J. Aquat. Plant Manage. 56s: 74–82

Using ^{14}C-labeled herbicides in aquatic plant management research

SCOTT J. NISSEN*

INTRODUCTION

Using radiolabeled herbicides to study absorption, translocation, and metabolism began shortly after the dawn of the "Green Revolution" and the "Nuclear Age." By the 1950s, scientists in the new field of weed science were using radiolabeled herbicides to understand herbicide behavior in terrestrial plants and a few years later work was initiated to examine herbicide absorption and translocation in aquatic plants (Aldrich and Otto 1959, Funderburk and Lawrence 1963, Frank and Hodgson 1964, Thomas and Seamen 1968).

What information can be derived from the use of ^{14}C-labeled herbicides that could not be obtained using nonradioactive compounds? The answer is not that radiolabeled herbicides provide information that is unobtainable by other means; it is more that using radiolabeled herbicides makes it easier and requires much less sample preparation and cleanup compared to using nonradioactive or "cold" herbicides.

Much of the earlier research relied the use of X-ray film to determine herbicide distribution in plants. While this can be informative, it is difficult to quantify the distribution and conduct statistical analyses. The reason many researchers choose to examine translocation using X-ray film was because the technology of counting radioactivity in plant tissue was not readily available. Converting a radioactive plant sample into a form that could be more easily quantified was difficult and at the time, equipment manufacturers were just beginning to understand research needs and to design equipment to address those needs. For example, the first Packard Tri-Carb Model 314 EX liquid scintillation counter (LSC) was commercialized in 1953 and was built specifically for the University of Chicago. Counting efficiency was low by today's standards (in the range of 15 to 20%), but improved scintillation cocktails and electronic counting have increased to the point that current instruments achieve $>$ 95% efficiency.

The purpose of this article is to provide some insights and general guidelines for conducting absorption, translocation, and metabolism experiments with aquatic plants using radiolabeled herbicides. Many of these suggestions or observations are based on personal experience and are presented as "rules of thumb" and should not be taken as absolutes. However, these basic guidelines are the result of years of trial and error and attention to these suggestions will increase the likelihood that your research project will be successful, provide meaningful results, and hopefully minimize the generation of radioactive waste.

*Professor of Weed Science, Department of Bioagricultural Sciences and Pest Management, Colorado State University, 115 Weed Research Laboratory, Fort Collins, CO 80523-1177. Corresponding author's E-mail: scott.nissen@colostate.edu.

THE BASICS

So what are the basics? The basics are things like understanding terminology, equipment requirements, and the simple calculations necessary to plan an experiment. What are the units used to measure radiolabeled compounds? How much radioactivity do you need in order to conduct the research? How much radioactivity do you need to apply to achieve your experimental objectives?

In aquatic plant management there are herbicides that are applied in parts per billion (ppb) or micrograms per liter, while others are applied in parts per million (ppm) or milligrams per liter. Herbicides like fluridone and penoxsalum are applied at very low use rates and will need to be applied solely as ^{14}C-labeled herbicides and not mixed with formulated product. These herbicides could also be applied with a formulation blank, meaning the company provides the solution or granular carrier without the target herbicide so that the researcher can reproduce the formulated product using only ^{14}C-labeled herbicide (Vassios et al 2014). You need to understand the concept of specific activity in order to make these determinations. The following is an introduction to some of the basic concepts needed to conduct research with ^{14}C-labeled herbicides.

Terminology

Several companies can provide common herbicides like 2,4-D or 2,4-D butoxy ethylester (BEE) as radiolabeled compounds. These companies will only ship ^{14}C compounds to universities/companies that have the necessary credentials to handle radioactive materials. Most universities have the capability and organization necessary to handle these compounds and your specific environmental health service or radiation control office should always be contacted in advance of ordering radiolabeled herbicides. Commercial companies (e.g., American Radiolabeled Chemicals,[1] PerkinElmer[2]) generally require that a radiation safety license be provided or be on file before an order will be shipped. For many other herbicides the primary manufacture will need to provide the ^{14}C-labeled material.

In the United States, the most common unit of measure for ^{14}C-labeled herbicides is the microcurie (μCi). Compounds that are available for purchase are generally sold in 50- to 100-μCi increments. So how much radioactivity does one μCi represent? One microcurie is equivalent to 2,220,000 disintegrations per minute (DPM). Next question,

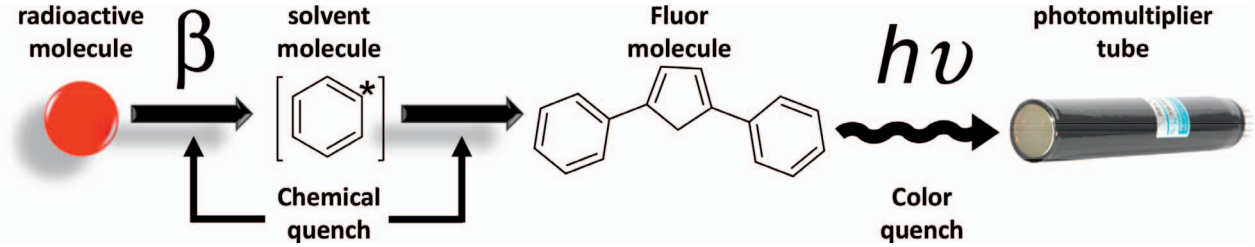

Figure 1. Principle of liquid scintillation counting (courtesy PerkinElmer, Inc., Waltham, MA).

what is a disintegration? A disintegration refers to the fact the ^{14}C is an unstable carbon isotope and is constantly decaying to a more stable compound, ^{14}N. The resulting decay releases a small amount of nonionizing radiation, often referred to as beta (β) radiation. The constant decay rate of ^{14}C to ^{14}N is the basis for radiocarbon dating.

Perhaps the next most important terminology to understand is specific activity. Specific activity is an estimate of how much of the carbon in the molecule is ^{14}C. The most common units in the United States are millicuries (mCi) per millimole (mmol). The amount of enrichment depends on where and how the compound is labeled; the more ^{14}C atoms, the higher the specific activity, so if a herbicide contains a benzene ring such as 2,4-D and all the carbon atoms on that ring are ^{14}C, it will have a higher specific activity than a 2,4-D molecule containing a single ^{14}C in the carboxylic acid side chain. The importance of where the compound is labeled will be discussed later.

Specific activity is important in determining if the amount of ^{14}C herbicide being added will significantly change the herbicide's application rate. As previously mentioned, this is highly dependent on the herbicide. For higher use-rate herbicides like 2,4-D or endothall, the addition of ^{14}C herbicide will have very little impact on the desired application rate, while for fluridone it could have a significant impact. So how do you make this determination?

A labeled herbicide with a specific activity of 30 mCi/mmol and a molecular weight of 266 (2,4-D) would have 0.113 mCi/mg or 0.113 µCi/µg. Converting 0.113 µCi to DPM would mean that there are approximately 250,000 DPM/µg. So for any herbicide applied in parts per million you could have a large number of DPM/ml in the water media without impacting the herbicide concentration. So let's say you wanted approximately 2,500 DPM/ml in the water column (about 1 µCi/L); you would be adding a little more than 10 µg/L to achieve the desired number of counts/ml. Adding 10 µg/L to a water column concentration of 1 to 3 ppm 2,4-D would not impact the concentration and the labeled herbicide could be mixed with formulated product to achieve the desired application concentration.

What about an herbicide like fluridone? With an application rate range from 5 to 30 ppb, higher molecule weight, and lower specific activity, the results would be significantly different. For fluridone the specific activity is more in the range of 18 to 20 mCi/mmol and with a molecular weight of 329, there are only 0.06 µCi/µg or 135,000 DPM /µg. So to achieve a water column concentration of 2,500 DPM/ml requires 18.5 µg of ^{14}C-fluridone. This amount of ^{14}C-fluridone could account for all the fluridone needed to achieve the desired application rate, so there would be no formulated product added. This is one limitation when conducting absorption, translocation, and metabolism research with aquatic herbicides that are applied at very low concentrations in the water column.

While microcuries and DPM are sometimes found in the literature and these units are often used to describe the quantity of radioactivity and specific activity, the International System of Units (SI) for measuring radioactive decay is not µCi or DPM. The SI unit for describing radioactive decay is the becquerel or disintegration per second. One microcurie is equal to 37,000 Bq or 37 kBq and one DPM equals 0.017 Bq. If you are comfortable with DPM and microcuries then sometimes it is just easier to continue with those units and then convert the values to becquerels for publication.

EQUIPMENT

What kind of equipment is needed to conduct absorption, translocation, and metabolism research with radiolabeled herbicides? The two most important pieces of equipment are a liquid scintillation counter (LSC) and biological sample oxidizer (BSO).

Liquid scintillation counter (LSC)

The basic principle behind liquid scintillation counting is shown in Figure 1. Because low-energy β-emitters like ^{14}C cannot be detected directly, determining or "counting" ^{14}C requires the interaction between a solvent that is excited by β-particles that are produced when ^{14}C disintegrates to form ^{14}N. The solvent molecule then transfers this energy to a scintillator sometimes referred to as the "phosphor" or "fluor." When the fluor returns to a lower energy state the excess energy is emitted as light and these light pulses can be detected by a photomultiplier system. Each light flash corresponds to the disintegration of one ^{14}C to ^{14}N, hence the term disintegrations per minute or DPM.

There are several companies currently manufacturing benchtop scintillation counters and a new instrument would often cost between US$40 and 60K. However, since the technology has not changed significantly in recent years and with the advent of online commerce sites, it is possible to purchase any number of used/refurbished LSCs for much less. One must consider that an LSC contains a significant amount of lead shielding and ^{137}Cs or ^{133}Ba as an external standard for determining sample quenching. Therefore,

moving and recalibrating a LSC needs to done by highly trained individuals.

A couple of things to remember about LSC: 1) counts per minute (CPM) must be corrected for background radiation in order to determine DPM and 2) DPM should always be corrected for changes in counting efficiency caused by sample quenching. Quenching simply refers to any factor that reduces the efficiency of energy transfer during the decay process. There are two types of quenching: chemical quenching is when electronegative compounds in the sample steal electrons away from the solvent molecule, thus reducing efficiency, and color quenching occurs when pigments in the sample reduce the efficiency of the fluor to produce light pulses that are detected by the photomultipier (Thomson 2014). LSCs are equipped to determine quench curves and adjust DPM based on counting efficiency. It is always a good idea to make sure that any LSC used is providing the most accurate DPM determinations.

Biological sample oxidizer (BSO)

What is a BSO and why is it so important in working with radiolabeled herbicides? Perhaps the main reason for its importance is that while it is theoretically possible to determine the amount of radioactivity in a piece of plant tissue by performing some kind of chemical digestion, the number of pigments and electronegative compounds in a plant sample would make accounting for sample quenching very difficult. The BSO eliminates this problem by converting all the ^{14}C in the samples to $^{14}CO_2$ and collecting the $^{14}CO_2$ so that DPMs can be accurately determined regardless of the sample matrix.

BSOs combust/burn dried plant tissue in a pure oxygen atmosphere at a temperature of 900 C, converting all the carbon to CO_2 plus water vapor. The combustion products are carried through a series of catalysts (680 C) and are then bubbled through a type of scintillation "cocktail" that contains a compound that efficiently traps CO_2. By converting plant tissue samples to CO_2, issues with sample quenching are eliminated or can be easily handled by establishing a quench curve for the LSC to adjust sample DPM for counting efficiency. There are a number of manufactures that make sample oxidizers ranging in price from US$60K for a PerkinElmer Model 307 to the RJ Harvey[3] OX-501 for about US$30K. There are fully automated systems that are even more expensive.

One limitation of a BSO is sample size. The Harvey Model OX-501 claims a 1-g dry plant material capacity while the PerkinElmer 307 claims to handle up to 1.2 g of dry material. However, from personal experience, 500 mg is a better sample size. Sample size is important because the instrument is operating near 900 C. Introducing too much plant material can cause minor explosions within the combustion tube that can loosen fittings and reduce percentage of recovery. It is always preferable to divide plants into relevant subsamples (i.e., shoots vs. roots) and then combust the entire subsample. When tissue samples exceed the oxidizer's capacity the sample can be oxidized in several steps or subsampled, although neither option is ideal. Multiple oxidations require more time and expense, while subsampling introduces more error and increases the potential for site contamination when plant tissue is homogenized. The best option is to manipulate plant size so that entire plant part can be oxidized. For aquatic plant research this is generally not a huge problem because aquatic plants are 90% water, so to exceed the 500-mg dry weight would mean starting with fresh weight of 5 g.

Oxidizer efficiency and potential carryover should be determined before each group of samples is oxidized. The easiest method for determining efficiency is to "spike" several pieces of filter paper (weighing 300 to 500 mg) with a known amount of radioactivity and process them through the oxidizer. Then take the same amount of radioactivity and add it directly to oxidizer cocktail (scintillation cocktail plus CO_2 trapping solution). Count both samples and divide DPM from the oxidizer sample by the DPM from the sample where radioactivity was added directly to the cocktail. This will provide an estimate of the oxidizer's efficiency. Dividing the DPM added by the DPM recovered will provide a correction factor.

Oxidizer efficiency should range from 85 to 94%. Efficiency below 85% indicates that the instrument is not operating properly. There are two main reasons for lower efficiency: 1) leaks between the combustion chamber and trapping solution and 2) breakdown/contamination of the catalysts. Both issues are reasonably easy to diagnose, but vary in repair costs. New catalysts for the Harvey OX-501 will cost approximately US$500 and a new combustion chamber will cost about the same. A squirt bottle filled with soapy water can be helpful in finding leaks that can be fixed by replacing O-rings or tightening fits.

"Carryover" or "memory" refers to the radioactivity remaining in the catalyst or combustion chamber between samples so that it adds counts to a subsequent sample. It is always to good idea to check carryover by burning spiked samples with high counts (50,000 to 100,000 DPM). Follow each radioactive sample by oxidizing a small piece of filter paper (nonradioactive) and determining how much radioactivity carryover or memory occurs between samples. The Harvey OX-501 claims no more than 50 CPM memory for a 100,000 CPM sample, while the PerkinElmer 307 claims no more than 0.08% memory. In any case, it makes sense to start each group of samples with those that are expected to have the lowest radioactivity (for aquatic work that would probably be roots) and oxidize samples with greatest radioactivity last (shoots exposed to the treated water column). This should minimize the impact sample carryover. The solution for sample carryover is to replace the three catalysts in the combustion chamber.

High-performance liquid chromatography (HPLC) with radioactivity detection and thin-layer chromatography (TLC)

Absorption and translocation studies can provide a great deal of useful information, but these types of determinations tell us very little about the form of the herbicide molecule. There is no way to know if the herbicide is intact and biologically active or if it has been converted to a metabolite that is less active or has no biological activity. So

to establish the herbicide's form requires some additional instrumentation.

Since all aquatic herbicides are water soluble to some degree, with many being weak acids, HPLC is by far the most versatile method of separating intact herbicides from metabolites. Aquatic herbicides like 2,4-D, 2,4-D BEE, fluridone, penoxsulam, triclopyr, imazamox, topramezone, imazapyr, carfentrazone, and flumioxazin do have chromophores, which would allow for UV detection; however, in the complex sample matrix of an aquatic plant the ability to detect and quantify intact herbicides and their early metabolites would require significant sample clean up. Studying herbicide metabolism becomes much more doable when plants are treated with ^{14}C-labeled herbicides and metabolism is monitored by combining HPLC with inline radioactivity detection.

In-line radioactivity detectors are available from several companies and range in price from US$20 to 35K. Berthold[4] and LabLogic Systems Inc[5] are two manufactures of in-line radioactivity detectors for HPLC that provide the option to mix HPLC elutes with scintillation cocktail or to use solid scintillation flow cells that do not require scintillation cocktail. What are the advantages and disadvantages of each system? The main advantage of systems that mix scintillation cocktail with the HPLC solvent is increased sensitivity. The main disadvantages are 1) the cost of a mixing pump, 2) the cost of scintillation cocktail, and 3) sample loss. Perhaps sample loss is the biggest disadvantage because once the sample is mixed with scintillation cocktail it is not possible to conduct additional analyses for identification.

The solid scintillation flow cells eliminate the need for a mixing pump and the cost of scintillation cocktail while preserving sample integrity. The biggest disadvantage is lower initial sensitivity and loss of sensitivity over time. A solid flow cell will need to be replaced periodically due to losses in sensitivity; however, the useful life span of a solid scintillation flow cell can be maximized by regular cleaning.

A low-cost technique known as TLC can provide useful information about herbicide metabolism without the expense of an HPLC system (Spangenberg et al. 2011). TLC is a "tried-and-true" method for separating compounds in a complex matrix and was the standard for many years; however, the speed and reproducibility of HPLC and now ultra-HPLC have replaced TLC for many applications.

There are a variety of TLC plates that will separate most herbicides from their metabolites. By spending time on method development, which usually means testing different solvent combinations for the mobile phase, it is possible to separate parent compounds from metabolites based on differences in migration relative to the solvent front (Rf). Most TLC plates come with fluorescent silica that allows compounds to be located when viewed under UV light. To quantify radioactivity the plates are often scraped using a razor blade and the silica that is removed is placed in scintillation vials and counted by LSC. They can also be analyzed by autoradiography or phosphorimaging (see section below).

Remember, when using UV light to view a TLC plate always wear eye protection. A face shield is a very good option because it protects the eyes and skin from exposure to strong UV light. It is always a good idea to spot a relatively large amount of herbicide standard on the far right and far left sides of the plate. That way you can draw a line between the two spots making it easy locate the parent compound. Try to keep the solution you spot at the origin of the plate as small as possible. This will improve the chromatography. In some cases, it is possible to purchase herbicide metabolites that could also be used to locate the metabolites on the plate. If your main objective is just to determine how much parent herbicide is remaining, then comparing the amount of radioactivity that was spotted on the plate to the amount recovered at the Rf corresponding to the parent herbicide should provide an accurate estimate of herbicide metabolism.

A few comments follow about the location of the ^{14}C label in the herbicide molecule and its importance in determining herbicide metabolism. In order to follow a herbicide molecule through multiple metabolic stages it is important to purchase or request a labeled herbicide where the ^{14}C atom or atoms are located in a major ring structure of the molecule. For example, ^{14}C-2,4-D that is uniformly labeled in the benzene ring will allow the molecule to be monitored through just about any metabolic process, potentially all the way to the opening of the benzene ring and the eventual release of $^{14}CO_2$. If the same molecule was labeled in one of the carbons of the acetic acid side chain, then as soon as the side chain was cleaved the metabolism of the remaining molecule could no longer be detected. In higher plants, most metabolic processes lead to some type of immobilization and do not result in complete metabolism. In many cases, simple ring hydroxylation will significantly reduce or eliminate biological activity due to changes in mobility or target-site interactions.

Metabolism is important for the bioactivation of several aquatic herbicides. One well-known example is 2,4-D BEE, for which the ester must be converted to free acid in order to be phytotoxic. The lipophilic nature of the ester provides for rapid absorption; however, the ester must be metabolized to the active form. Using ^{14}C–ring-labeled 2,4-D BEE and in-line radioactivity detection it would be possible to follow this bioactivation and subsequent metabolism over time.

Autoradiography versus phosphorimaging

Both autoradiography and phosphorimaging are very useful for detecting radioactivity in plant and animal tissue. As previously mentioned, much of the early research (late 1950s and early 1960s) utilized autoradiographs to visualize the distribution of radiolabeled herbicides in aquatic plants (Aldrich and Otto 1959, Funderburk and Lawrence 1963, Frank and Hodgson 1964). This technique does not require expensive equipment and can provide useful information (Rogers 1979); however, phosphorimaging has many advantages over autoradiography.

While both techniques work in a similar manner, phosphorimaging is more sensitive and can provide more data in less time when compared to autoradiography (Van Kirk et al. 2010). Both techniques work on the principle that energy emitted from a decaying radioactive substance, in

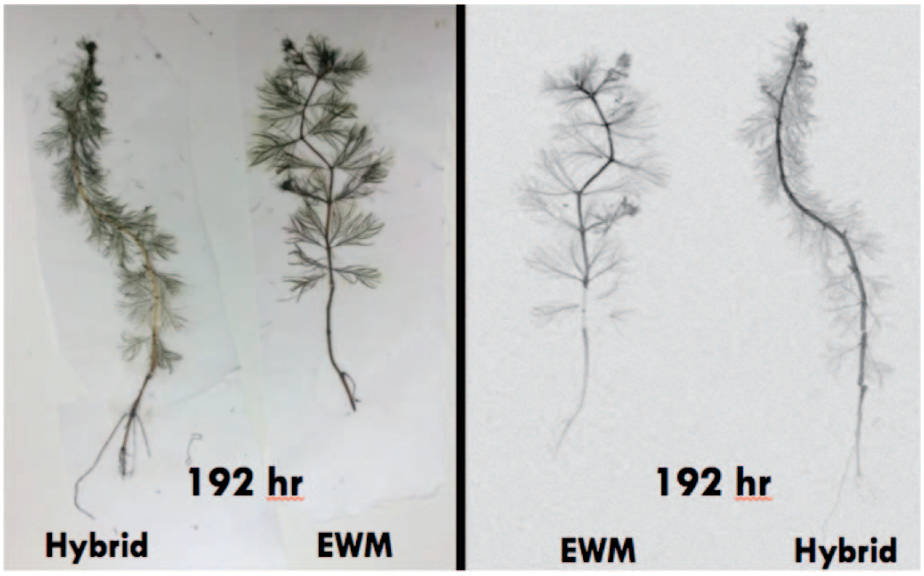

Figure 2. Phosphorimage of Eurasian watermilfoil (EWM) and hybrid milfoil following ^{14}C-2,4-D application. Image on the left is photograph, while the image on the right is the phosphorimage showing the distribution of radioactivity (courtesy K. Kessler, Colorado State University).

this case ^{14}C-labeled herbicides, can be used to produce an image associated with the location of the radioactive material. In the case of autoradiography, the decaying ^{14}C interacts with silver crystals to darken the X-ray film in areas with high radioactivity. The phosphorimaging plates contain crystals of europium-activated barium fluorohalide (BaFBr:Eu^{2+}). When exposed to radiation from the decaying ^{14}C, an electron from Eu^{2+} is ejected to form Eu^{3+}. The ejected electron is trapped by the bromine in the BaFBr:Eu^{2+} crystal. When the imaging plate is exposed to visible light from a laser, the trapped electrons are released from the bromine and Eu^{3+} is converted back to Eu^{2+}. As the electrons return to their ground energy state photons are emitted. The energy released by these photons is a different wavelength than the laser used to stimulate the conversion of Eu^{3+} back to Eu^{2+}; therefore, the emitted energy can be used to quantitatively and spatially reconstruct the distribution of radioactivity in the sample, producing an image similar to an autoradiograph.

An autoradiograph is a piece of X-ray film that provides a permanent record of the original sample, while a phosphorimage is a digital image generated by computer software. There are several software programs that are designed specifically to allow the digital image to be analyzed, providing more quantifiable information compared to autoradiography. A two-step process is required to quantify an X-ray film image. First, the image is exposed and developed, and then densitometric quantification is conducted. Some basic information about densitometric quantification can be found at the following website: https://imagej.nih.gov/nih-image/more-docs/Engineering/ImgEngr.html. If the X-ray film was not properly exposed, then multiple exposures are required to ensure that the image falls within the X-ray film's dynamic range. Phosphorimaging eliminates the need for this multi-step process and the dynamic range for the phosphorimage is five orders of magnitude compared to only two orders of magnitude for X-ray film. This means that the intensity of the signal is directly proportional to the amount of radioactivity over a much larger range, avoiding the issue of signal saturation.

While phosphorimaging has many advantages over autoradiography, including 1) speed (10× shorter exposure times), 2) greater sensitivity (10 to 250× greater sensitivity), and 3) greater dynamic range (1,000× greater dynamic range), autoradiography is significantly less expensive and provides greater resolution when working at the cellular level. This small-scale resolution provided by autoradiography is not important when trying to visualize the distribution of ^{14}C-labelded herbicides on a whole-plant basis. Selecting one technique over the other comes down to cost and availability. Many universities have shared equipment facilities that can provide access to a phosphorimager and charge a per-scan fee. Phosphorimaging plates cost around US$1,600 each, but can be used multiple times and "erased" by exposure to strong visible light. Always remember to take a regular photo of your plant material just as it will be imaged with the phosphorimage or autoradiograph. The more carefully the plant material is arranged, the more compelling the visual will be when comparing the phosphorimage or autoradiograph to the photo (Figure 2).

PLANT MATERIAL

Studying the behavior of radiolabeled herbicides in aquatic plants generates relatively large quantities of low-level radioactive waste water that is also considered hazardous waste due to herbicide contamination, so it becomes a mixed waste. For purposes of minimizing waste generation and ease of sample oxidation, it works well to "miniaturize" the target plant. Depending on water quality, many aquatic species can be several meters long with

Figure 3. Hydrilla, curly leaf pondweed, and hybrid watermilfoil apical shoot cuttings were grown for several weeks to develop adventitious roots and transferred to vials filled with fine, washed silica sand. Plants were allowed to acclimate for several days before treatment. The white material at the top of the glass vial is eicosane wax. (Frank and Hodgson 1964) (courtesy M. Ortiz, Colorado State University).

extensive root systems composed of root crowns and other perennating structures (i.e., tuber, turions). However, for reasons previously mentioned, it is not practical to work with plants this large. For research purposes, generating plants that have all of the important structures (shoot, roots, turion, tubers), but are small enough to be treated in containers that are only a few liters in volume is a reasonable compromise.

For plants that root adventitiously, like Eurasian watermilfoil (*Myriophyllum spicatum* L.) and hydrilla [*Hydrilla verticillata* (L.f.) Royle], rooted shoot cuttings work well. Ten centimeters of apical meristem can be planted in a medium-textured field soil (loam to silt loam, 1 to 3% organic matter) by inserting the basal 3 cm into the soil and then "capping" the soil with a sand layer. These shoot cuttings can be planted in flats, pots, or even 50-ml plastic tubes (falcon tubes) and after 2 to 3 wk adventitious roots will establish. This technique provides rooted shoot cuttings that can be used to measure absorption and translocation.

Since clean, uniform roots are an advantage, it is a good idea to remove rooted cuttings from the field soil, gently remove the soil with water, select the most uniform cuttings, and then replant them in washed silica sand in the vials to be used in the experiment. To partition shoots from roots, eicosane wax can be used to seal the top of the vial (Figure 3) (Frank and Hodgson 1964). Eicosane wax has a melting point of 35 C, which means it is possible to seal the roots away from the water column without harming the plant stem. These rooted cuttings can be used for shoot-to-root and root-to-shoot translocation studies. When evaluating root-to-shoot translocation it is a good idea to eliminate soil in the root zone and simply fill the vial with some kind of diluted nutrient solution. To apply the ^{14}C-labeled herbicide to the root system a hot needle will make a small hole through the wax layer and allow the treatment solution to be added to rooting media. Capping the vials first and then adding the labeled herbicides is a little safer and less likely to spill. Eliminating soil from the root system means that less radioactivity is needed and the roots are exposed uniformly to the herbicide (Figure 4).

Plants like curlyleaf pondweed (*Potamogeton crispus* L.), hydrilla, and sago pondweed [Stuckenia pectinata (L.) Börner] can be easily propagated from perennating organs like turions and tubers to produce small plants suitable for small tank experiments. Plants grown from turions or tubers would have structures very similar to field-grown plants and would provide additional information about the potential for herbicides to translocate to these perennating structures. Since these structures allow the plant to persist, herbicides that translocate to these organs could provide better long-term control than herbicides that do not.

For evaluations of herbicide absorption and desorption, nonrooted shoot tissue works well. Healthy apical shoot pieces (10 cm) can be used to evaluate shoot absorption and subsequent desorption when transferred to clean water. These types of experiments provide information about the impacts of high water-exchange conditions where the external herbicide concentration would fluctuate dramatically. Nonrooted shoots can also be useful for studying herbicide metabolism. Healthy shoots can be treated with

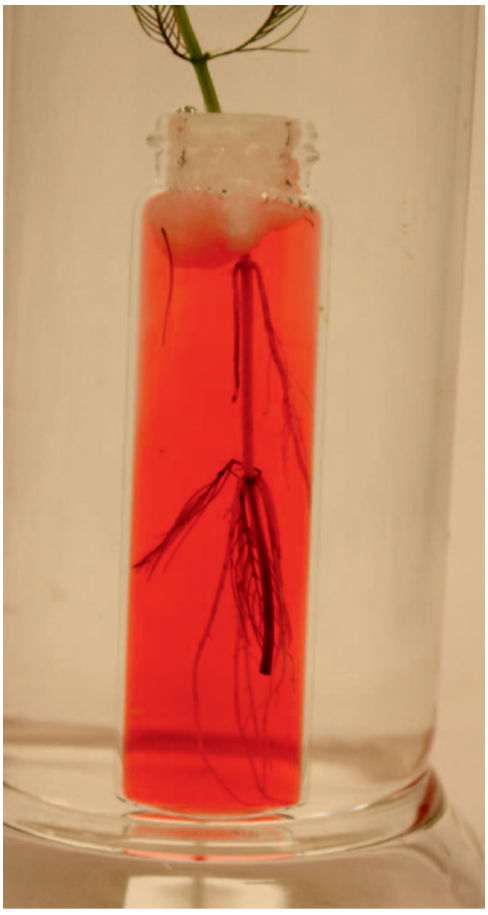

Figure 4. Vial showing the soil-free system used to evaluate herbicide translocation from root to shoot. The plant is Eurasian watermilfoil with several adventitious roots isolated from the water column with eicosane wax. The red color is food dye, used just for demonstration purposes (courtesy J. Vassios, United Phosphorus, INC).

more radioactivity in smaller water volumes to ensure that the shoots have sufficient radioactivity to monitor herbicide metabolism using HPLC and in-line radioactivity detection or TLC.

Each weed species of interest will present challenges when it comes to producing a "realistic" plant the can be manipulated in these highly controlled, small-tank experiments. Sago and horned pondweed (*Zannichellia palustris* L.) are interesting cases of plants that require some special growing conditions. While sago pondweed is easily propagated from tubers, the plants morphology is very different when grown in quiescent water compared to flowing water. So to produce a plant that mimics a sago pondweed that grows in an irrigation canal, plants need to be grown under conditions that simulate flowing water. Any kind of trough in which recirculating water can simulate water movement can be used to produce plants that are more representative of field-grown plants.

Horned pondweed presents an additional challenge. Horned pondweed reproduces from seed; however, the seeds are so small that the only practical way to propagate horned pondweed is to find a heavy infestation (generally from an irrigation canal), wait for the canal to be dewatered, and with a flat shovel remove a thin layer of surface soil in the infested area. That soil becomes a source of "inoculum" that will make it possible to produce plants in the greenhouse. By adding a thin layer of soil containing the horned pondweed seeds to the surface of small pots in a flowing water system, it has been possible to produce small plants that are very representative of field-grown plants.

Propagating plants that are suitable for these highly controlled experiments requires a significant amount of trial and error. There is no right or wrong way to produce these miniature plants, only the way that works for your particular situation. See article 1 for more detail on plant propagation for research purposes. Remember that these types of experiments do not account for UV degradation of the herbicide or dilution due to water exchange; however, even with these limitations small-tank experiments with small plants can provide valuable insights into operational behavior of aquatic herbicides.

EXPERIMENTAL DESIGN

The most important thing to keep in mind when planning any experiment is that it must be repeated in time or space to be considered for publication. To quote Dr. Mike Netherland from the 2016 Aquatic Plant Management Society meeting, "If you don't publish your research, it is as if you never did the work," and therefore any research project worth doing is worth publishing. Aquatic plant management research often faces issues in which true treatment replication is not possible. Each lake, pond, stream, or irrigation canal is unique and therefore it is difficult (or sometimes impossible) to replicate field-scale treatments. Treatments in large lakes are often applied to coves or inlets where it is possible to limit mixing of the water column and off-target movement in order study herbicide impacts on weeds and desirable native species. These studies are given some latitude because of the difficulty in replicating large-scale treatment applications. The same is not true for mesocosm research evaluating herbicide efficacy and behavior. These types of research projects are not difficult to repeat in time or space and should always be established with the idea that the experiment will be repeated as soon as possible. That means generating sufficient plant material and repeating the experiment when environment conditions are similar to the initial experiment. Waiting too long to repeat an experiment means that changes in day length and temperature could have significant impacts on the research results. Obviously, an easy way to avoid these issues is to conduct the research in a growth chamber that has been approved for experiments with radiolabeled herbicides.

The importance of conducting small preliminary experiments cannot be overemphasized. There is no better way to find flaws or issues with the experimental design, procedures that are likely to result in contamination, or ways to streamline treatment applications and harvest procedures. Sacrificing a small amount of radioactivity can help to establish the most appropriate time points for absorption experiments. Aquatic herbicides vary significantly in absorption rates due to differences in the octanol/water

partitioning coefficient (log K_{ow}) (de Carvalho et al. 2007) and therefore one set of time points will not capture the most important absorption parameters for every herbicide. For regression analysis, doubling the time between time points makes it easier to fit the most appropriate regression models. Time points that are the most inconvenient are often useful in establishing important model parameters. Being completely prepared to initiate the time course early in the morning means a 12-h time point will not be in the middle of the night.

Several recent reports demonstrate the wide variation in the most appropriate time course for absorption experiments. In one case, in order to get the best fit for 2,4-D BEE absorption by Eurasian watermilfoil the total time course was less than 1 h (Ortiz et al. 2016), while endothall absorption by hydrilla continued in a nearly linear fashion past the last time point of 192 h after treatment (Ortiz et al. 2017). Every time a new herbicide is evaluated for a new weed species a preliminary study should be conducted to establish the most informative time points for that species–herbicide combination.

One common mistake in statistical analyses with both terrestrial and aquatic research is using ANOVA and means separations to analyze structured data. Structured data in this case refers to time in hours after treatment or days after treatment. Kniss et al. (2011) found that in a literature search for 2006 to 2010 using the key words "herbicide" and "absorption," 27 papers examined herbicide absorption as a function of time, with 56% (15) using means and standard errors rather than regression analyses. As a result of this literature review on herbicide absorption, Kniss et al. (2011) has suggested that more biologically relevant and easily comparable information could be generated by using an asymptotic regression, also called the asymptotic rise to maximum or the rectangular hyperbolic model. The reader is referred to the original manuscript for the annotated R code needed to perform the analysis of maximum absorption and time required for 90% of maximum absorption. This type of regression analysis has been used to describe imazamox absorption in Eurasian watermilfoil (Vassios et al. 2011) and with slight modification, liquid versus granular triclopyr absorption, also in Eurasian watermilfoil (Vassios et al. 2011).

Herbicide metabolism studies should follow similar experimental design parameters. Again conducting small preliminary experiments is critical to making sure that the time course selected captures the most useful information. For most herbicides simply following the reduction in the parent molecule may provide a significant part of the information necessary to establish differences in sensitivity between weeds or impacts on native species. As previously mentioned, for a herbicide like 2,4-D BEE, it would be important to follow at least the first two metabolic process: 1) conversion of the ester to the free acid and 2) metabolism of the free acid to some hydroxylated metabolite.

One additional parameter to consider in an experiment following herbicide metabolism is establishing metabolite profiles for each species or biotype evaluated. The rate at which the herbicide is converted to some inactive or immobilized form is important, but the number and types of major metabolites are also important. Identifying herbicide metabolites can be labor intensive and for most herbicides there is considerable information on common metabolites. It is difficult to obtain ^{14}C-labeled metabolites for most herbicides; atrazine might be one exception. It is more common to find cold (nonradioactive) metabolites that could be available for purchase or by request from the manufacturer. By combining a UV detector and in-line radioactivity detector and accounting for the delay time between the two instruments, it is possible to use cold metabolite standards to establish retention times for the ^{14}C-labeled metabolites. To positively identify a herbicide metabolite would require mass spectral and nuclear magnetic resonance profiles.

CONCLUSIONS

Since the late 1950s radiolabeled herbicides have been used to study herbicide behavior in terrestrial and aquatic plants (Aldrich and Otto 1959). The information generated by this research has been useful in understanding differences in herbicide absorption, translocation, and metabolism. These studies have also provided insights into the impacts of plant morphology on herbicide absorption (Vassios et al 2017), the importance of herbicide formulation (liquid vs. granular, free acid vs. ester) (Vassios et al 2014, Ortiz et al 2016), relationship between log K_{ow} (de Carvalho et al. 2007) and herbicide behavior, and most recently establishing conclusively that endothall is a systemic herbicide (Ortiz et al. 2017). When used in accordance with establish protocols, ^{14}C-labeled herbicides are powerful tools for understanding herbicide behavior in aquatic plants.

SOURCES OF MATERIALS

[1]American Radiolabeled Chemicals, Inc., St. Louis, MO 63146.

[2]PerkinElmer, Inc., Waltham, MA 02451.

[3]R.J. Harvey Instrument Corp., Tappan, NY 10983.

[4]Berthold Technologies U.S.A. LLC, Oak Ridge, TN 37830.

[5]LabLogic Systems Inc, Brandon, FL 33511.

ACKNOWLEDGEMENTS

The author would like to thank SePRO, United Phosphorus, Inc., Lonza, and AERF for supporting graduate student research projects that were discussed in the manuscript. In addition, the author would like to thank the graduate students who have worked on numerous projects designed to understand herbicide behavior in aquatic plants: Dr. Joe Vassios, Kallie Kessler, and Mirella Ortiz.

LITERATURE CITED

Aldrich FD, Otto ND. 1959. The translocation of 2,4-D-1-C^{14} in *Potamogeton pectinatus*, a submersed aquatic plant. Weeds 7:295–299.

de Carvalho RF, Bromilow RH, Greenwood R. 2007. Uptake of pesticides from water by curly waterweed *Lagarsiphon major* and lesser duckweed *Lemna minor*. Pest Manag. Sci. 63:798–802.

Frank PA, Hodgson RH. 1964. A technique for studying absorption and translocation in submersed plants. Weeds 12:80–82.

Funderburk FF, Lawrence JM. 1963. Preliminary studies on the absorption of C^{14}-labeled herbicides in fish and aquatic plants. Weeds 11:217–219.
Kniss AR, Vassios JD, Nissen SJ, Ritz C. 2011. Nonlinear regression analysis of herbicide absorption studies. Weed Sci. 59:601–610.
Ortiz MF, Kessler KC, Nissen SJ, Ratajczyk W, Wersel R. 2016. Absorption rates of 2,4-D butoxy ethyl ester and 2,4-D amine by Eurasian watermilfoil. p. 16 In: Proceedings of 35th Annual Meeting of the Western Aquatic Plant Management Soceity, Del Mar, CA.
Ortiz MF, Nissen SJ, Gray C. 2017. Translocation of ^{14}C-endothall in Eurasian watermilfoil and two hydrilla biotypes. p. 21 In: Proceedings of 37th Annual Meeting of the Midwest Aquatic Plant Management Society, Milwaukee, WI.
Rogers, AW. 1979. Techniques of autoradiography. 3rd ed. Elsevier North Holland, New York. 429 pp.
Spangenberg B, Poole CF, Weins P. 2011. Quantitative Thin-Layer Chromatography: A Practical Survery, Springer-Verlag, Berlin.
Thomas TM, Seaman DE. 1968. Translocation studies with endothal-^{14}C in *Potamogeton nodosus* Poir. Weed Res. 8:321–326.
Thomson J. 2010. Use and preparation of quench curves in liquid scintillation counting. Application note: liquid scintillation. Perkin-Elmer, Inc. Waltham, MA. 6 pp.
Van Kirk C, Feinberg LA, Robertson DJ, Freeman WM, Vrana KE. 2010. Phosphoimager. In: Encyclopedia of life sciences (ELS). John Wiley & Sons, Ltd: Chichester. DOI: 10.1002/9780470015902.a0002973. pub2
Vassios JD, Nissen SJ, Brunk GR. 2011. Imazamox absorption, desorption, and metabolism by Eurasian watermilfoil. J. Aquat. Plant Manag. 49:44–49.
Vassios JD, Nissen SJ, Koshnick TJ, Heilman MA. 2014. Triclopyr absorption and translocation by Eurasian watermilfoil (*Myriophyllum spicatum*) following liquid and granular applications. Weed Sci. 62:22–28.
Vassios JD, Nissen SJ, Koshnick TJ, Heilman MA. 2017. Fluridone, penoxsulam, and triclopyr absorption and translocation by Eurasian watermilfoil (*Myriophyllum spicatum*) and hydrilla (*Hydrilla verticillata*). J. Aquat. Plant Manag. 55:58–64.