron flow allows for the production of ATP, and both ATP and $NADPH_2$ are used in the light-independent reactions in carbon fixation. Excess energy that cannot be utilized in the light reactions is passed from chlorophyll to carotenoids and/or to anthocyanins, which subsequently reradiate this energy as infrared energy (heat) and/or the production of reactive oxygen species (ROS).

Chlorophyll molecules can also dissipate energy directly through a process called fluorescence, and can be easily detected through the use of a fluorometer. Although elevated fluorescence can be the result of a myriad of photosynthetic stresses (both light and light-independent systems), measuring fluorescence over time following a herbicide treatment can provide valuable insight into the effects of a herbicide. For example, the photosynthesis-inhibiting herbicides, such as diuron or simazine, show elevated fluorescence within 30 min after exposure (Lichtenthaler et al. 1997), whereas fluridone or topramezone might not show elevated fluorescence for several hours or days (Berger et al. 2015). MacDonald et al (1993) used

fluorescence to elucidate the mechanism of action of endothall, and Berger et al (2015) effectively utilized this technique to rapidly screen for fluridone resistant biotypes of Eurasian watermilfoil (*Myriophyllum spicatum* L.). This technique has also been used to detect herbicide phytotoxicity in algae (Eullaffroy and Vernet 2003). Chlorophyll fluorescence is most useful for those herbicides that directly affect photosynthetic activity, including fluridone, topramezone, flumioxazin, carfentrazone, and diquat, but should be coupled with other measurements such chlorophyll and carotenoid content. As mentioned, elevated fluorescence is indicative of photosynthetic stress and might not always directly correlate with herbicide treatment. However, this technique can rapidly and nondestructively measure large numbers of samples over a short period of time, providing managers with valuable insight into the reaction of plant species to a particular treatment. Netherland and Jones (2015) successfully utilized fluorescence to assess hydrilla susceptibility to fluridone over a 28,500 ha of central Florida lakes, proving large scale applicability of this technique.

CONDUCTIVITY

Conductivity is another indirect measurement of plant stress, and this technique measures the concentration of ions of a solution. Typically, tissues are placed in a bathing solution containing the herbicide and distilled water. Leaf tissue generally consists of uniform-sized leaf discs that are cut from representative plants, or shoot tips from submersed plants. The selection of plant material is critical; shoot tips should be of uniform length and similar number of leaves and leaf area. Leaf discs should be taken from leaves of uniform size and age, and larger leaf veins should be avoided. Due to the inherent variability of leaf tissues, several replications might be needed, with a single disc per replicate. After cutting or severing, discs and shoot tips are placed in distilled water for 30 to 60 min to allow for the formation of callus tissue. Callus formation is important to avoid false leakage associated with a treatment, because tissue can show a large ion efflux from cut surfaces. Tissue is then placed in the treatment solution for measurements. The solution amount (ml) must be similar for each treatment, and conductivity should be measured prior to adding the tissue to the treatment solution because different treatments will inherently have different ion levels. At the termination of the study the tissue should be heated in boiling water or subjected to a freeze/thaw cycle three times to release all ions. Activity is determined as % percent ion leakage as a function of total conductivity of each individual treatment replicate.

Depending on the activity of the herbicide, ions in the cells will leak out over time, displaying an increase in conductivity (μohms). Time-course measurements provide valuable insight into activity, especially if conducted under light versus dark conditions. Rapid ion leakage (within 24 h) is generally linked to herbicides that cause plants to generate radical oxygen, singlet oxygen, hydrogen peroxide, hydroxyl radicals, and lipoxides (Dayan et al. 2015). Leakage that is more rapid under light conditions suggests photosynthetically active compounds such as diquat or flumioxazin, whereas accelerated ion leakage in the dark indicates effects on respiration. Conductivity measurements have been used in several aquatic plant studies; to determine activity of endothall (MacDonald et al. 1993, MacDonald et al. 2002), assay for herbicide resistance (Berger et al. 2011), and to predict impacts of herbicides on aquatic plants (Glomski and Netherland 2013).

OXIDATIVE STRESS

Because ion leakage, and often plant death, is generally the result of oxidative stress, conductivity and assays to measure antioxidant activity are often linked. In this section we will discuss assays to measure the activity of enzymes that quench oxidative stress and the levels of antioxidants. Assaying for enzymatic activity or antioxidants directly can provide clues into the specific activity of a herbicide (Dayan et al. 2015). This technique has not been extensively used for aquatic herbicides, but could be a valuable complement to help explain how certain species react (succumb to or tolerate) herbicides.

Reactive oxygen species (ROS) is the term used to collectively refer to molecules that are formed as the result of stressors within plants cell and organelles. ROS are a part of normal cell functions and have been linked to cell signaling and programmed cell death (PCD) (Petrov et al. 2015). However, in excess, these compounds are powerful oxidizers and react with DNA, proteins, lipids, and most often cellular membranes. Plants quench (reduce) these radicals through a broad range of mechanisms, but fall under two categories—enzymatic and antioxidants. The review article by Das and Roychoudhury (2014) provides an excellent overview of the types of ROS, how these species are formed within plant cells, and the mechanisms to defend against ROS stress and damage. Some of the more routine enzymatic assays include superoxide dismutase (SOD) that converts O_2^- to hydrogen peroxide, catalase (CAT) that converts hydrogen peroxide to water and oxygen, and glutathione reductase which utilizes $NADP^+$ to reduce oxygen radicals (Das and Roychoudhury 2014). Other enzymes include those that reduce the common antioxidant ascorbic acid, such as monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR). Antioxidants that are often measured include ascorbic acid (vitamin C), glutathione, α-tocopherol (vitamin E), carotenoids, phenolics, and flavonoids such as anthocyanins.

Oxidative stress is a normal response of plants to several abiotic and biotic stresses. Therefore, it is important to run assays in absence or at least minimizing stresses with the exception of the parameter being tested. Plant tissues should be allowed to equilibrate for several hours, days prior to the introduction of the herbicide. In addition, more replications will likely be needed to demonstrate a statistically different response due to the high level of variability. Utilizing isolated chloroplasts, or single cells such as algae will reduce variability but might not work for all herbicides.

OXYGEN EVOLUTION/CONSUMPTION

Submersed aquatic plants are ideal for the use of oxygen evolution/consumption methodology. In fact, the submersed aquatic plant, Brazilian egeria (*Egeria densa* Planch.) was used to study photosynthetic rates by counting the number of bubbles of oxygen evolved as a function of treatment parameter (Hartman and Brown 1967). Today, a simple probe for measuring dissolved oxygen is utilized to monitor the change in oxygen levels over time. Plant tissues (generally shoot tips) are placed in sealed vials with treatment solution and minimal head space above the solution; the aim is to keep the oxygen in the solution and not diffused into the open head space. Maximizing the amount of shoot tissue per volume of treatment solution is critical to generate meaningful and measurable differences. A tight seal between the probe and the vial is also important when performing measurements to avoid gas exchange with outside air. Gas bubbles can be present in tissues prior to treatment, and might not reflect actual oxygen generation from photosynthesis or carbon dioxide from cellular respiration. Vacuum infiltration of treatment solution helps to pull gas bubbles trapped inside intercellular spaces, but care must be taken not to damage the tissue. The removal of gas from the solution requires the "adding back" of oxygen or carbon for respiration or photosynthesis, respectively. Light is needed to facilitate photosynthetic responses, whereas oxygen consumption via cellular respiration should be conducted in the dark by wrapping the vials in foil to exclude light. This is needed to negate the oxygen generation by photosynthesis.

Although monitoring oxygen levels is a relatively simple and easy approach to measure herbicide activity, it should be used only as a crude measurement to show differences between species, populations, and/or herbicides because photosynthesis is not being directly measured (MacDonald et al. 1993). For those aquatic species with emerged leaves, several options exist for measuring photosynthesis directly (Nielsen 1993, Beer et al. 2001, Sorrell and Brix 2003). Precise measurements of photosynthesis and/or respiration from underwater aquatic tissues are complicated and will not be covered in the scope of this article. A thorough and detailed review for performing underwater photosynthesis measurements was published by Pedersen et al. (2013).

ENZYME ACTIVITY

Glyphosate and the acetolactate (ALS)-inhibiting herbicides imazamox, imazapyr, penoxsulam, and bispyribac sodium are slow-acting herbicides, and typically do not show the rapid cascade of effects that are detected by the assays discussed previously. These herbicides block the ability of the plant to produce essential amino acids, which are the primary components of proteins and enzymes. These herbicides are weak acids and are readily translocated to regions of new growth within the plant. The formation of new amino acids is inhibited, resulting in a cessation of growth and eventual plant death. As such, assays to detect activity should be conducted on new-growth tissue. These assays could be particularly useful when resistance or tolerance of a target plant population is suspected (Rattray et al. 1993).

Glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), preventing the conversion of shikimate to shikimate-3-phosphate, and blocking the formation of the aromatic amino acids tyrosine, phenylalanine, and tryptophan. In glyphosate-treated plants a notable increase in the levels of shikimate is observed, due to the inability to convert shikimate to shikimate-3-phosphate. Shikimate can be extracted using the methods of Shaner et al. (2005) and Cromartie and Polge (2000) where leaf tissue (often uniform leaf discs) are incubated with ammonium phosphate with or without glyphosate herbicide for 24 h. The tissue and solution is frozen to cease metabolic activity and hydrochloric acid is added to completely lyse the cells. The resulting solution is then added to 0.25% (w/v) periodic acid + 0.25% (w/v) *m*-periodate and allowed to incubate for 90 min. Sodium hydroxide (0.6 N) and sodium sulfite (0.22 M) is then added and the solution measured spectrophotometrically at 380 nm. Measurements should be taken within 30 min and quantified using a standard curve. A shikimate standard curve is developed by adding known amounts of shikimate to leaf tissue during the incubation step with ammonium phosphate in the absence of herbicide. This assay method can be used to determine possible glyphosate herbicide resistance in suspected plants, or to determine if plants have been exposed to glyphosate. The latter determination is difficult because plant stress can also show an increase in shikimate. Therefore, to obtain reliable and meaningful data, it is important to use healthy, untreated control plants and also to assay plants soon after suspected exposure.

The acetolactate-inhibiting herbicides, as the name implies, block the enzyme acetolactate synthase. This enzyme catalyzes two parallel reactions—the conversion of two molecules of pyruvate to form 2-acetolactate and the conversion of a molecule of 2-ketobutyrate and a molecule of pyruvate to form 2-acetohydroxybutyrate. The latter reaction denotes the alternative naming of these herbicides—AHAS inhibitors. The first reaction blocks the formation of valine and leucine, whereas inhibition of the second blocks isoleucine. ALS activity can measured through the levels of acetoin using the methods of Gerwick et al. (1993). Acetoin results from decarboxylation of accumulated 2-acetolactate and CPCA (1,1- cyclopropanedicarboxylic acid) is used to block ketoacid reductoisomerase (KARI) to allow for this accumulation. Typically, plant tissues are incubated with CPCA for 6 to 12 h, homogenized, and filtered. The solution is then acidified to 0.5% with sulfuric acid (H_2SO_4) and warmed for 30 min to cause the acetolactate to decarboxylate to acetoin. The resulting acetoin is then derivitized with α-naphthol (creatine is also added to enhance color) to form a red complex that can be quantified spectrophotometrically at 540 nm. Because ALS herbicides block the formation of 2-acetolactate, low levels of acetoin indicate herbicide activity compared to levels in untreated tissues. Acetoin-based assays have been extensively used to determine ALS-resistant terrestrial weeds; with increasing use patterns for submersed, emergent, and floating plants, the need to distinguish between tolerant and

potentially resistant populations of plants will increase in the future.

There are several auxinic herbicides registered for use in aquatic situations, and assays to determine activity at the tissue level is difficult. Auxinic herbicides interfere with the normal hormone balance within plants, causing a cascade of effects including ethylene generation, increase in abscisic acid levels, and subsequent growth cessation and cell death. Elevated ethylene has been suggested as a method of determining auxinic herbicide activity (Grossmann 2003; Kraft et al. 2007). However, detection and measurement is difficult and qualitative only; and has not been used extensively. Moreover, in a review of assays for herbicide mode of action, Dayan et al. (2015) point out the lack of assays for the auxinic herbicides.

MOLECULAR ASSAYS

Molecular assays have become increasingly important tools in aquatic plant management. The hybridization of myriophyllums and the development fluridone-resistant hydrilla populations have resulted in plants that are phenotypically indistinguishable, yet respond differentially to herbicide treatment. Comparative growth studies and visual evaluations provide the ability to separate these populations, but these assays take considerable time and effort. Moreover, many situations (lakes, water bodies) could contain several spatially independent infestations, thus increasing the level of complexity of sampling and testing to determine population differences prior to treatment. Molecular assays afford the ability to quickly ascertain population identity and thus susceptibility to a particular herbicide treatment. This capacity has been realized at the commercial level for determining the susceptibility of hydrilla to fluridone (GenTEST 2017). Thum et al (2006) has also developed assays for differentiating the hybrids of watermilfoils which vary widely in herbicide susceptibility.

Another molecular technique that has been utilized for aquatic herbicides is immunoassays. Immunoassays provide a manager with the ability to detect herbicide concentrations without the use of complicated analytical techniques. These tests use antibody/antigen responses to reflect herbicide levels. Antibodies specific to a particular herbicide are created and adhered to a test strip or plastic well. The sample containing the herbicide is exposed to the antibodies, where the individual herbicide molecules will bind to an individual antibody. A chromophore-linked antigen specific to the antibody is also in the solution; thus, the herbicide and the antigen "compete" for binding sites within the plastic well/test strip. After a period of time the solution is removed and the assay is measured based on chromophore absorbance. The higher level of chromophore absorbance indicates a lower concentration of herbicide due to the increased number of binding sites occupied by the antigen-linked chromophore. Higher herbicide concentrations show less absorbance due to reduced chromophores because the herbicide is binding the antibody sites rather than the antigen-chromophore. Other types of tests link the chromophore to the herbicide antibody complex; in these tests, the greater the absorbance the higher the herbicide concentration.

Immunoassays are often not reliable measures to determine concentrations in $<$ ppb (parts per billion), compared to analytical techniques employing gas or liquid chromatography due to the inherent variability of the antibody/antigen reaction. However, this technique is rapid and can be performed in the field, providing managers with immediate results that can be used for operational decisions.

Collectively, these assays provide researchers with methods to evaluate and assess the impacts of herbicides on aquatic plants, both target and nontarget species. We have chosen assays that can be conducted with routine and relatively inexpensive laboratory techniques and equipment. More refined assays to quantify herbicides, metabolites, and other compounds by high performance liquid chromatography (HPLC) or gas chromatography (GC) can be found in Dayan et al. (2015). Methods to directly measure enzyme activity are also discussed in this review. Other methods such as the use of ^{14}C radiolabeled herbicides, molecular techniques to measure gene regulation in response to herbicides, or quantifying herbicide activity on a larger, field scale level are also not discussed.

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