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TOXICITY OF THE HERBICIDES BROMACIL AND SIMAZINE TO THE AQUATIC MACROPHYTE, *VALLISNERIA AMERICANA* MICHX

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Abstract—*Vallisneria americana* Michx. (tapegrass) is an ecologically important submersed, vascular aquatic plant that provides food and shelter for many aquatic and waterfowl species. This plant often occurs close to land areas where herbicides are used. Nontarget exposure of these plants to herbicides may compromise ecological structure and function. The objective of the present study was to evaluate the suitability of several endpoint measurements for determining no-observable-adverse effect concentrations (NOAECs), lowest-observable-adverse effect concentrations (LOAECs), and median effective concentration values (EC50s) for tapegrass exposed to the herbicides bromacil (0–0.092 mg/L) and simazine (0–0.592 mg/L) following a 13-d single-pulse exposure and 15-d (bromacil) or 14-d (simazine) postexposure periods. The NOAEC/LOAEC/EC50 for fresh weight gains, new leaf production, and total leaf growth after 13-d exposure to bromacil were 0.020/0.036/0.032, 0.036/0.054/0.036, and 0.036/0.054/0.043 mg/L, respectively. The same respective NOAEC/LOAEC/EC50s for simazine were <0.058/0.058/0.067, 0.229/0.344/0.154, and 0.058/0.116/0.081 mg/L. Reductions in quantity and fresh weight of daughter plants produced and stolon fresh weights occurred at bromacil concentrations ≥0.077, 0.020, and 0.036 mg/L, respectively; and simazine concentrations ≥0.344, >0.592, and ≥0.116 mg/L, respectively. Neither herbicide affected leaf greenness, total chlorophyll concentrations, or carbohydrate allocation. Although toxicity was shown for many endpoints, most EC50 values were greater than aquatic life benchmark values for algae used by the U.S. Environmental Protection Agency (U.S. EPA), but less than for aquatic plants, indicating that *V. americana* would likely be protected by use of the algal benchmark criteria. Environ. Toxicol Chem. 2010;29:201–211. © 2009 SETAC

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INTRODUCTION

Vallisneria americana Michx. (common names, American tapegrass, eel-grass, or wild celery) is a submersed, vascular aquatic plant that reproduces vegetatively and by seed. It has tape-like leaves 20 to 50 mm wide, grows up to several meters in length, and is commonly found in both still and running fresh to brackish waters [1,2]. Within Florida (USA), it is more commonly found in hard water lakes and streams, but can also be found in soft water systems [1]. The known distribution of this plant includes most of eastern North America, with a few records in western areas of the United States [2]. All parts of the plant are important food sources for many waterfowl species, especially for canvasback ducks during their migratory flights [2]. Korschgen and Green [2] estimated that 75% of the migrating canvasback populations use this food resource each fall for the three eastern North American migratory flyways. *V. americana* also provides refuge for ecologically important aquatic invertebrates and fish populations [1,2].

Many of the streams, rivers, and lakes where this species naturally occurs are surrounded by land-uses that use herbicides for controlling weeds or receive runoff/drainage water from those areas. Information regarding the potential negative impact of herbicides typically associated with runoff water from these areas is needed given the ecological importance of this species. Two herbicidal active ingredients of possible concern in areas of North America are bromacil (5-bromo-3-*sec*-butyl-6-methyluracil) and simazine (6-chloro-N^2,N^4-diethyl-1,3,5-triazine-2,4-diamine). Bromacil is registered for annual and perennial weed control in many agricultural and horticultural landscapes, as well as for nonselective control of brush in noncrop land areas. Simazine is registered for selective and nonselective control of grassy and broadleaf weeds in fruit, nut, and field crops. Both herbicides are photosystem II inhibitors. Bromacil (substituted uracil chemical family) is very soluble in water (700–815 mg/L) and is relatively nonvolatile (4.1×10^{-2} mPa at 25°C) [3]. Simazine (*s*-triazine chemical family) is moderately soluble in water (6.2 mg/L at 22°C) and is relatively nonvolatile (2.9×10^{-9} kPa at 25°C) [3].

Contamination of surface water with simazine has been documented in many parts of the USA. Glotfelty et al. [4] reported simazine loadings into the Wye River Estuary (Chesapeake Bay, MD, USA) were directly related to the amount applied in the watershed and the timing of rainfall after application. Other researchers have reported simazine in the Patuxent River (USA) at concentrations of up to 2.7 μg/L [5,6]. Domagalski et al. [7] monitored basin yields of simazine in five different watersheds located in Nebraska, Indiana, Washington, Maryland, and California (USA). They estimated basin yields of 0, 0.06, 0.2, 1.1, and 1.5 g/ha, respectively [7]. Outside of the

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USA, Louchart et al. [8] estimated annual loads of 0.56 g/ha simazine at a watershed outlet in France; with 83% of the losses resulting from fast transmission of overland flow drainage/runoff water exiting fields through a network of ditches. They documented edge-of-field simazine concentrations of 0.1 to >580 μg/L. Wilson et al. [9] measured simazine concentrations ranging from 18 to 646 μg/L in two successive runoff events following application in bedded citrus groves in Florida, with total losses accounting for 2.9 to 3.8% of the active ingredient applied. Simazine has also been reported in 22 Michigan (USA) streams, with mean concentrations ranging from 0.02 to 0.83 μg/L ([10]; http://pubs.water.usgs.gov/sir20075077/). Miles and Pfeuffer [11] reported simazine and bromacil concentrations of up to 2.5 μg/L in 71, and 7.4 μg/L in 72 surface water samples, respectively, collected from 1991 to 1995 in South Florida drainage canals. They reported spatial trends in pesticide detections followed use patterns, and that bromacil and simazine were detected frequently at monitoring sites near citrus groves [11]. Fewer data documenting bromacil in surface water are available, even though its chemical and physical properties favor its movement. However, it can readily leach into ground water in areas with well drained soils [12,13]. By extension, bromacil may be expected to move with surface water drainage/runoff in areas with poorly drained soils, which may account for the frequent detections in the South Florida Water Management District's (SFWMD) pesticide monitoring program, in place since 1998 (https://my.sfwmd.gov/portal/page?_pageid=2235,4688634, 2235_4688260&_dad=portal&_schema=PORTAL, accessed October 31, 2008).

Given that these herbicides have the potential to be detected often in nontarget water bodies, and that no information was available regarding the toxicity of bromacil and simazine to *V. americana*, the present studies were designed to evaluate appropriate toxicity endpoints and characterize effects of these two herbicides on *V. americana* health, growth, and reproduction. No-observable-adverse effect concentrations, LOAECs, and EC50s for a variety of endpoints were estimated following a 13-d exposure and a 14-d (simazine) or 15-d (bromacil) post-exposure period.

MATERIALS AND METHODS

Plant culturing

All plants used in these assays were offspring from stock plants originally collected from Lake Okeechobee (FL, USA). Original stock plants were collected from northern Lake Okeechobee in November 2001 by the SFWMD staff. Plants were transplanted into 1,135 L RubberMaid® cattle watering tanks located inside of an enclosed greenhouse (UF/IFAS IRREC, Fort Pierce, FL, USA). Fine-grade play sand was added to each tank to serve as a substrate (5.1-cm depth). Stock plants were fertilized using Nutricote® Total (13-13-13) controlled release fertilizer (Florikan). Stock tanks were filled with aged tap water, originating from the Fort Pierce Utilities Authority (Fort Pierce).

Subcultures of the stock plants were established under controlled laboratory conditions in 38-L glass aquaria. Daughter plants produced vegetatively from these plants were used in each toxicity assay. This arrangement allowed plants to acclimate to testing conditions for several weeks before tests were initiated. These plants were grown under a 16:8 h light:-dark photoperiod using General Electric plant and aquarium wide-spectrum fluorescent lights (~76 $\mu mol \cdot s^{-1} \cdot m^{-2}$ at a 30-cm height). Water temperature was maintained at 25°C using aquarium heaters. Prior to use, the sand substrate was rinsed with 0.2 N hydrochloric acid, followed by rinsing with reverse-osmosis water until a pH of 5.5 to 6 was achieved in the rinsate. Reconstituted, very hard water was used for culturing and testing plants [14]. General characteristics of the reconstituted water included: pH 8.2 ± 0.2, electrical conductivity $= 826 \pm 14$ μS, alkalinity $= 207 \pm 7$ mg/L as $CaCO_3$, and hardness $= 226 \pm 6$ mg/L as $CaCO_3$.

Toxicity testing procedures

Toxicity tests were conducted on individual plants grown within individual glass cylinders (6.5-cm outer diameter, 45 cm high). Plants of relatively uniform size and appearance were chosen for the assay. Fresh weights of the test plants ranged from 0.34 to 1.63 g for bromacil, and 0.22 to 1.97 g for simazine. The average length of plant leaves was 9.4 cm for the bromacil treatments and 8.4 cm for simazine. Herbicides were administered to four (bromacil) or five (simazine) individual, replicate plants for each treatment concentration. Replicates were restricted by the number of daughter plants available at the time of the assay. Plants were carefully removed from testing stock cultures, taking care not to damage roots. Following collection of nondestructive data, plants were assigned an individual identification code and placed in unspiked assay water until placement in testing cylinders.

For placement in individual glass cylinders, 100 ml of acid-washed sand was first poured into each cylinder. One gram of slow-release fertilizer (Nutricote 13-13-13) was then added, followed by approximately 100 ml of treatment solution. Fertilizer sources and rates were based on recommendations from SFWMD staff, University of Florida/Institute of Food and Agricultural Science's Center for Aquatic Plants, and aquatic plant nurseries. Each plant was then placed within the cylinder using a glass tube. While holding the plant roots above the previously poured sand, an additional 100 ml of sand was added to secure the plant roots within the substrate. The balance of the 1-L treatment volume was then added to the cylinder. The final water level was marked on the outside of each container. Water volume within each cylinder was maintained by daily additions of deionized water to the marked level.

For all assays, plants were exposed to both herbicides (separate experiments) for 13 d using a single-pulse exposure experimental design. Following collection of nondestructive data after exposure periods, individual plants were rinsed with deionized water and planted in community 18.9-L containers (substrate and media same as above) along with other plants from the same treatment concentration to observe possible latent effects or recovery. After 2 d, half of the water was replaced with newly reconstituted water to dilute pesticide concentrations that may have resulted from depuration. At the end of this recovery/depuration period (15 d, bromacil; 14 d, simazine), all of the nondestructive endpoints were remeasured, with the addition of total chlorophyll, total soluble sugars, and starch measurements.

Range-finding assays were conducted to narrow concentration ranges used in these studies. For those studies, nominal

concentrations of 0.001, 0.01, 0.1, 1, and 10 mg/L were evaluated (data not shown). Based on the results, definitive tests were initiated to determine the NOAEC and LOAEC. Measured concentrations evaluated for NOAEC/LOAEC determination included: 0, 0.020, 0.036, 0.054, 0.077, and 0.092 mg/L for bromacil; and 0, 0.058, 0.116, 0.229, 0.344, 0.457, and 0.592 mg/L for simazine. Herbicide concentrations were measured at the beginning of each assay using a Hewlett-Packard 5890 Series II gas chromatograph (Agilent Technologies) equipped with a flame ionization detector and DB-5 column (Agilent Technologies). For confirmations, two 200-ml sample aliquots (from excess treatment solution) for each treatment concentration were extracted using activated C_{18} columns following a modified version of U.S. Environmental Protection Agency (U.S. EPA) Method 3535A: Solid Phase Extraction ([15]; http://www.epa.gov/epawaste/hazard/testmethods/sw846/online/index.htm). In this case, cartridges were activated with 10 ml acetone, followed by 10 ml methanol, and 10 ml deionized water prior to loading the samples. The solid phase extraction columns were air dried under vacuum for approximately 1 h prior to elution. Herbicides were eluted with 2 ml of pesticide grade acetone. Recoveries were greater than 90% for both herbicides.

Measured endpoints

Growth. Several nondestructive growth measurements were taken before exposures and after the exposure and postexposure periods. For all of the nondestructive measurements, plants were gently removed from the media and blotted with paper towels to remove excess moisture before taking measurements. As soon as measurements were taken, plants were then immediately rewetted to avoid desiccation. Fresh weight measurements were taken using an Ohaus (Pine Brook) top-loading balance (Model TS 400s). Fresh weight gains during the exposure periods were calculated by subtracting the initial fresh weight from that measured following exposure. Likewise, fresh weight gains following the exposure + postexposure periods were calculated by subtracting the fresh weights at the end of the study from the initial fresh weights. The number of leaves on each plant was also measured at the previously described time intervals. New leaf production was calculated by subtracting the initial number of leaves from the number measured at the end of the exposure and postexposure periods. Lengthwise growth of plants was also measured at the same time intervals. In this case, the lengths of all of the leaves per plant were summed together as an aggregate measure. Net increases in lengthwise growth were calculated by subtracting the initial length from that measured following exposure and postexposure.

Destructive analyses were conducted at the end of the study, where all plants were divided into shoots, roots, and stolons. Plant parts were freeze-dried over a one-week period and dry weights of each were recorded. Subsamples of these tissues were then used for destructive chlorophyll and soluble sugars and starch analyses.

Vegetative reproduction. At the end of the exposure + postexposure period, daughter plants produced were removed from the parent plant, counted, and fresh weights taken. Additionally, stolons were detached from the parent and daughter plants and fresh weights taken. Tissues were combined with respective parent plants for analysis of dry weights, soluble sugars, and starch.

Leaf greenness and total chlorophyll. Nondestructive measurements of leaf greenness (SPAD) were taken on the inner and outer three leaves (six total) using a Minolta (Konica Minolta Holdings) chlorophyll meter (SPAD-502) before treatment and after the exposure and post exposure periods. Measured SPAD values, defined by Minolta, provide an indicator of the relative amount of chlorophyll present in plant leaves, based on the amount of light transmitted by the leaf in two wavelength regions in which the absorbance of chlorophyll is different [16]. The SPAD readings increase as chlorophyll content increases. Correlations between SPAD readings and chlorophyll content have been reported with r^2 values ranging from 0.82 to 0.90 [17–19]. Total chlorophyll was extracted from plant tissues (0.9 cm^2 from inner and 0.9 cm^2 from outer leaves) using *N,N*-dimethylformamide over a 48–72-h period, in darkness, and at 5 to 7°C [20]. This measurement was not made following the initial exposure because of its destructive nature and the limited quantity of available plants. Absorbance of extracts was then measured at 664 and 647 nm using a Cary 300 (Varian) spectrophotometer. Total chlorophyll was calculated using the method outlined by Moran [21].

Soluble sugars and starch. Sucrose, fructose, glucose, and starch were measured in roots and shoots to identify possible effects on plant metabolism. Detailed descriptions of each are provided by Hall and Rao [22].

Procedures for total soluble sugars and starch determination in each individual replicate plant were modified as previously described [23,24]. Glass Pasteur pipettes with glass wool plugs were loaded with 25 to 50 mg of the freeze-dried, finely chopped root and leaf tissue (separately) from each replicate plant. Three extractions with 1.5 ml of 12 methanol: 5 chloroform: 3 water (by volume, MCW) were carried out for soluble sugar extraction before the residue was used for starch extraction.

Total soluble sugars were analyzed using a Waters 2695 high-pressure liquid chromatograph (HPLC; Waters Technological Corporation) equipped with a Waters 2414 refractive index detector (Waters Technological Corporation) and a Bio-Rad Aminex HPX-87C column (BioRad Laboratories). Column and detector temperatures were maintained at 80°C and 50°C, respectively. High-pressure liquid chromatography grade water was used as the mobile phase, at a flow rate of 0.6 ml/min.

For starch analysis, the tissue residue was dried overnight at 60°C, suspended in 4 ml NaOAc buffer (100 mM, pH 4.5) and placed in a boiling water bath for 30 min. After cooling to room temperature, 1 ml amyloglucosidase solution (from Rhizopus mold; Sigma-Aldrich) (50 units/assay in 0.1 M pH 4.5 NaOAc buffer) was added to each test tube. Samples were incubated for 48 h at 55°C with occasional agitation. Glucose determinations via the glucose oxidase and peroxidase enzymatic method were completed on 100-μl samples [23]. Absorbance at 450 nm was assessed using a Beckman DU-64 spectrophotometer (Beckman Coulter) and starch content was calculated based on the regression equation of the glucose calibration curve (0.0–0.6 μmol).

Statistical analysis

Four individual replicate plants for each treatment concentration were evaluated for bromacil, and five were evaluated for

simazine. Replicate plant quantities were limited due to space and resource constraints. The present study used a completely randomized statistical design. Toxicity data were ranked and analyzed by analysis of variance. Treatment means were separated from control means using calculated least significant differences ($p = 0.05$) for each endpoint (SAS Institute software). In addition, four-parameter logistic functions were fitted to the treatment means for each endpoint using SigmaPlot version 9 (Systat Software). The EC50 point estimates were calculated by solving for the 50% effect concentration, relative to untreated controls.

RESULTS

Growth

Fresh weight production. A summary of the effects of bromacil and simazine on fresh mass production, new leaf production, and total leaf lengthwise growth is shown in Figure 1. Significant reductions in fresh weight gains were observed for plants exposed to both herbicides following the initial 13-d exposure and 13- to 14-d postexposure periods. Following the exposure periods, fresh weight gains were significantly reduced to 40, 24, 20, and 1% of the controls at 0.036, 0.054, 0.077, and 0.092 mg/L bromacil, respectively (Fig. 1A). Plants exposed to 0.092 mg/L bromacil produced very little fresh mass during the 13-d exposure period. The EC50 following the exposure period was 0.032 mg/L (Table 1). Some recovery was exhibited by all of the affected treatments as indicated by net increases in fresh mass following the postexposure periods. Fresh weight gains were 67, 44, 51, 39, and 21% of the controls at each respective concentration at the end of the 28-d study. The LOAEC for bromacil was 0.020 mg/L, indicating that the NOAEC is <0.020 mg/L under the present study conditions. The postexposure EC50 was 0.040 mg/L.

Fresh weight gains following the simazine exposures were reduced to 56, 25, 4, 6, and −6% of the controls at 0.116, 0.229, 0.344, 0.457, and 0.592 mg/L, respectively (Fig. 1B). Plants exposed to concentrations of simazine greater than 0.344 mg/L exhibited loss of tissue due to necrosis, beginning at the tips and moving inward, accounting for the negative increases (Fig. 1B). The EC50 following the exposure period was 0.067 mg/L (Table 1). As with bromacil, some recovery was exhibited by all of the affected treatments as indicated by net increases in fresh mass following the postexposure period. Fresh weight gains at the end of the 27-d study were 76, 51, 38, 29, 34, 26, and 20% of the controls at each respective concentration. A NOAEC of <0.058 mg/L, LOAEC of 0.058 mg/L, and EC50 of 0.086 mg/L was observed for plants at the end of the 27-d study.

New leaf production. Results for new leaf production for both herbicides were highly variable during the initial exposure phase, with coefficients of variation (CVs) ranging from 0.27 to 1.28 for bromacil and 0.7 to 2.2 for simazine (data not shown). A significant reduction in new leaf production was observed after exposure to bromacil concentrations 0.054 and 0.077 mg/L, but not at 0.092 mg/L (Fig. 1C). Production of new leaves was 126, 67, 27, 43, and 50% of the controls at the 0.020, 0.036, 0.054, 0.077, and 0.092 mg/L bromacil treatment levels, respectively, after the 13-d exposure period; with a corresponding NOAEC = 0.036, LOAEC = 0.054, and EC50 of 0.036 mg/L (Table 1). Following the postexposure period, the only statistically significant reductions in cumulative new leaf production occurred at 0.036, 0.077, and 0.092 mg/L bromacil, where new leaf gains were 59, 53, and 29% of the controls (Fig. 1C). The corresponding NOAEC, LOAEC, and EC50 were 0.054, 0.077, and 0.071 mg/L, respectively.

A significant reduction in new leaf production was observed after 13-d exposure to simazine concentrations ≥0.344 mg/L (Fig. 1D). Production of new leaves was 79, 67, 30, 12, 0, and 12% of the controls at the 0.058, 0.116, 0.229, 0.344, 0.457, and 0.592 mg/L simazine treatment levels, respectively, after the exposure period. As with fresh weight gains, some new leaf production measurements were negative for individual replicate plants due to desiccation of affected leaves. This EC50 was estimated at 0.154 mg/L. New leaf production was similar across all treatments following the postexposure period, except for the 0.058 mg/L treatment, which was greater than the controls. An estimate of EC50 was not possible because responses never reached 50% of the controls. The mean number of leaves produced ranged from 7.4 to 10.8 (16.8 for 0.058 mg/L treatment) at the end of the study, indicating significant recovery potential once the herbicide was removed.

Total leaf growth (cm). Following the 13-d exposure to bromacil, total leaf growth (length increase) was statistically reduced to 34 and 27% of the controls at 0.054 and 0.077 mg/L, respectively (Fig. 1E). Although not statistically different, mean lengthwise growth of plants exposed to 0.092 mg/L bromacil was only 37% of the controls. The EC50 following exposure was 0.043 mg/L. Following the recovery period, leaf growth was 58, 54, 71, 34, and 26% of the controls for the 0.020, 0.036, 0.054, 0.077, and 0.092 mg/L treatments, respectively. However, the leaf growth at 0.036 and 0.054 mg/L was not statistically different from the controls. The EC50 was slightly higher, although less precise, at 0.053 mg/L (Table 1).

Total leaf growth for plants exposed to simazine was significantly lower than the controls at concentrations ≥0.116 mg/L following exposure (Fig. 1F), with a corresponding EC50 of 0.081 mg/L. In this case, total leaf growth relative to the controls was 28, 5, −7, −13, and −2%, respectively. The negative values observed were due to losses of leaf tissue, beginning at the tips of older leaves. Following the 14-d postexposure period, no changes in the NOAEC (0.058 mg/L) and LOAEC (0.116 mg/L) were observed, whereas the EC50 increased slightly to 0.096 mg/L (Table 1). Leaf growth was reduced by 10 to 102% at simazine concentrations ≥0.116 mg/L at the end of the study.

Vegetative reproduction. The data for vegetative reproduction are summarized in Figure 2. At the end of the exposure + postexposure periods, plants exposed to the 0.092 mg/L bromacil treatment vegetatively produced an average of 75% fewer daughter plants, relative to the controls (Fig. 2A). Reductions in the number of daughter plants produced at the other concentrations ranged from 17 to 40%. Daughter plant counts were highly variable as indicated by CVs ranging from 0 to 2.00 (data not shown), which also accounts for the less precise ($r^2 = 0.907$) EC50 estimate of 0.076 mg/L (Table 1). The fresh weight of daughter plants produced was a more sensitive indicator of potential herbicide effects (Fig. 2C). In this case, the NOAEC and LOAEC was <0.020 and 0.020 mg/L bromacil, respectively; with the EC50 = 0.016 mg/L. Mean fresh weights of daughter plants were generally reduced by 55 to 89% at

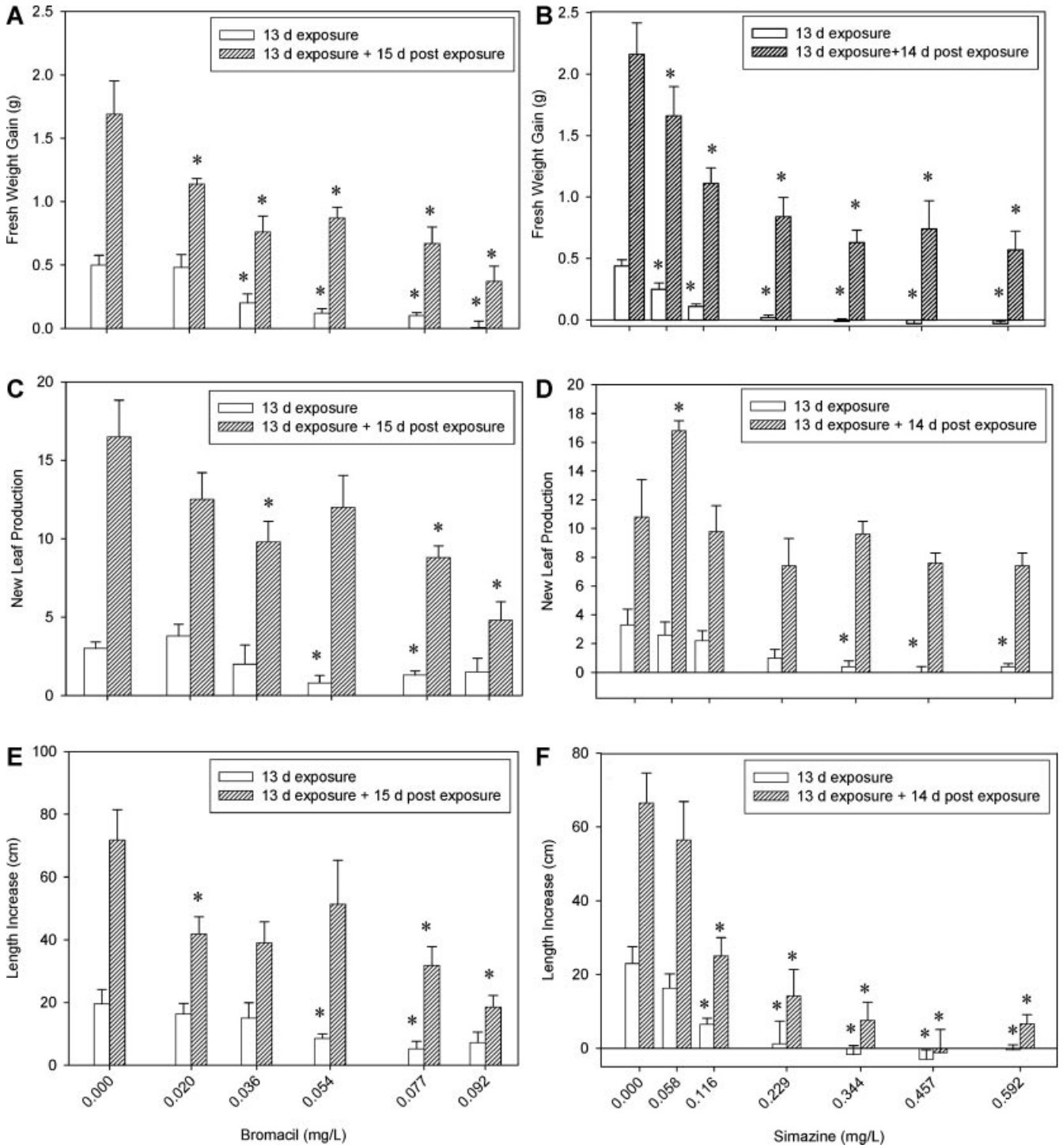

Fig. 1. *Vallisneria americana* Michx. (American tapegrass) growth measurements following 13-d exposure and 15-d (bromacil) or 14-d (simazine) postexposure periods to various concentrations (mg/L) of bromacil and simazine. (**A–B**) total fresh weight gains (whole plant), (**C–D**) total quantity of new leaves produced, including daughter plants, and (**E–F**) total increase in cumulative leaf length (whole plant). *Indicates mean is significantly different from control; analysis of variance of ranked data ($p = 0.05$). $n = 5$ for all simazine treatments and $n = 4$ for all bromacil treatments. Lines = standard error.

concentrations $\geq$0.020 mg/L. Stolon fresh weights were also reduced by bromacil exposures, with a realized NOAEC, LOAEC, and EC50 of 0.020, 0.036, and 0.022 mg/L, respectively. Stolon fresh weights were reduced 59 to 82% relative to controls.

Significantly fewer daughter plants were produced for plants exposed to simazine concentrations $\geq$0.344 mg/L (Fig. 2B); with an EC50 = 0.144 mg/L. Significant reductions in daughter plant counts ranged from 66 to 89% relative to the controls. The fresh weights of daughters produced were highly variable with CVs ranging from 0 to 1.40% (data not shown). As a result, there were no statistical differences in fresh weights of offspring produced. However, the estimated EC50 was 0.114 mg/L. Mean daughter plant fresh weights were reduced 50 to 90% at

Table 1. Summary of no-observable-effects concentrations (NOAECs), lowest-observable-effects concentrations (LOAECs), and nonlinear regression estimates of median effective concentration (EC50s) and corresponding EC50 estimate r^2 values for *Vallisneria americana* Michx. (American tapegrass) exposed to bromacil and simazine (separately) for 13 d followed by a 15-d (bromacil) or 14-d (simazine) recovery period

	Bromacil				Simazine			
Endpoint	NOAEC (mg/L)	LOAEC (mg/L)	EC50 (mg/L)	r^2	NOAEC (mg/L)	LOAEC (mg/L)	EC50 (mg/L)	r^2
Growth								
Fresh weight gain—exposure	0.020	0.036	0.032	0.990	<0.058	0.058	0.067	0.989
Fresh weight gain—postexposure[a]	<0.020	0.020	0.040	0.989	<0.058	0.058	0.086	0.998
New leaf production—exposure	0.036	0.054	0.036	0.982	0.229	0.344	0.154	0.990
New leaf production—postexposure[a]	0.054[b]	0.077	0.071	0.981	0.592	>0.592	>0.592	—
Length increase (cm)—exposure	0.036	0.054	0.043	0.993	0.058	0.116	0.081	0.983
Length increase (cm)—postexposure[a]	<0.054[b]	0.077	0.053	0.968	0.058	0.116	0.096	0.991
Vegetative reproduction (postexposure[a])								
Offspring quantity	0.054[b]	0.077	0.076	0.907	0.229	0.344	0.144	0.928
Offspring fresh weight	<0.020	0.020	0.016	0.973	0.592	>0.592	0.114	0.982
Stolon fresh weight	0.020	0.036	0.022	0.988	0.058	0.116	0.102	0.989
Dry weights (postexposure[a])								
Total plant	0.092	>0.092	>0.092	—	0.229	0.344	0.106	0.994
Shoot	0.092	>0.092	>0.092	—	0.229[b]	0.334	0.186	0.994
Root	0.020	0.036	0.033	0.990	0.457[b]	0.592	0.080	0.992
Stolon	0.020	0.036	0.020	0.988	0.229[b]	0.344	0.156	0.989
Leaf greenness/chlorophyll a								
SPAD units (Greenness)—exposure	0.092	>0.092	>0.092	—	<0.058	0.058[c]	>0.592	—
SPAD units (Greenness)— postexposure[a]	0.092	>0.092	>0.092	—	0.592	>0.592	>0.592	—
Total chlorophyll— postexposure[a]	0.092	>0.092	>0.092	—	0.592	>0.592	>0.592	—
Soluble sugar and starch allocation (postexposure[a])								
Roots and shoots								
Total soluble sugars	0.092	>0.092	>0.092	—	0.592	>0.592	>0.592	—
Hexose	0.092	>0.092	>0.092	—	0.592	>0.592	>0.592	—
Sucrose	0.092	>0.092	>0.092	—	0.592	>0.592	>0.592	—
Starch	0.092	>0.092	>0.092	—	0.592	>0.592	>0.592	—

— = not applicable.
[a] Postexposure = 13-d exposure + 15-d postexposure period (bromacil) and 13-d exposure + 14-d postexposure period (simazine).
[b] One lower concentration was significantly different from the control (analysis of variance of ranked data, $p = 0.05$).
[c] Effects were not negative relative to the control.

concentrations ≥0.116 mg/L, relative to the controls. Stolon fresh weights were reduced by 50 to 87%, relative to controls, at concentrations ≥0.116 mg/L. The stolon fresh weight reduction EC50 was 0.102 mg/L.

Dry weights. The data for dry weight measurements are summarized in Figure 3. Total dry weights for plants exposed to bromacil were 73, 75, 71, 70, and 68% of the controls for the 0.020, 0.036, 0.054, 0.077, and 0.092 mg/L treatments, respectively (Fig. 3A). Although the mean dry weights for the treatments were numerically similar, statistically only the 0.020 and 0.036 mg/L treatments were different from the controls, yielding a NOAEC of 0.092 and a LOAEC of >0.092 mg/L. It was not possible to estimate an EC50. No significant differences were seen in shoot dry weights (range: 100.6–125.7 mg) of plants exposed to bromacil at the end of the study (Fig. 3C). However, root dry weights were significantly reduced by 53 to 77% at concentrations ≥0.036 mg/L (Fig. 3E), with an EC50 = 0.033 mg/L (Table 1). Although not statistically different, mean root dry weights for plants exposed to 0.020 mg/L bromacil were 36% lower than the controls. Stolon dry weights were reduced by 66 to 80% at bromacil concentrations ≥0.036 mg/L (Fig. 3G), with an EC50 = 0.020 mg/L.

Total dry weights for plants exposed to simazine were 76, 53, 57, 40, 39, and 36% of the controls for the 0.058, 0.116, 0.229, 0.344, 0.457, and 0.592 mg/L treatments, respectively (Fig. 3B). All of the treatments were significantly different from the controls except for 0.058 and 0.229 mg/L, yielding an NOAEC = 0.229 and an LOAEC = 0.344 mg/L. The EC50 was 0.106 mg/L. Significant reductions of 47, 60, 64, and 64% in shoot dry weights occurred at the 0.116, 0.344, 0.457, and 0.592 mg/L simazine treatments, respectively (Fig. 3D). Although not statistically different from controls, mean shoot dry weight for the 0.229 mg/L treatment was only 58% of the controls. The EC50 was 0.186 mg/L. Root dry weights were reduced by 59 and 58% at the 0.344 and 0.592 mg/L treatments, respectively (Fig. 3F). Although not statistically different, mean root dry weights for the 0.116, 0.229, and 0.457 mg/L treatments were only 56 to 60% of the controls. The root dry weight EC50 was 0.080 mg/L. Stolon dry weights were reduced by 46, 50, 74, 67, and 87% for the 0.116, 0.229, 0.344, 0.457, and 0.592 mg/L treatments, respectively (Fig. 3H), with an estimated EC50 = 0.156 mg/L.

Leaf greenness and total chlorophyll. No adverse effects were seen for leaf greenness or total chlorophyll measurements for either herbicide (data not shown). Leaf greenness ranged from 11.2 to 20.5 and 12.1 to 16.7 SPAD units for bromacil following the exposure and postexposure periods, respectively. Leaf greenness for plants in the simazine study ranged from 10.9 to 19.5 and 14.1 to 18.5 for the exposure and postexposure periods, respectively. Interestingly, following the exposure period for simazine, leaf greenness was significantly higher for all of the treatment concentrations relative to the controls, ranging from 137 to 178%. These plants were noticeably darker in color. Following the postexposure period, leaf greenness

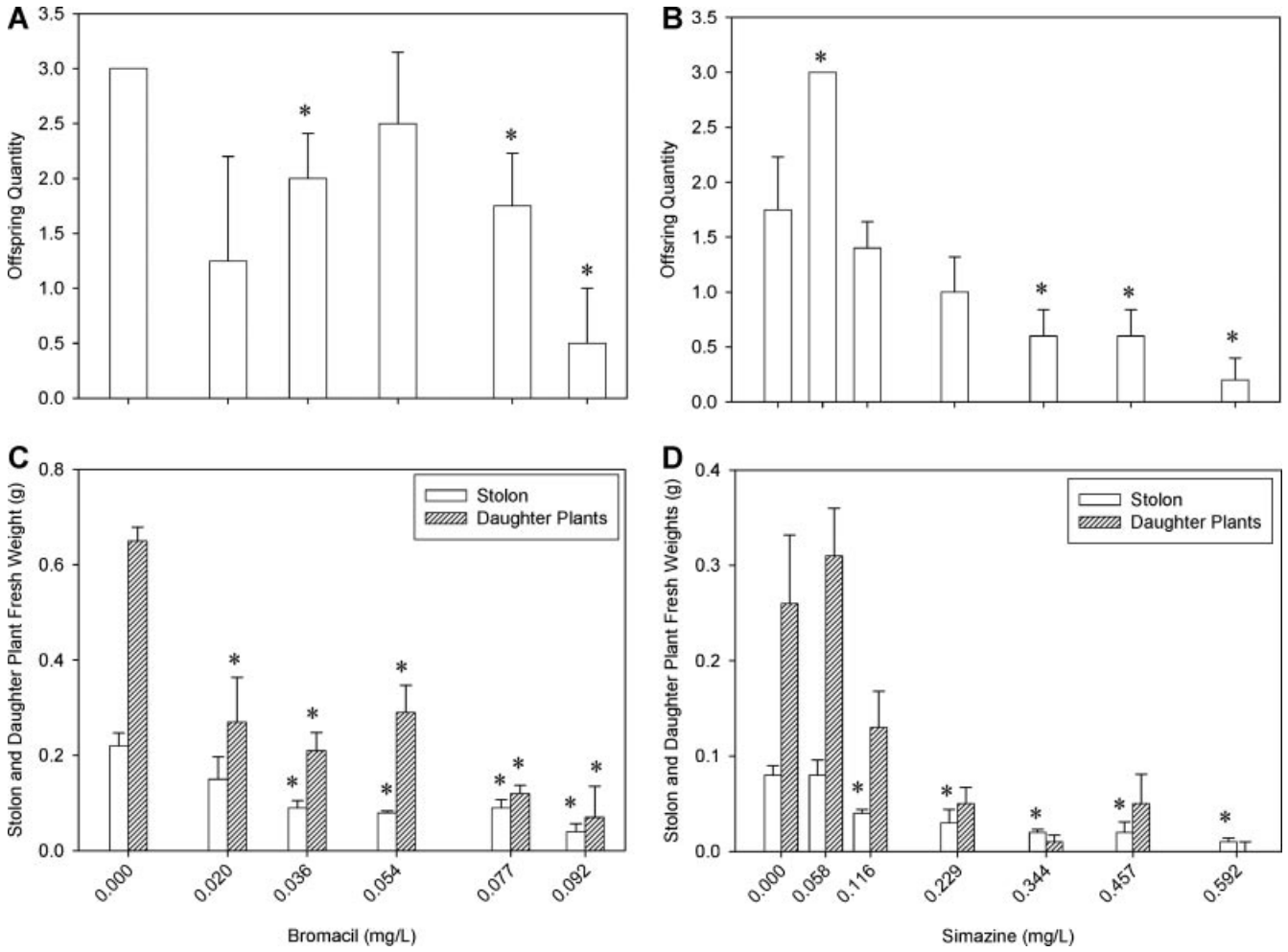

Fig. 2. *Vallisneria americana* Michx. (American tapegrass) vegetative reproduction measurements following 13-d exposure + 15-d (bromacil) or 14-d (simazine) postexposure periods to various concentrations (mg/L) of bromacil and simazine. (**A–B**) offspring quantity, (**C–D**) stolon and offspring fresh weights. *Indicates mean is significantly different from control; analysis of variance of ranked data ($p = 0.05$). $n = 5$ for all simazine treatments and $n = 4$ for all bromacil treatments. Lines = standard error.

levels for all treatment concentrations were similar to the controls. Total chlorophyll concentrations ranged from 8.5 to 11.5 μg/g and 6.8 to 10.5 μg/g for the bromacil and simazine treatments, respectively.

Soluble sugars and starch. Among all bromacil treatments, average sucrose levels ranged from 5.3 to 8.7 mg/g dry weight in shoots and 0.7 to 4.3 mg/g dry weight in roots (Table 2). Relative to the controls, sucrose levels in plant shoots and roots were not significantly reduced when exposed to any of the bromacil treatments, given the 2-week recovery period. Average hexose levels (glucose and fructose) ranged from 14.8 to 29.4 mg/g dry weight in shoots and 5.7 to 13.5 mg/g dry weight in roots (Table 2). Relative to the controls, hexose levels in plant shoots and roots were not significantly reduced when exposed to any of the bromacil treatments. Average total soluble sugars (TSS; sucrose, D-glucose, D-fructose) ranged from 20.1 to 36.8 mg/g dry weight in shoots and 6.4 to 15.3 mg/g dry weight in roots (Table 2). Total soluble sugars levels in plant shoots and roots were not significantly reduced with any of the bromacil treatments. Average starch (insoluble polymer of glucose) levels were variable, ranging from 2.8 to 26.4 mg/g dry weight in shoots and 35.3 to 86.3 mg/g dry weight in roots, but were not significantly different when compared to untreated controls (Table 2).

Among simazine treatments, average sucrose levels ranged from 3.8 to 5.7 mg/g dry weight in shoots and fell below detectable levels in roots (Table 2). Relative to the controls, sucrose levels in plant shoots were significantly reduced when exposed only to the 0.116 mg/L simazine treatment. Average hexose levels (glucose and fructose) ranged from 7.7 to 16.2 mg/g dry weight in shoots and 1.9 to 5.2 mg/g dry weight in roots (Table 2). Hexose levels in plant shoots were significantly reduced when exposed to 0.229 mg/L simazine, but not the other concentrations. Average TSS ranged from 12.0 to 21.3 mg/g dry weight in shoots with the 0.058 and 0.229 mg/L treatments reducing TSS by 21 to 31%, respectively, compared to untreated controls. A comparable reduction (23%) was evident with the 0.116 mg/L treatment, although not statistically different from the controls. Starch levels ranged from 2.8 to 16.9 mg/g dry weight in shoots and 12.5 to 34.8 mg/g dry weight in roots (Table 2). When plants were treated with 0.344 mg/L simazine, shoots had 2.6 times more starch than untreated controls.

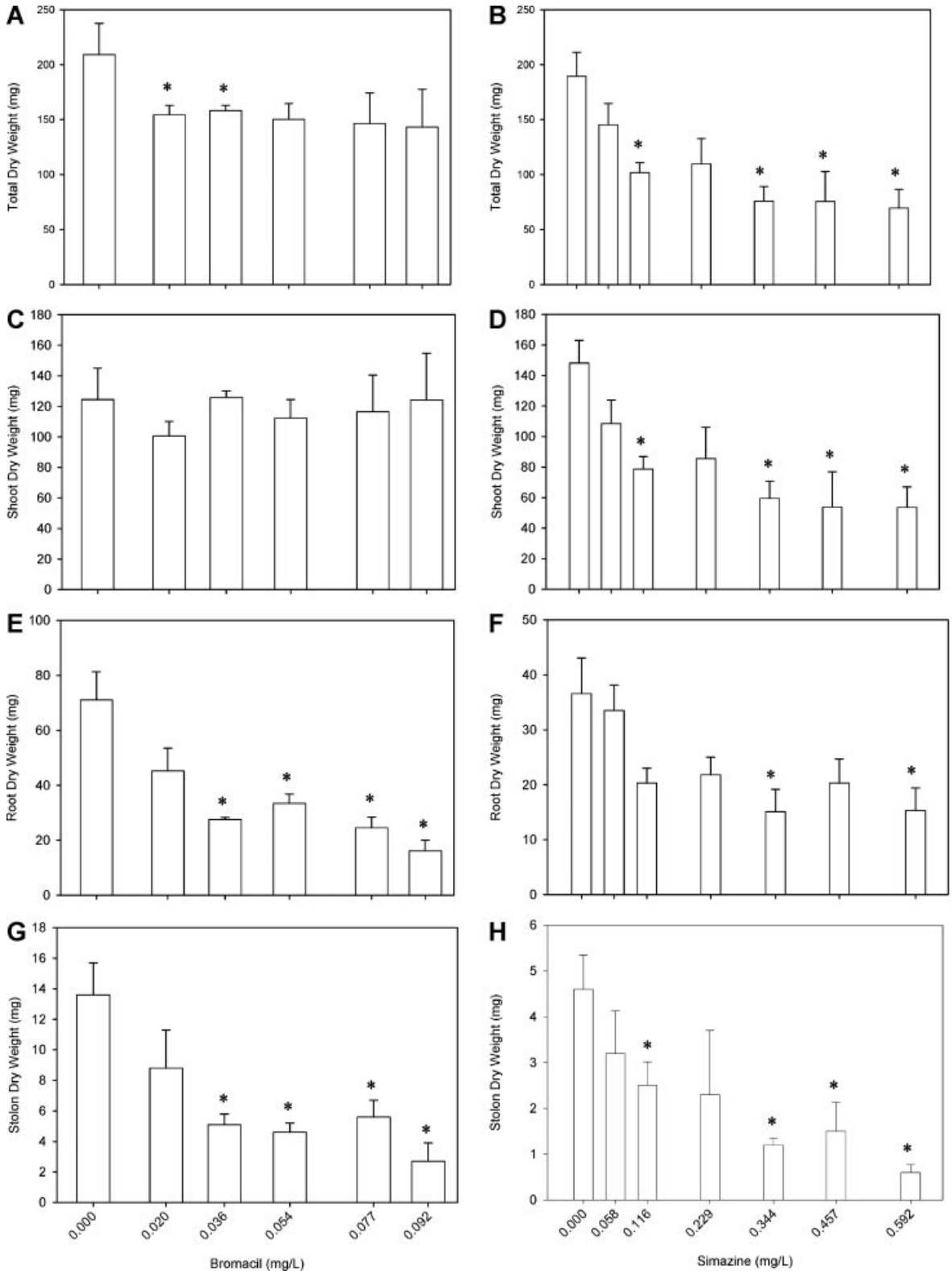

Fig. 3. *Vallisneria americana* Michx. (American tapegrass) dry weights following 13-d exposure + 15-d (bromacil) or 14-d (simazine) postexposure periods to various concentrations (mg/L) of bromacil and simazine. Dry weights for: (**A–B**) total plant, (**C–D**) shoots, (**E–F**) roots, and (**G–H**) stolons. *Indicates mean is significantly different from control; analysis of variance of ranked data ($p = 0.05$). $n = 5$ for all simazine treatments and $n = 4$ for all bromacil treatments. Lines = standard error.

Table 2. Summary of sucrose, hexose, total soluble sugar (TSS), and starch content in *Vallisneria americana* Michx. (American tapegrass) following 13-d exposure + 15-d (bromacil) or 14-d (simazine) postexposure periods to varying concentrations (mg/L) of bromacil and simazine

	Sucrose (mg/g-dry wt)				Hexose (mg/g-dry wt)				TSS (mg/g-dry wt)				Starch (mg/g-dry wt)			
	Shoots		Roots		Shoots		Roots		Shoots		Roots		Shoots		Roots	
Bromacil (mg/L)	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV
0	7.3	0.32	4.3	0.84	29.4	0.18	9.1	0.43	36.8	0.21	13.4	0.20	25.8	0.63	41.9	1.50
0.020	8.7	0.26	1.2	1.05	26.6	0.11	8.7	0.38	35.3	0.12	9.9	0.45	21.3	1.35	60.1	3.77
0.036	5.3	0.48	0.7	0.46	14.8	0.37	5.7	0.25	20.1	0.40	6.4	0.26	10.2	1.15	57.6	1.52
0.054	7.4	0.33	1.8	0.7	20.3	0.37	13.5	0.75	27.7	0.35	15.3	0.72	2.8	0.61	35.3	1.44
0.077	6.7	0.08	1.3	0.7	21.7	0.11	10.1	0.19	28.4	0.09	11.1	0.25	8.0	0.53	64.9	3.85
0.092	8.6	0.25	1.9	0.3	21.2	0.36	12.8	0.18	29.7	0.25	14.2	0.18	26.4	2.15	86.3	2.79
	Sucrose (mg/g-dry wt)				Hexose (mg/g-dry wt)				TSS (mg/g-dry wt)				Starch (mg/g-dry wt)			
	Shoots		Roots		Shoots		Roots		Shoots		Roots		Shoots		Roots	
Simazine (mg/L)	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV	Mean	CV
0	5.2	0.16	—	—	12.2	0.15	4.6	0.36	17.4	0.15	4.6	0.36	6.6	0.61	14.7	0.41
0.058	4.1	0.09	—	—	9.7	0.13	5.2	0.22	13.8[a]	0.10	5.2	0.22	8.6	0.26	17.4	0.84
0.116	3.8[a]	0.13	—	—	9.6	0.16	4.8	0.36	13.4	0.15	4.8	0.36	2.8	0.90	13.6	0.62
0.229	4.3	0.32	—	—	7.7[a]	0.37	2.9	0.57	12.0[a]	0.35	2.9	0.57	6.3	0.54	12.5	0.79
0.344	5.7	0.29	—	—	13.2	0.15	3.3	1.05	18.9	0.17	3.3	1.05	16.9[a]	0.74	32.3	0.56
0.457	5.1	0.42	—	—	16.2	0.60	4.2	0.28	21.3	0.54	4.2	0.28	13.1	0.49	26.3	0.65
0.592	4.4	0.23	—	—	11.8	0.31	1.9	0.95	16.2	0.27	1.9	0.95	5.1	0.79	34.8	1.62

— = nonmeasurable levels.
CV = coefficient of variation.
$n = 5$ for all treatments (simazine).
$n = 4$ for all treatments (bromacil).
[a] Indicates mean is significantly different from control; analysis of variance of ranked data ($p = 0.05$).

DISCUSSION

Given the lack of data regarding herbicide toxicity to *V. americana*, one goal of these studies was to identify which measurements were most appropriate and useful for characterizing toxic thresholds and effects levels. Many potential endpoints can be measured for estimating the NOAEC, LOAEC, and EC50 of pesticides on target organisms. However, the most protective estimates are those with the lowest values relative to the plant aspect (i.e., growth, reproduction, etc.) to be protected. Ideally, all treatment concentrations greater than the LOAEC are statistically different from the controls, and all treatment concentrations less than or equal to the NOAEC are statistically similar to the controls.

Under the experimental conditions used in the present study, measured endpoints varied in their usefulness for consistently estimating the NOAECs and LOAECs (Table 1). These also varied with each herbicide. Overall measurements of fresh weight gains following the exposure and postexposure periods were the only growth endpoint that provided consistent estimates for both herbicides (i.e., effects were significant at all concentrations greater than or equal to LOAEC). Measurements of new leaf production and total leaf growth for simazine treatments provided definitive NOAECs and LOAECs, although they were higher (less protective) than the fresh weight gain estimates. New leaf production and total leaf growth for bromacil estimates were less consistent due to having at least one treatment concentration greater than the LOAEC being not significant from the controls. Measurements of daughter plant and stolon fresh weights provided good estimates of the effects of bromacil on vegetative reproduction, whereas only stolon fresh weights were the most consistent predictor of the simazine NOAECs and LOAECs. Dry weight measurements also tended to be protective, with the additional advantage of isolating the effected plant organs (i.e., shoot, root, and stolons). For the reproductive measurements, EC50 estimates were most protective for daughter plant and stolon fresh weight measurements at the end of the study. Simply counting the daughters produced tended to be less sensitive. Measurements of total chlorophyll, leaf greenness, and soluble sugars/starch allocation were not useful for estimating EC50s due to a lack of response.

Estimates of NOAEC, LOAEC, and EC50 are useful for comparing relative toxicities of a compound between endpoints and species. This can be especially useful for evaluating the level of protection provided for a target species by use of other surrogate species. The EC50 estimates varied greatly depending on the endpoint measured (Table 1). As with the NOAEC/LOAEC, estimates of fresh weight gains were the most consistent and sensitive indicators for total plant growth effects (Bromacil EC50 = 0.032 mg/L; Simazine EC50 = 0.067 mg/L). Measurements of leaf production and length-wise growth tended to be less protective. These EC50 values are lower than those reported by Fairchild et al. [25], who reported a NOAEC of 0.6 mg/L, LOAEC of 1.2 mg/L, and an EC50 of 1.240 mg/L (1,088–1,393) for *Selenastrum capricornutum* biomass production (measured by fluorescence) while exposed to simazine for 96 h. Additionally, they reported an NOAEC of 0.075 mg/L, an LOAEC of 0.150 mg/L, and an EC50 of 0.166 mg/L (102–230) for the same exposures to *Lemna minor* [25]. These results indicate that use of *Selenestrum* as a surrogate for *Vallisneria* may not provide protection. However, the *Lemna* results were more similar and protective of *Vallisneria* exposed to simazine.

Other researchers reported NOAEC and LOAEC values for *Myriophyllum aquaticum* [26], *Canna hybrida* [27], and *Typha latifolia* [28] following a 7-d exposure + 7-d postexposure period. The most sensitive NOAEC/LOAEC values reported were 0.1/0.3 mg/L for branch production in *M. aquaticum* and 0.3/1.0 mg/L for fresh weight gains in both *C. hybrida* and *T. latifolia* exposed to simazine by root contact only [26–28]. The simazine Aquatic Life Benchmark Values for acute nonvascular and vascular plants used by the U.S. EPA for risk assessments are 36 μg/L and 140 μg/L, respectively (http://www.epa.gov/oppefed1/ecorisk_ders/aquatic_life_benchmark.htm; accessed 2/19/2009). Use of the U.S. EPA nonvascular benchmark may be more protective of *V. americana* for risk management under longer term exposures. Schafer et al. [29] reported an NOAEC of 0.045 mg/L and an EC50 of 0.097 mg/L for growth inhibition in the green algae, *Scenedesmus subspicatus*, exposed to bromacil for 72 h. The U.S. EPA acute nonvascular aquatic plant benchmark is 6.8 μg/L and the acute vascular plant benchmark is 45 μg/L (http://www.epa.gov/oppefed1/ecorisk_ders/aquatic_life_benchmark.htm; accessed 2/19/2009). As with simazine, use of the nonvascular benchmark should be more protective of *V. americana*. In both cases, the Aquatic Life Benchmark values for vascular plants were less protective than the benchmark values for nonvascular plants.

Within the confines of these studies, effects caused by both herbicides appeared to be lasting, relative to the controls. The 13-d exposure to bromacil resulted in a fresh weight gain LOAEC of 0.036 mg/L, whereas the postexposure period LOAEC was 0.020 mg/L, indicating latent effects. In contrast, the postexposure NOAEC and LOAEC for simazine were the same as following the exposure period ($<$0.058/0.058), indicating no latent effects. However, some recovery was observed for all treatments for the fresh weight gain, new leaf production, and length increase endpoints. Although these measures indicate that some recovery was in progress and that the effects were at least partially reversible, there is no way to predict how long it would take for health, growth, and vegetative reproduction to be restored to nonexposed levels, if at all, based on these studies. Longer term studies are needed to assess whether affected plant endpoints could possibly recover to control levels. The fact that some recovery was evident at all treatment concentrations suggests that toxicity tests incorporating a recovery period may be more useful than exposure-only tests for predicting effects in an environment likely to receive pulsed pesticide inputs.

Both of these herbicides are photosynthesis inhibitors, binding to the Q_B-binding niche on the D1 protein of the photosystem II complex in chloroplast thylakoid membranes, effectively blocking electron transport from Q_A to Q_B [3]. Although this blockage ultimately can inhibit CO_2 fixation and production of energy resources for the plant, actual death usually is due to cell desiccation and loss of pigments caused by free radical reactions [3]. The free radicals, triplet chlorophyll, and singlet oxygen, are formed in response to the inability to reoxidize Q_A [3]. Plant sensitivity to bromacil was much greater than simazine, as evidenced by the occurrence of adverse effects at much lower concentrations. The reductions in stolon and offspring fresh weights at $\geq$0.036 and $\geq$0.020 mg/L bromacil, respectively, and $\geq$0.116 mg/L simazine for stolons indicates that plants exposed to these levels of the herbicides likely diverted resources from asexual reproduction to maintenance and reconstruction of roots and shoots on the parent plant.

Interestingly, although significant negative impacts to plant growth were seen after 13-d exposure to simazine, leaf greenness measurements indicated that there were significantly more green pigments in all exposed plants relative to the controls. These plants were noticeably darker green than the controls. However, at the end of the study, leaf greenness was similar to the controls for all treatments. It is possible that the plants exposed to simazine increased chlorophyll content in response to the interference with photosystem II to help compensate for the reduced photosynthetic efficiency. Although this seems to reasonably explain this manifestation, no studies have confirmed this response.

Measurements of soluble sugar/starch allocation were also not sensitive endpoints under the conditions evaluated. The lack of consistent dose-dependent changes in TSS, hexose, and starch for bromacil and simazine at the end of the study suggests that soluble sugar/starch production and metabolism had established a new steady state following removal of the herbicides. This observation is counter to that reported by Wilson et al. [30] for *V. americana* response to the herbicide norflurazon, a carotenoid biosynthesis inhibitor. In that study, lower amounts of TSS and hexose in the shoots seemed to indicate increased use of the D-glucose, D-fructose, and sucrose for regeneration of photosynthetic machinery and for new growth. This was supported by the observation that the significant reductions in TSS and hexose occurred at the same treatment levels where plants showed visual signs of bleaching after the 14-d exposure period [30]. They also observed higher amounts of sucrose in the roots at the 0.1 mg/L treatment level, which likely was associated with increased transport into the roots from the shoots to support root regeneration. The observation of no differences in the present study likely indicates that the plants had reached a new steady state relative to carbohydrate metabolism after adjusting to the losses of root and shoot tissue observed with the other measurements once the herbicides were removed. This is in contrast to plants exposed to norflurazon, where no reductions in fresh weight production, new leaf production, or total leaf growth were observed [27]. The norflurazon toxicity exhibited as bleaching of pigments from the leaves (youngest first) during exposure, followed by regeneration of pigments once the norflurazon was removed. The simazine and bromacil exposures actually resulted in destroyed tissues, which would necessitate a readjustment of carbohydrate metabolism to a new level to support the reduced biomass. The re-establishment of steady-state carbohydrate metabolism is further supported by the increases in growth measurements for all treatments at the end of the study.

Variability in measurements was the most significant factor limiting the usefulness of many of the endpoints for estimation of NOAEC, LOAEC, and EC50 values. It is possible that increased replicates of the treatment plants could eliminate some of the inconsistencies in the estimates by reducing variability. Additionally, use of asexually produced plants originating from a single parent plant could have significantly reduced variability, but may have resulted in misrepresentation of responses in genetically diverse *V. americana* communities. However, time, facilities, labor, and financial resources were limiting. These studies were designed to fit all replicates under

two light fixtures (outer dimensions: 61 × 122 cm) and used all daughter plants produced in four 37.9 L aquaria stocked with at least 25 stock plants each.

CONCLUSIONS

These studies illustrate the value of including a recovery period following an exposure period. With an exposure only test, no information is available to assess the onset or potential for recovery of the affected plant. Tests that include a recovery period may also more realistically represent pulsed exposures common in the environment.

Based on these results, measurements of fresh weight gains after the exposure and postexposure periods were the most reliable and sensitive estimator of bromacil and simazine effects on *V. americana* growth and asexual reproduction. Frequent 2-week exposures of plants to 0.020 to 0.036 mg/L bromacil or higher would likely result in reduced plant growth and recruitment via asexual reproduction. Frequent two-week exposures of plants to ≥0.058 mg/L simazine would likely result in reduced plant growth, and possibly recruitment via asexual reproduction. Within the context of the published bromacil and simazine environmental concentrations, some negative effects may be possible, especially if the plants are located in close proximity to the edge of field where the losses originate. Maximum reported simazine concentrations of ≥0.580 mg/L [7,9] would almost certainly impact growth and reproduction given a 2-week exposure period. Less severe or no effects might be expected in areas where the concentrations are more diluted due to drainage from a larger watershed as reported earlier; where simazine concentrations were significantly lower than 10 μg/L [5,6,10,11]. However, much uncertainty exists regarding actual herbicide concentrations present throughout the year due to the limited number of samples analyzed in those studies. Additionally, because plants were destructively analyzed at the end of the assay, no conclusion can be made regarding the effects on recruitment through sexual reproduction. It is also likely that differences in the other measured endpoints may become more pronounced under frequent 2-week exposure scenarios because more metabolic resources would be devoted to plant maintenance and reconstruction at any given time. Additional studies are needed to better characterize the effects of these herbicides on sexual reproduction and seed germination, and for assessing the full recovery potential for affected plants.

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