Toxicity of the Herbicides Norflurazon and Simazine to the Aquatic Macrophyte, *Lemna minor* L.

Michelle Ouellette
SOS # Research Project Report

Introduction

Some herbicides may be mixed (following label instructions) in the spray tank to increase the toxic effects on undesirable weeds or to provide broader spectrum weed control (i.e. exploit different modes of action). Tank mixtures of simazine and norflurazon are commonly used in citrus production systems. Norflurazon and simazine are both pre-emergent herbicides. They are also commonly detected in surface water associated with South Florida agricultural canals and drainage water (Wilson and Boman 2011), indicating their movement from sites of application into the surface water drainage systems. These herbicides may be applied together as a tank-mix in order to enhance weed control in the terrestrial environment. Given their application together, some concerns exist over their potential effects on non-target aquatic plant species. Before the effects of herbicide mixtures can be assessed for non-target species, effect threshold concentrations must be known for each individual herbicide. These experiments characterized the toxic concentrations of these two herbicides on the surrogate aquatic macrophyte, *Lemna minor* L. This information is needed before evaluation of the potential interactions can proceed.

Simazine (6-chloro-N^2, N^4-diethyl-1,3,5-triazine-2,4- diamine) is registered for selective and nonselective control of grassy and broadleaf weeds in fruit, nut, and field crops, turf grasses, vegetables, orchards, and vineyards (Knuteson et al., 2002). Simazine production may result in localized releases into the environment through various point-source waste streams (Tomlin 1997). The use of simazine as a selective systemic herbicide for controlling annual grasses and broad-leaved weeds results in its direct release into the environment (Tomlin 1997). Simazine is moderately soluble in water (6.2 mg/L at 25 °C)(Vencill 2002) and is relatively non-volatile (Wilson and Wilson 2010). Photo-chemically produced hydroxyl radicals in the atmosphere may react with the trace amounts of simazine that may be present in the atmosphere, with an estimated half-life of 2.8 hours (US EPA National Primary Drinking Water Regulations 2010). With a vapor pressure of 2.9×10^{-9} kPa at 25 deg C (Vencill 2002), low log K_{ow} (2.17) and low Henry's Law constant of 4.63×10^{-9} atm m^3 mol^{-1} (Knuteson et al, 2002) simazine is not expected to volatilize from dry and moist soil surfaces, respectively . Based on its low K_{oc} value, simazine does not adsorb strongly to soil organic materials. Also, if released into water, some adsorption of simazine to suspended solids and sediment in the water column is expected (US EPA 1988). The average half life of simazine in ponds where it has been applied is 30 days, with the actual half life

dependent on the level of algae present, the degree of weed infestation and other factors (WSSA 1989). Simazine may undergo hydrolysis in water at a lower pH, but does not readily undergo under hydrolysis when water is at a pH of 7 (WSSA 1989). Volatilization of simazine from water surfaces is not expected to occur based upon its estimated Henry's Law constant. Simazine has a BCF of 221 which suggests potential bioconcentration in aquatic organisms is moderate (Schuler 2008).

Norflurazon (4-chloro-5-(methylamino)-2-(alpha, alpha, alphatrifluoro-m-tolyl)-3(2H) pyridazinone) is a selective pre-emergent herbicide used to control germinating annual grasses and broadleaf weeds in cranberries, cotton, soybeans, almonds, apples, apricots, cherries, citrus and non-crop areas such as storage areas, airports, and rights-of-way. Norflurazon is more soluble than simazine in water (28 mg/L at 25°C) (Vencill 2002). The physicochemical properties of norflurazon (Table 1) indicate potential to move offsite with water (Troiano et al., 1999). With a vapor pressure of 2.66×10^{-9} kPa at 20 deg C, norflurazon is not expected to volatilize from dry soil surfaces (Vencill 2002). Norflurazon is a persistent and mobile compound. When it is applied to soil it is expected to be moderately mobile based on an estimated K_{oc} of 700 (Ware 1992). The primary route of dissipation for norflurazon in water and on soil surfaces is photodegradation to desmethyl-norflurazon, with a half-life of 2-3 days and 12-15 days, respectively (US EPA 1996). Norflurazon is stable to hydrolysis and degrades slowly in aerobic soil with an estimated half-life of 130 days. In an aerobic aquatic study, norflurazon degraded to desmethyl-norflurazon with a half-life of 6-8 months. Under anaerobic conditions, norflurazon is also persistent with a half life of approximately 8 months (US EPA 1996). Hydrolysis is not expected to be an important environmental fate process since this compound lacks functional groups that hydrolyze under environmental conditions. An estimated BCF of 28 suggests the potential for bioconcentration in aquatic organisms is low (Schuler 2008).

Simazine is a photosynthesis inhibitor and norflurazon is a pigment inhibitor. Often, specific herbicidal mechanisms-of-action produce unique symptoms of toxicity in susceptible plants exposed to lethal or sub-lethal concentrations. Simazine is a photosystem II inhibitor. The transfer of electrons from photosystem II to photosystem I is essential for the production of photosynthetic energy. This herbicide binds to the D_1 protein of the photosystem II complex in thylakoid membranes, blocking electron transport. This inhibition of electron transport stops the synthesis of ATP and NADPH in the chloroplast, resulting in an inability to fix CO_2 and produce carbohydrates needed for respiration. This blockage of electron transfer also causes oxidative stress through the generation of free radicals (Roe et al, 1997). This herbicide is described as a “bleaching herbicide” because the primary symptom of toxicity is bleaching of green tissues making them appear yellow or white (Dayan et al. 2012). This herbicide is

absorbed by roots and translocates to the shoot tissue where it inhibits carotenoid synthesis. Without carotenoids, chlorophyll may be destroyed due to the inability to effectively dissipate excess energy absorbed by the plant. This herbicide does not destroy carotenoids already formed, but prevents the formation of new ones. Norflurazon inhibits carotenoid biosynthesis by inhibiting phytoene desaturase (Dayan et al. 2012). Norflurazon can be combined with simazine to provide control of a broader spectrum of weeds than either herbicide alone (Dayan et al. 2012).

Significant amounts of simazine and norflurazon are used in Florida. From 2007-2009, 313,400 total pounds of simazine was used for grapefruit, orange, tangelo, and tangerine production (Florida Department of Agriculture and Consumer Services, 2008). Norflurazon use from 2003-2006 was 317,532 total pounds for production of grapefruit, orange, tangelo, tangerine, temple, and cotton (Florida Department of Agriculture and Consumer Services, 2010).

Table 1. Summary of herbicide characteristics

Herbicide	Chemical Class/Use	CAS #	Molecular Weight (g/mol)	Solubility	Vapor Pressure	Henry's law constant ($atm\ m^3\ mol^{-1}$)	log Kow	Koc (mL/g)
Simazine	Triazine	122-34-9	201.66	6.2 mg/L	2.9×10^{-9} kPa at 25 deg C	4.63×10^{-9}	2.17	130
Norflurazon	Pyridazinone	27314-13-2	303.7	28 mg/L	2.66×10^{-9} kPa at 20 deg C	2.49×10^{-10}	2.45	700

(Essington 2004; Knuteson et al, 2002; US EPA 1999; Vencill 2002; Ware 1992)

Given the widespread use of these herbicides in Florida, and the high likelihood of them moving into surface water bodies and drainage systems where non-target macrophytes grow, these studies were designed to evaluate the effects of norflurazon and simazine on the surrogate macrophyte, *Lemna minor*. Duckweed (*Lemna minor* L.) is often used in water quality studies to monitor effects of pollutants on aquatic macrophytes. The small size, rapid growth rates between pH 5 and 9, and vegetative propagation make them an ideal test system for monitoring biological effects (Radic´, et al, 2009). This information is needed before effects of mixtures of the herbicides on non-target aquatic macrophytes can be assessed.

Materials and Methods

Herbicide stock solutions

Two stock solutions of norflurazon were made using *Predict Herbicide* (norflurazon, 78.6%), a commercially available formulation. A primary stock solution was made by adding 1276.6 mg of the formulation to 1000 ml of reagent-grade water. A secondary stock solution was made by diluting 1 ml of the primary stock solution into 1000 ml of reagent-grade water. Likewise, two stock solutions were made with simazine using the commercially available formulation, *Simazine 4L* (simazine, 42.1%). The primary stock solution was made by diluting 2.086 ml of Simazine 4L into 998 ml of R-G water. One (1) ml of the primary stock solution was diluted with 999ml of R-G water to make the secondary stock solution. Both of these stock solutions were suspensions, requiring constant stirring using a magnetic stir plate.

Growth media

For culturing and testing, reconstituted moderately hard water (RMH) (15 L) was made as follows: 15 L R-G water, 1.44 g sodium bicarbonate ($NaHCO_3$), 0.9 g magnesium sulfate (MgSO4), 0.6 g potassium chloride (KCL), and 0.9 g calcium sulfate ($CaSO_4 \times 2H_2O$). RMH water had a pH of 7.4-7.8, hardness between 80-100 mg/L as $CaCO_3$ and alkalinity of 60-70 mg/L as $CaCO_3$. Hoagland's nutrient solution (20%) was made by adding the required nutrients (Table 2) to 15 L of RMH water.

Table 2. Salts added to RMH water to make 15 L of 20% Hoagland's nutrient solution.

Salt	Stock Concentration	Volume of stocks needed (in 1L) (ml)
1. KH2PO4	1M (136.09 G)	3
2. KNO3	1M (101.1 G)	15
3. Ca (NO3)2	1M (236.15 G)	15
4. MgSO4	1M (120.37 G)	6
5. Supplementary Micronutrient Solution		3
H3BO3	2.86 (g/L)	
MnCl2 ·4H2O	1.81 (g/L)	
ZnSO4 ·7H2O	0.22 (g/L)	
CuSO4 ·5H2O	0.08 (g/L)	
NaMoO4 ·H2O	0.02 (g/L)	
6. Iron Supplementary Solution	1000 ppm EDTA- FeIII sodium salt hydrate (1000 mg/L)	15

Herbicide dosing

Treatment concentrations were achieved by addition of stock solutions to RMH/ Hoagland's solution in a 2000 ml beaker (stock volumes shown in Table 3). Initially, range-finding studies were conducted using concentrations of 0, 0.01, 0.025, 0.05, 0.1, 0.25, and 0.5 mg/L for each herbicide. Based on those results, definitive studies were conducted using a narrower range of concentrations in order to determine effects-concentrations. Seven (7) norflurazon treatments and eight (8) simazine treatments were prepared from the primary (1000 ppm) and secondary (1 ppm) stock solutions. For the controls (0 ppm) of both herbicides, no stock solution was added to the nutrient solution.

Table 3. Simazine and norflurazon target concentrations and volumes of primary and secondary standards used to formulate treatment solutions.

Herbicide Treatment	Target Concentration (mg/l)	Primary Stock Solution (ml)	Secondary Stock Solution (ml)	Total Volume (ml)
*Norflurazon**				
1	0	-	0	1000
2	0.01	-	10	1000
3	0.025	-	25	1000
4	0.05	0.05	-	1000
5	0.1	0.1	-	1000
6	0.25	0.25	-	1000
7	0.5	0.5	-	1000
*Simazine***				
1	0	-	0	2000
2	0.05	50	-	2000
3	0.1	100	-	2000
4	0.2	200	-	2000
5	0.4	400	-	2000
6	0.6	600	-	2000
7	0.8	800	-	2000
8	1	1000	-	2000

*Norflurazon stock solution was added to 1000 ml of RMH/ Hoagland's solution mixture in a 2000 ml beaker

**Simazine stock solution was added to 2000 ml of RMH/ Hoagland's solution mixture in a 2000 ml beaker

Confirmation of herbicide concentrations

Herbicide concentrations were measured at the beginning of each assay to confirm that initial exposure concentrations were accurate. Duplicate volumes of each treatment solution (from extra volume of each treatment solution) ranging from 20 – 1000 mLs were extracted to achieve a 1 mg/L target concentration in the extract, assuming 100% recovery. The designated treatment solution volumes were transferred into corresponding separatory funnels. Methylene chloride was added to each separatory

funnel (10 -30 mls for norflurazon extraction, 30 mls for simazine extraction), which was then shaken. The solvent layer was then collected into a 250 ml flask. This procedure was repeated twice more. The methylene chloride was evaporated completely in a water bath (60°C). Each flask was rinsed with MTBE three times, six (6) ml each time, and the rinsate was transferred into a concentrator tube. The concentrator tubes were placed in a RapidVap system to reduce the liquid volume to less than 1 ml. The liquid in each concentration tube was then brought back up to 2 ml with MTBE and transferred into a labeled gc vial. Herbicide concentrations were quantified using a Hewlett-Packard 5890 Series II gas chromatograph (Agilent Technologies) equipped with dual electron capture detectors and a RXi-5ms-Sil column (Restek, Bellefonte, PA, USA) and RXi35ms-Sil column (Restek, Bellefonte, PA, USA). The GC was operated under the conditions outlined by Wu et al. (2010). Recoveries of each herbicide were 80% (61-97%, simazine) and 85.2% (70-97%, (norflurazon).

Plant exposures

All assays were conducted in glass crystallization dishes covered with glass petri dishes. All glassware was sterilized in an autoclave for 30 minutes and dried for 10 minutes before they were taken out. For treatment solutions, beakers (2000 mL) were labeled and taken to the tissue culture lab. Five glass crystallization/petri dishes were labeled for each treatment (e.g. T1-1 = treatment 1, exposure dish 1). One hundred milliliters of each treatment solution was transferred into each respective exposure dish, with five replicates per treatment concentration for simazine and four replications per treatment for norflurazon. Plants of relatively uniform size and appearance were chosen for the assay. Between 8-11 duckweed fronds were transferred into each petri dish from an algal-free culture maintained in the Environmental Toxicology and Chemistry laboratory. To prevent cross contamination within treatments, forceps were decontaminated in between each petri dish using ethanol and a Bunsen burner flame. Acetone rinsing and a Bunsen burner flame were used for decontamination in between each treatment series. The plant-loaded replicates were then randomly placed on lighted-racks under a 16:8 h light:-dark photoperiod using plant and aquarium wide-spectrum fluorescent lights (General Electric, ~76 $\mu mol \cdot^{-1} \cdot^{-2}$ at a 30-cm height). Replicates were re-randomized every other day to distribute the lighting evenly between them.

Plant measurements

Measurements of growth parameters are commonly used to determine effects of contaminants on ecological resources such as macrophytes. Several destructive and nondestructive growth measurements were taken before exposures and after the 7 and 15 d exposure periods. These

measurements included: growth as measured by counts of new fronds produced (FN), functioning of electron transport in PSII (chlorophyll fluorescence, Fv/Fm), and fresh weights (FW).

Frond production

Counts for the norflurazon experiment were conducted once every other day at the same time, while counts for the simazine exposures were conducted at 7-d intervals. Each petri dish was taken from the fluorescent light rack and duckweed counts were performed with the lids on to prevent possible contamination of the duckweed fronds. After the sets of duckweed fronds were counted, all replicates were then placed back under the fluorescent light rack in a completely randomized order. New frond production was calculated by subtracting the initial number of fronds from the number measured at the end of the exposure periods.

Fv/Fm measurements

Light energy absorbed by chlorophyll molecules in a leaf can experience three fates: it can be used to drive photosynthesis, excess energy can be dissipated as heat, or excess energy can be re-emitted as light (chlorophyll fluorescence) (Maxwell and Johnson, 2000). These three processes occur in competition. An increase in the efficiency of one process will result in a decrease in the yield of the other two processes. By measuring the yield of chlorophyll fluorescence information can be gained on the changes in efficiency of photosynthesis and heat dissipation (Maxwell and Johnson, 2000).

Chlorophyll fluorescence (Fv/Fm) measurements were taken on the 7^{th} and 15^{th} days for all assays. On the 7^{th} day for each experiment, one petri dish from each treatment was chosen and divided into two (2) small glass vials. Using a plastic Pasteur pipette, water from each petri dish was used to fill each glass vial to the top. The duckweed fronds from each petri dish were then divided equally among the two (2) glass vials for each treatment. The glass vials were labeled, placed in a vial rack, and placed in a drawer in the lab to be kept in the dark for 30 min. After 30 minutes, the lights were turned off. Each glass vial was removed from the drawer and the fluorescence measurements (Fo, Fm, Fv/FmFv/Fm) were taken using an OptiSciences OS-500 chlorophyll fluorometer (OptiSciences, Hudson, HH, USA). On the 14^{th} day the contents of each petri dish were divided in two (2) glass vials and Fv/Fm was measured as previously described. Following measurements, plants were returned to their respective petri dishes so their fresh weights could be measured.

Final Fresh weights

Final fresh weight measurements were taken at the end of the studies using an Ohaus (Pine Brook, state) top-loading balance (Model TS 400s). At the end of the assays, plants were collected by pouring each replicate through a sifter. Plants were then measured on a tared dish placed on the scale. Fresh weight gains during the exposure periods were calculated by subtracting the initial fresh weight from that measured following exposure to both herbicides. Fresh weight measurements following post-exposure periods were calculated by subtracting the fresh weights at the end of the study from the initial fresh weights.

Statistical analysis

ANOVA with mean comparison's using Dunnett;s test were utilized via IBM SPSS Statistical Software was used for the statistical analysis. When a set of new treatments is compared with a control, Dunnett's two-tailed t test is usually used under the equal variances assumption (Koenig et al; 2008). Dunnett's test was used to determine the NOEC (No Observed Effect Concentration) and LOEC (Lowest Observed Effect Concentration) for each destructive and nondestructive growth measurement (frond production, fluorescence, and final wet weight).

Results and Discussion

Frond production

A significant reduction in new frond production was observed following 7 and 15 d exposure to norflurazon. After 7 d exposure, new frond production was similar to the controls at the 0.01 and 0.025 mg/L concentrations, with averages of 57.8 (control), 58 (0.01 mg/L), 55.6 (0.025 mg/L), 43 (0.05 mg/L), 35.6 (0.1 mg/L), 28.8 (0.25 mg/L), and 26.2 (0.5 mg/L) fronds produced. Mean frond production was reduced by 26%, 38%, 50%, and 55% percent, relative to controls, at the 0.05, 0.1, 0.25, and 0.5 mg/L norflurazon treatment concentrations. After 15 d exposure, some recovery was evident in the 0.05 and 0.1 mg/L treatments (Fig 1). The average production of new leaves after 15 d exposure was 221 (control), 273 (0.01 mg/L), 210 (0.025 mg/L), 186 (0.05 mg/L), 146 (0.1 mg/L), 58 (0.25 mg/L), and 50 (0.5 mg/L). Mean frond production was reduced by 16, 34, 74, and 78%, relative to controls, at the 0.05, 0.1, 0.25, and 0.5 mg/l norflurazon treatment concentrations, respectively. After the 15 d exposure period the NOEC and LOEC was 0.1 and 0.25 mg/L, respectively. Partial discoloration was first noticed after 5 d exposure and steadily increased through 15 d exposure at 0.025 and 0.05 mg/L. Full discoloration was very minimal at 0.025 and 0.05 mg/L compared to the other treatment concentrations. Full discoloration

distinctly started after 7 d exposure and steadily affected more fronds at 0.1, 0.25 and 0.5 mg/L through the 15 d exposure period.

A significant reduction in new leaf production was observed following 7 and 14 d exposure to simazine. After 7 d exposure, new frond production was similar to the controls at the 0.05 and 0.1 mg/L concentrations with an average of 47 (control), 55.6 (0.05 mg/L), 48.6 (0.1 mg/L), 35 (0.2 mg/L), 27.4 (0.4 mg/L), 27.4 (0.6 mg/L), 20.4 (0.8 mg/L) and 15.6 (1.0 mg/L). However, mean frond production was reduced by 26, 42, 42, 57 and 67%, relative to controls, at the 0.2, 0.4, 0.6, 0.8 and 1.0 mg/L simazine treatment concentrations, respectively. After 14 d exposure, it is evident that the 0.05 and 0.1 mg/L concentrations were minimally affected, while the higher concentrations significantly affected frond production (Fig 2). The average production of new leaves after 14 d was 283 (control), 294 (0.05 mg/L), 267 (0.1 mg/L), 201 (0.2 mg/L), 102.6 (0.4 mg/L), 79.4 (0.6 mg/L), 49 (0.8 mg/L) and 31.4 (1.0 mg/L). Mean frond production was reduced by 29, 64, 72, 83, and 89%, relative to controls, at the 0.2, 0.4, 0.6, 0.8 and 1.0 mg/L simazine treatment concentrations, respectively. After the 14 d exposure period the NOEC and LOEC was 0.1 and 0.2 mg/L, respectively. Partial discoloration started after 7 d exposure at 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/L, continuing to affect more fronds through the 14 d exposure period. Full discoloration started after 7 d exposure with minimal effects at the 0.2 mg/L concentration and steadily affected more fronds at 0.2, 0.4, 0.6, 0.8 and 1.0 mg/L through the 14 d exposure.

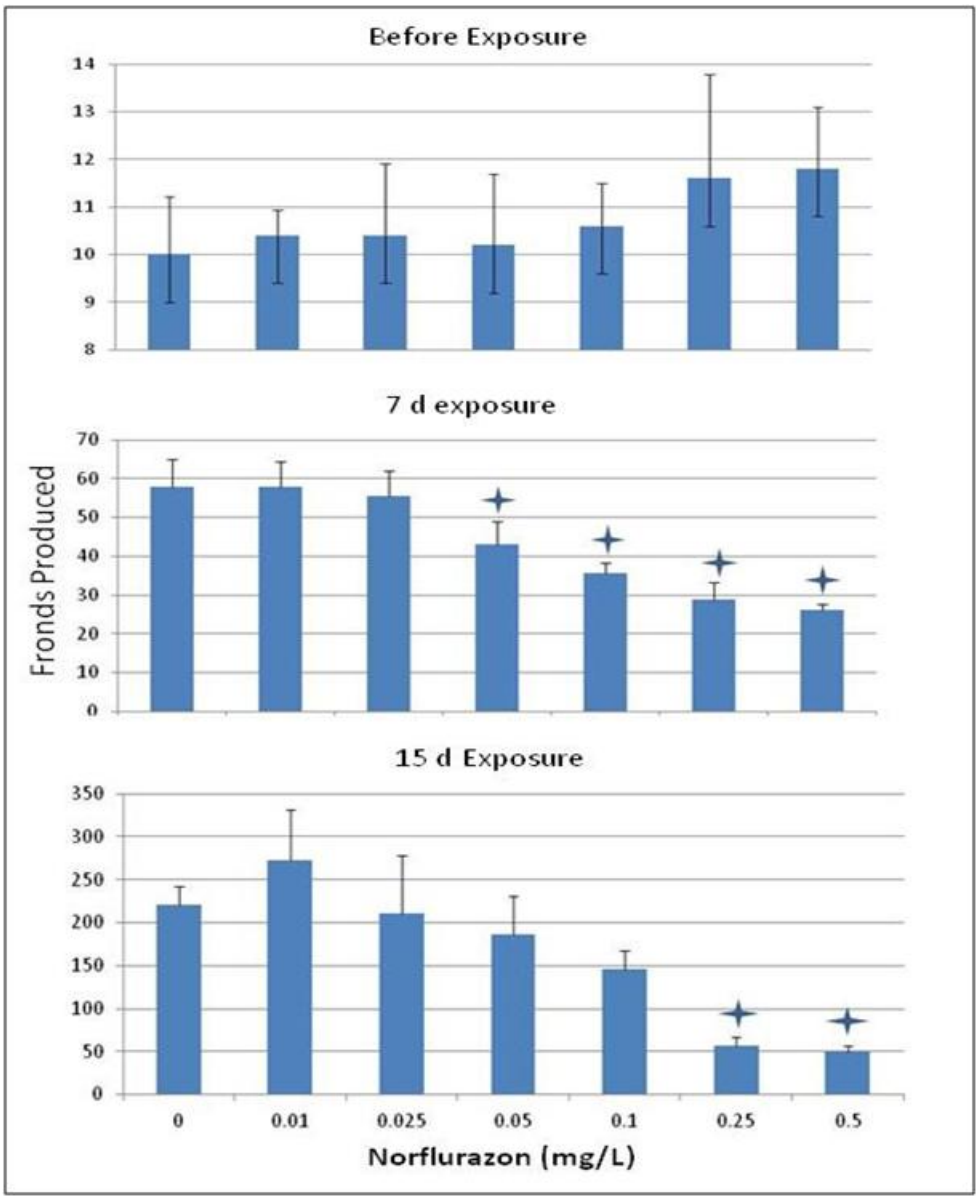

Figure 1. Frond production after 0, 7, and 15 days exposure to norflurazon. *indicates treatments for which the mean difference is significant at *P*= 0.05 based on ANOVA and Dunnett's test.

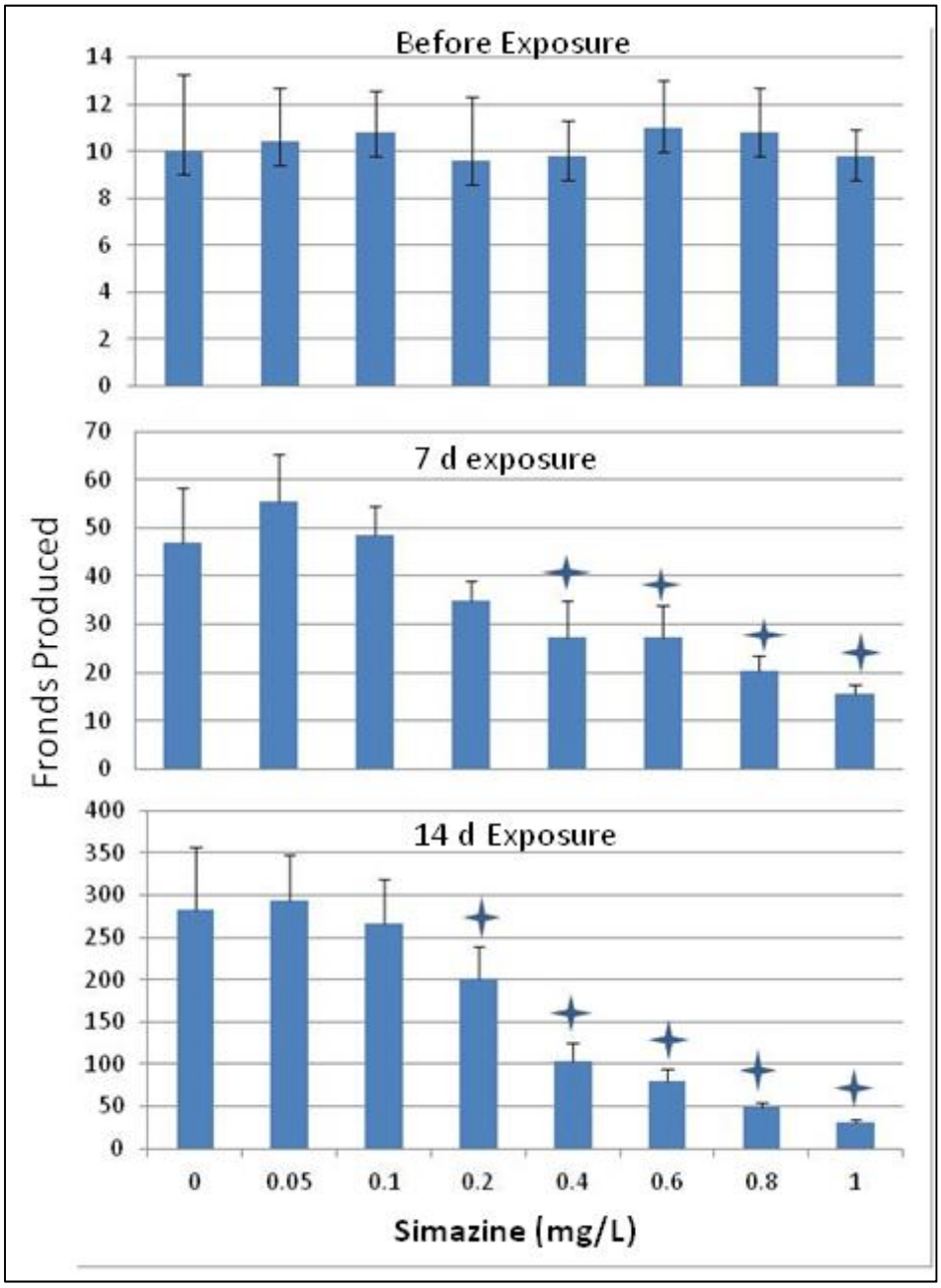

Figure 2. Frond production after 0, 7, and 14 days exposure to simazine. *indicates treatments for which the mean difference is significant at *P*= 0.05 based on ANOVA and Dunnett's test.

Final Fresh weights

Fresh weights following exposure to norflurazon decreased as exposure concentrations increased. Final fresh weights after 15 d exposure were 0.261 g (control), 0.257g (0.01 mg/L), 0.172 g (0.025 mg/L), 0.115g (0.05 mg/L), 0.045g (0.1 mg/L), 0.033 (0.25 mg/L), and 0.025 (0.5 mg/L). After the 15 day exposure period the observed NOEC and LOEC was 0.01 and 0.025, respectively. Plants exposed to concentrations of norflurazon greater than 0.025 mg/L exhibited loss of tissue due to necrosis, beginning at the tips and moving inward, accounting for loss of weight. Fresh weights following the simazine exposures also decreased with increasing concentrations. Measured fresh weights were 0.164g (control), 0.156g (0.05 mg/L), 0.148g (0.1 mg/L), 0.090g (0.2 mg/L), 0.039g (0.4 mg/L), 0.019 (0.6 mg/L), 0.013g (0.8 mg/L) and 0.005g (1 mg/L). After the 14 day exposure period the observed NOEC and LOEC was 0.1 and 0.2 mg/l, respectively. Plants exposed to concentrations of simazine greater than 0.2 mg/L exhibited loss of tissue due to necrosis.

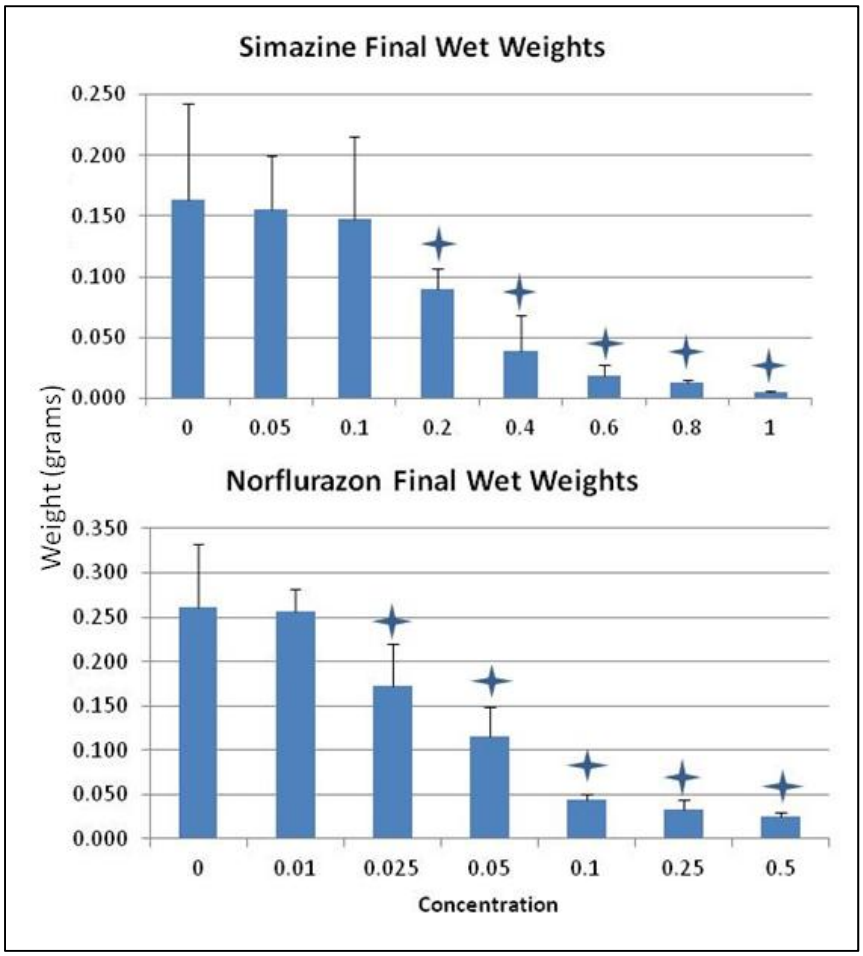

Figure 3. Final wet weight measurements after 15 days of exposure to simazine and norflurazon. *indicates treatments for which the mean difference is significant at *P*= 0.05 based on ANOVA and Dunnett's test.

Fv/Fm measurements & chlorophyll fluorescence

No adverse effects were seen for Fv/Fm measurements for norflurazon after 7 and 15 d exposure. Fv/Fm measurements were highly variable, ranging from 0.568 to 0.747 on day 7 and 0.390 to 0.715 Fv/Fm on day 15, compared to the control group's 0.662. Fv/Fm for plants exposed to simazine were also variable, ranging from 0.445 to 0.614 Fv/Fm after 7 d exposure and 0.656 to 0.704 after 14 d exposure, compared to 0.656 for the control group. Resulting NOEC and LOEC was 0.8 and 1, respectively, for simazine.

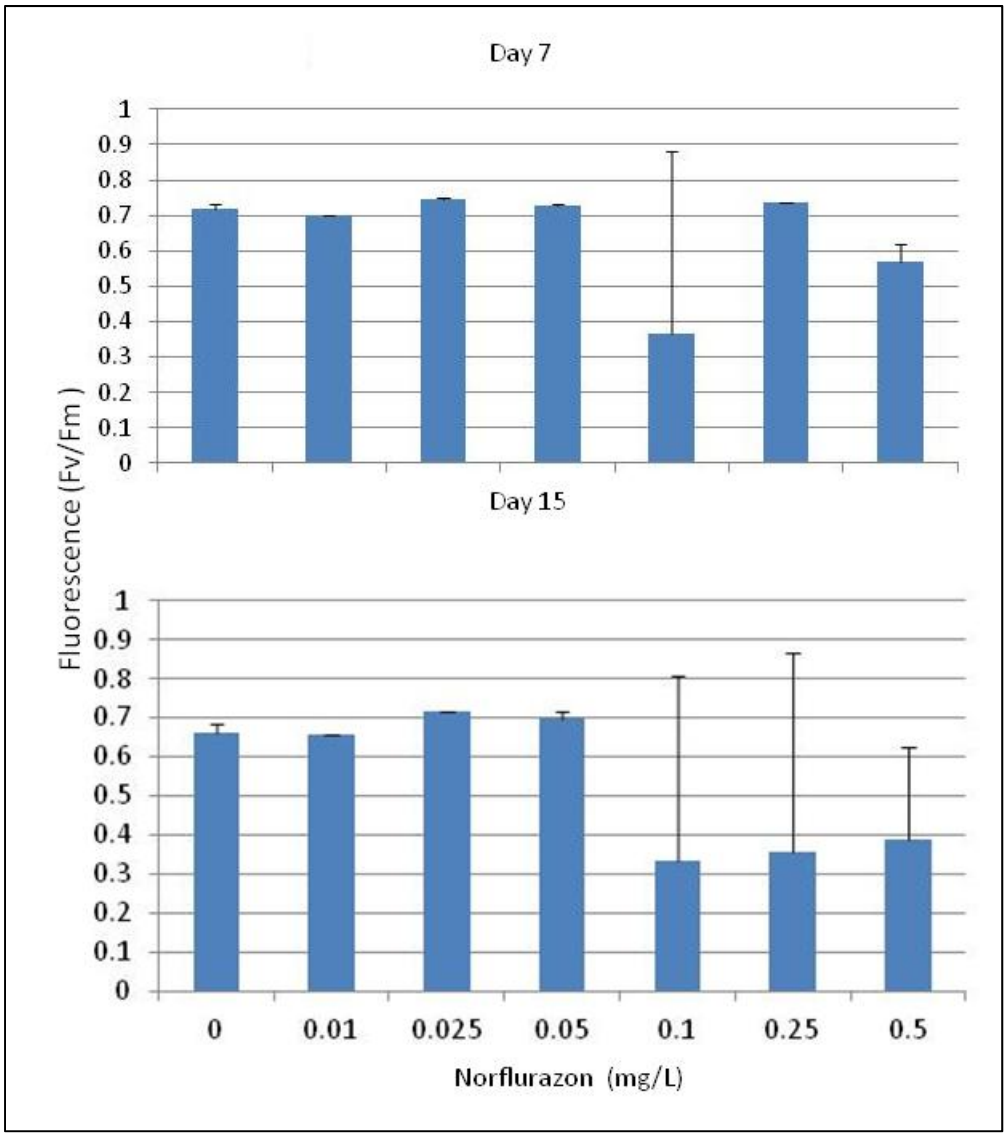

Figure 4. Fluorescence measurements (Fv/Fm) after 7 and 15 days of exposure to norflurazon.

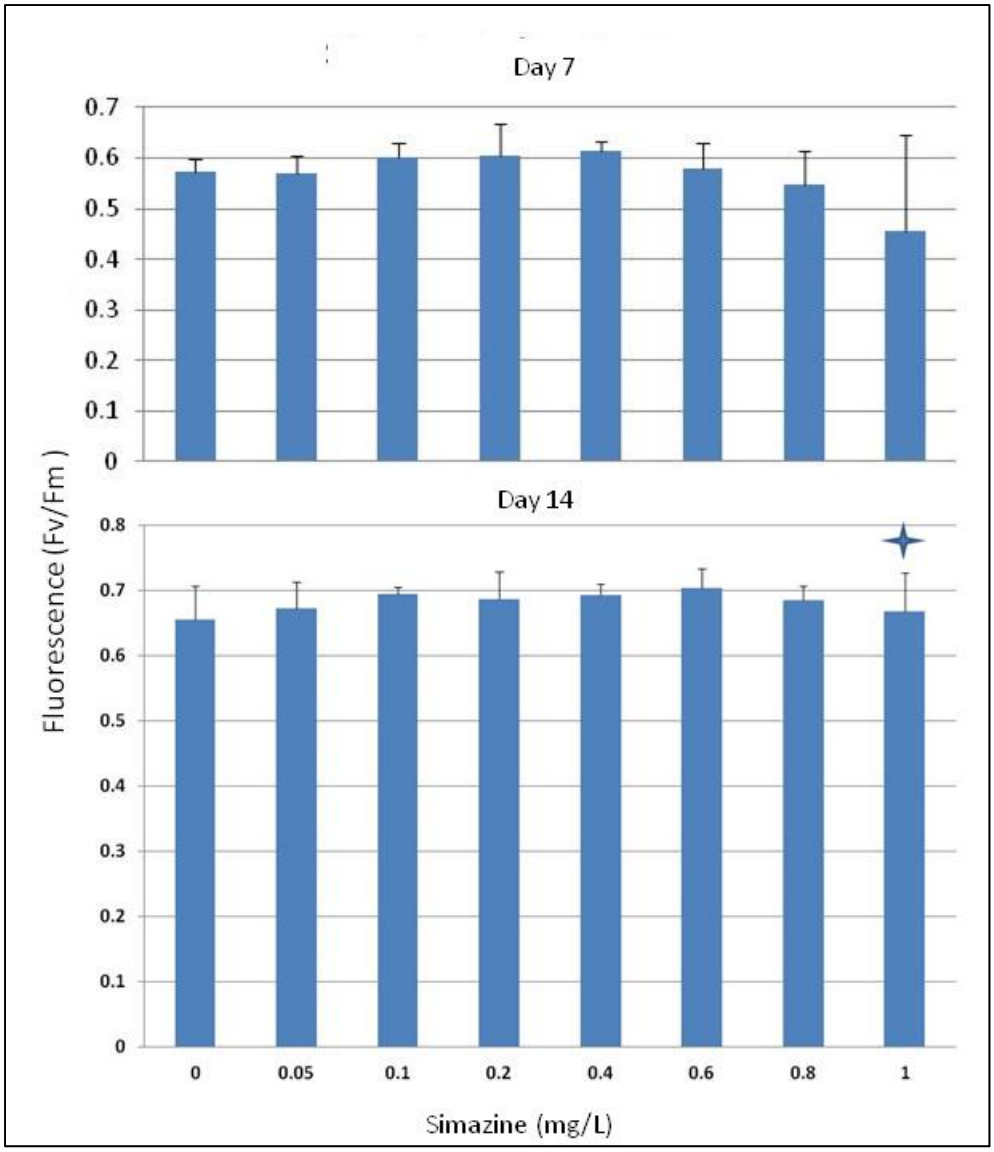

Figure 5. Fluorescence measurements (Fv/Fm) after 7 and 14 days of exposure to simazine. *indicates treatments for which the mean difference is significant at *P*= 0.05 based on ANOVA and Dunnett's test.

Table 4. Summary of NOEC & LOEC's

Norflurazon (mg/L)					
	Total Count Day 7	Total Count Day 15	Fv/Fm Day 7	Fv/Fm Day 14	Final Fresh Weights
NOEC	0.025	0.1	N/A	N/A	0.01
LOEC	0.05	0.25	N/A	N/A	0.025
Simazine (mg/L)		(Day 14)			
NOEC	0.2	0.1	N/A	0.8	0.1
LOEC	0.4	0.2	N/A	1	0.2

Conclusion

Before the effects of herbicide mixtures can be assessed for non-target species, effect threshold concentrations must be known for each individual herbicide. This experiment characterized the toxic concentrations of these two herbicides on the surrogate aquatic macrophyte, *Lemna minor* L. using destructive and nondestructive growth measurements (frond production, , chlorophyll fluorescence, and final wet weight). Due to the destructive analysis at the end of the experiment (final wet weight), post-exposure periods were not observed for possible recovery from herbicide exposures. Following a two week exposure period, a NOEC of 0.1 mg/L and LOEC of 0.25 mg/L for norflurazon was observed for total frond production. Simazine had a common NOEC of 0.1 mg/L and LOEC of 0.2 mg/L for total frond production and final wet weights. Using LOEC concentrations for both simazine and norflurazon would almost certainly impact growth given a two week exposure period. NOEC's might be expected in areas where dilution occurs due to drainage from the surrounding watershed. These NOEC and LOEC thresholds found are indicative of effective concentrations that can be used in future experiments to observe the effects of both simazine and norflurazon in mixtures.

References

Dayan, Franck E. Zaccaro, Maria Leticia de M. "Chlorophyll fluorescence as a marker for herbicide mechanisms of action". *Pesticide Biochemistry and Physiology*, 102.3(2012):189–197. http://dx.doi.org/10.1016/j.pestbp.2012.01.005

Essington, Michael E. Soil and Water Chemistry: An integrated Approach. Technology and Engineering. 2004.

Knuteson S.L., Whitwell T., Klaine S.J. "Wetlands and Aquatic Processes: Influence of Plant Age and Size on Simazine Toxicity and Uptake." *J Environ Qual* 31 (2002):2096–2103.

Koenig, Franz; Brannath, Werner; Bretz, Frank; Posch, Martin. "Adaptive Dunnett tests for treatment selection." *STATISTICS IN MEDICINE*. 27 (2008):1612–1625.

Maxwell, Kate; Johnson, Giles N. "Chlorophyll Fluorescence- a practical guide". *Journal of Experimental Botany*, 51.345(2000): 659-668.

Mohan BS; Hosetti BB. “Aquatic plants for toxicity assessment”. *Environ Res*. 81(1999):259–274.

Radic´, Sandra; Stipanic˘ev, Draz˘enka; Cvjetko, Petra; Mikelic´, Ivanka; Lovrenc˘ic´, Rajc˘ic´; Marija, Marijanovic´; S˘irac, Sinis˘a; Pevalek-Kozlina, Branka; Pavlica, Mirjana. “Ecotoxicological assessment of industrial effluent using duckweed (Lemna minor L.) as a test organism” *Ecotoxicology* 19(2010):216–22.

Roe, R.M.; Burton ,J.D.; and Kuhr, R.J. (eds.) Herbicide Activity: Toxicology, Biochemistry, and Molecular Biology. (1997) I.O.S. Press, Inc., Amsterdam

Florida Department of Agriculture and Consumer Services. “Summary of Agricultural Pesticide Use in Florida: 2007-2009.” Division of Agricultural Environmental Services Bureau of Pesticides Tallahassee, Florida 32399-1650 Phone Number: (850) 617-7917. October 2010.

Schuler, Lance J., Rand, Gary M. “Aquatic Risk Assessment of Herbicides in Freshwater Ecosystems of South Florida.” *Arch Environ Contam Toxicol* (2008) 54:571–583.

Tomlin CDS, ed; The Pesticide Manual, 11th ed. The British Crop Protection Council (1997)pg 1106.

Troiano, J., J. Marade, and F. Spurlock. “Empirical Modeling of Spatial Vulnerabilitiy Applied to a Norflurazon Retrospective Well Study in California”. *J. Environ. Qual*. 28(1999):397-403. http://www.cdpr.ca.gov/docs/empm/pubs/ehapref.htm

U.S. Environmental Protection Agency. “EPA Method 3535: Solid phase extraction (SPE). SW- 846 online: Test methods for evaluating solid waste, physical, chemical methods. Report SW-846, Ch 4.2.1 (Rev. 0).” Washington, DC. 1996.

U.S Environmental Protection Agency. “Reregistration Eligibility Decision: Norflurazon List A Case 0229.” Environmental Protection Agency Office of Pesticide Programs Special Review and Reregistration Division. 1999. http://www.epa.gov/oppsrrd1/REDs/0229.pdf. Accessed on October 18, 2013.

U.S. Environmental Protection Agency. “Simazine: Health Advisory.” Office of Drinking Water, US EPA, Washington, DC. 1988.

U.S. Environmental Protection Agency. “Technical Factsheet on: SIMAZINE” taken from EPA National Primary Drinking Water Regulations 40 CFR part 142. 2010. http://www.epa.gov/safewater/pdfs/factsheets/soc/tech/Simazine.pdf

Vencill WK .Herbicide Handbook, 8th edn. Weed Science Society of America, Lawrence . 2002.

Ware GW (Ed). The SCR/ARS/CES pesticide propertiesdatabase for environmental decision-making. Rev Environ Contam Toxicol 123:164 (1992)

Wilson, P.C. and Boman, B.J. “Characterization of selected organo-nitrogen herbicides in South Florida canals: exposure and risk assessments.” *Sci. Total Environ.* 412.13(2011):119-126.

Wilson, P. Chris; Wilson, Sandra B. “Toxicity of the herbicides Bromacil and Simazine to the Aquatic Macrophyte, *Vallisneria Americana* Michx” *Environmental Toxicology and Chemistry*, 29.1(2010): 201-211. DOI: 10.1002/etc.22

WSSA Herbicide Handbook Committee. Herbicide Handbook of the Weed Science Society of America, 6th Ed. WSSA, Champaign, IL. 1989.

Wu, J., J. Lu, P.C. Wilson, Y. Lin, and H. Lu. “Effective liquid-liquid extraction (LLE) method for analysis of pyrethroid and phenylpyrazole pesticides in emulsion-prone surface water samples.”*Journal of Chromatography A*. 1217.41(2010): 6327-6333.